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# **Uptake, Accumulation, and Toxicity of Silver Nanoparticles to Juvenile Bivalve Mollusc's Hard Clam *Mercenaria Mercenaria***

Hanaa Radhi Jolan Al Hameed

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UPTAKE, ACUUMULATION, AND TOIXICITY OF SILVER NANOPARTICLES TO  
JUVENILE BIVALVE MOLLUSC'S HARD CLAM *MERCENARIA MERCENARIA*

by

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## DEDICATION

I dedicate my work to soul my father (Radhi Jolan Ebrahim ALjezany), my mother (Bahyia Oun Myzail), and my sisters, brothers for their inspiration, guidance and support in my endeavors.

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## ABSTRACT

Nanoscience and nanotechnology have gained attention in the last 20 - 30 years. Manufactured nanoparticles (NPs), which are defined as having at least one dimension between 1 and 100 nm, are the main products of nanotechnology. Nanoscale materials often have novel properties due to their size and this has made them so attractive for a range of processes and sectors. These attractive properties have enhanced their use in a wide range of consumer products and applications including electronic, biomedical, and pharmaceutical. The overall objective of this dissertation was: 1) to investigate the transformation of well-characterized AgNPs, at environmentally relevant concentrations (1-100  $\mu\text{gL}^{-1}$ ) in complex media, primarily seawater, and 2): to quantify the accumulation, and toxicity AgNPs to juvenile bivalve's mollusc hard clam *Mercanria mercenaria*. Literature searches to understand the relationship between NP properties and biological effects revealed that there was a lack of data leading to substantial uncertainty about their risk, including their fate, behavior and toxicity AgNPs to clams species. The majority of these studies were performed in concentration ranges higher than environmental concentrations. However, some studies showed that smaller sized NPs have more biological influence and toxicity, likely due to their ability to be up-taken and translocate more rapidly, and depurate more slowly, than larger particles.

Due to the lack of data, this study aimed to quantify uptake and toxicity of Ag NPs, using in-house synthesized AgNPs, which were characterized prior to quantification of their uptake, accumulation, and toxicity to juvenile hard clam *M. mercenaria*. Result indicated that AgNPs were spherical and monodisperse on synthesis and were stable in ultrapure water. However, when added to either a synthetic or a natural marine water, there were changes in their physico-chemical properties in the both. PVP- AgNPs showed more stability than Cit. AgNPs in the both seawater types. The result of our study emphasized the importance of use multi methods in order to obtain sufficient characterization of NPs to be get interpret other data.

Toxicity studies found that exposure of AgNPs to *M. mercenaria*, was toxic to juvenile hard clams but was not always more toxic than conventional silver nitrate ( $\text{AgNO}_3$ ). Initial statistical comparisons of both the acute and chronic toxicity of the nanoparticles (Cit. AgNPs in NFSW, Cit. AgNPs in SSW, PVP-AgNPs in NFSW and PVP- AgNPs in SSW) with conventional silver nitrate ( $\text{AgNO}_3$  in NFSW and  $\text{AgNO}_3$  in SSW) using Trimmed Spearman Karber analysis, suggested there were no significant ( $p > 0.05$ ) differences in acute toxicity among treatments. However, further statistical analysis using multiple comparison tests (Mann Whitney) comparing replicate  $\text{LC}_{50}$  values for each treatment, indicated that there were significant ( $p < 0.05$ ) differences among treatments in both the acute and chronic toxicity tests. In the acute toxicity tests,  $\text{AgNO}_3$  in SSW was the most toxic compound which was only more toxic than Cit. AgNPs in NFSW and PVP- AgNPs in SSW. None of the other comparisons in the acute toxicity tests were significantly different. In the chronic toxicity tests Cit. AgNPs in NFSW was the most toxic compound which was more toxic than  $\text{AgNO}_3$  in SSW and both of these compounds were more toxic

than AgNO<sub>3</sub> in NFSW, Cit. AgNPs in SSW, PVP- AgNPs in NFSW, and PVP-AgNPs in SSW. Comparisons of No Observable Effects Concentrations (NOEC) and Lowest Observable Effects (LOEC) in both acute and chronic toxicity tests were not significantly different in comparisons between conventional AgNO<sub>3</sub> and all Ag NPs treatments. This indicated that initial onset of toxicity thresholds were similar for both conventional forms of Ag (e.g. AgNO<sub>3</sub>) and the different types of AgNPs tested, regardless of the seawater type (filtered or unfiltered). In this study, Ag accumulated inside clam's tissues in all concentrations of exposure and in all treatments. The AgNPs accumulated into clam tissues more than AgNO<sub>3</sub>. The largest percentages mass mostly was in the lowest concentrations for all treatments, and the Ag accumulation was decreased with increased Ag concentrations exposure. This data of Ag accumulation because of AgNPs at higher concentration aggregated and showed less accumulation due to less dissolution. While at the lower concentration AgNPs showed less aggregation, more dissolution and higher accumulation. After the 24h depuration period, Ag accumulation decreased in all treatments. In the highest concentrations, histological changes were shown. From the result of this study, it is highly important continuing investigation AgNPs uptake and accumulation in bivalves species especially in hard clam *M. mercenaria*. Because of the lack of studies, that dealing with this topic and studying the interaction AgNPs with clam species as well as to have better understanding to the mechanisms of AgNP uptake and accumulation and the subsequent effect on bivalves' species and marine ecosystem. Therefore, this study was designed to investigate the effects of AgNPs on marine bivalve.



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# CHAPTER 1

## INTRODUCTION

### **1.1 Nanoscience, nanotechnology, and the environment**

Nanoscience define as the study of structures at the nanoscale, which is defined as having at least in one dimension between 1 and 100 nm [1-3]. However, the American Society for Testing and Materials defines, NPs as a particle that has two or three dimensions between 1–100 nm [4]. Nanotechnology refers to creating, manufacturing, and manipulating materials at a scale that falls within this range. A more generalized description of nanotechnology, which is defined nanotechnology as the manipulation of matter with at least one dimension sized from (1 to 100) nanometers [5]. The famous lecture that was introduced by Richard Feynman in which he said his phrase “There’s plenty of room at the bottom” and he explained the importance of nanotechnology in a wide numbers of scientific fields by dressmaking material properties at the atomic scale [5]. Nanoparticles (NPs) as a building block for the rapid development of nanotechnology [6]. Nanoparticles (NPs) are materials with at least one dimension between 1 and 100 nm, which exhibit novel properties relative to their bulk material and dissolved counterparts due to their size [1-6]. For the reason that of their unique properties, NPs have been increasingly used in a variety of applications, in different scientific and industrial fields [7-9]. NPs have been used in

consumer products [10], including, food packaging [11], medicine [12], and environmental remediation [13] and based on the NP composition, size, and coating [14-16]. These applications depend on their novel properties. However, fast-growing of nanotechnologies has led to increasing applications of (NPs). With the rapid increase in the production of NPs with approximately more than (1100) consumer products containing NPs are listed which there are (379) reported products including AgNPs (35%) and making AgNPs the most commercialized NP in the market due to their high electrical and thermal conductivity [17-19]. Consequently, the release of NPs in the environment is also expected to increase; the concern is arising because of the large discharge NPs products that might harm organisms and possible novel environmental behavior of some NPs in the environment [20]. In fact, the NPs are released into the aquatic environment either directly through industrial and domestic discharges or indirectly via aerial deposition, and run-off [21, 22].

## **1.2 Silver nanoparticles AgNPs**

### **1.2.1 Synthesis of Ag NPs**

In recently years, Ag NPs synthesis have been the focus of attention due to the biocidal properties. Two different approaches were used in the synthesis Ag NPs “Top- down” and “bottom-up” approaches. [23-25]. In the top-down method, bulk-sized materials are divided into nano-sized structures. [19]. In this process, the mechanical, physical or chemical grinding, or etching of bulk silver to form the metal nanomaterials actions are involved. Usually the size of this type of fabrication between in range 10-100 nm [25]. NPs produced using a top down approach have unpredictable and broad physical properties and present defects in the surface morphology of the nanoparticle, the production is highly complex nanostructures [26]. The “bottom-up” approach is defined as the construction of

nanostructures molecule by molecule. The NPs produced by this method have fewer structural defects, and are characterized by more homogeneous and stable physical and chemical compositions [27-29]. A typical bottom up approach synthesis of Ag NPs would be the reduction of a precursor salt. Then, this reaction will be terminated with a stabilizer, (donor ligands, polymers, or surfactants) or capping agent which would ensure the chemical and physical homogeneity and stability of NPs in the media formed [30]. There are three synthetic approaches have been utilized in synthesis AgNPs, physical, chemical, and biological approach. The chemical methods tend to be the more laboratory intensive use compared to the physical and biological techniques, AgNPs synthesized by this approach exhibits attractive properties, such as high yield, solubility, and stability [30]. AgNPs are chemically synthesized mainly through the Brust–Schiffrin synthesis (BSS) or the Turkevich method [32- 35]. Several considerations should be taken when choosing the type of reducing agents and stabilizer for the safety and effectiveness of the method by use environment-friendly reducing agent and selection of relatively non-toxic substances. Nucleation phase and growth of NPs are controlled by various reaction parameters, including reaction temperature, pH, concentration, type of precursor, reducing and stabilizing agents, and molar ratio of surfactant/stabilizer and precursor [36]. The chemical reduction of silver nitrate by reducing agents such as sodium citrate and sodium borohydride is an important bottom-up synthesis procedure of AgNPs were used in this study. Silver ion ( $\text{Ag}^+$ ) is reduced in aqueous solution, receiving an electron from a reducing agent (sodium citrate) to switch from a positive valence into a zero-valent state ( $\text{Ag}^0$ ), followed by nucleation and growth. This step leads to coarse agglomeration into oligomeric clusters to yield colloidal AgNPs. Stabilizing dispersive NPs during a course of

AgNP synthesis is critical. The most common strategy is to use stabilizing agents that can be absorbed onto the surface of AgNPs, avoiding their agglomeration [37].

### **1.2.2 Ag NPs characterization**

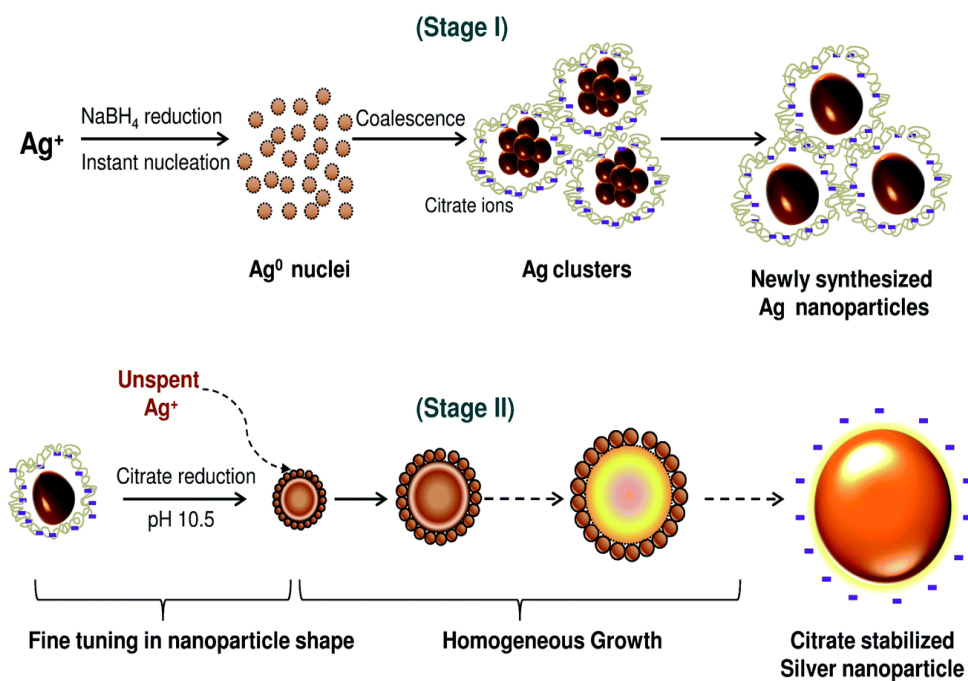
In order to determine the intrinsic and extrinsic properties, it is critical to perform an appropriate physical and chemical characterization. Thus, size, surface chemistry and charge, crystallinity, phase purity, solubility and shape are crucial to elucidate the stability, reactivity, bioavailability of NPs in different media [38]. NP size is the one of the most vital physico-chemical properties of NPs, which can be correlated with their behavior. It is as well one of the main factors that influence bioavailability, distribution and retention of the NPs in target tissues [39, 40]. One of the advanced techniques that is provided an accurate valuation of the size and shape of NPs making surface images by scanning the NPs using a physical probe [41]. For example, Transmission Electron Microscopy and Scanning (TEM and SEM) allow the identification of structure and morphology of NPs [42]. However, it needs complex sample preparation, which could lead to imaging artefacts due to previous sample treatment and as well to vacuum conditions. The technique, which is the Atomic Force Microscopy (AFM) delivers quantitative and qualitative data on physical properties such as size, morphology, surface texture and roughness [43]. This technique is depended on van der Waals forces and could be applied in liquid media [41].

One of the commonly technique often use the Dynamic Light Scattering (DLS) is commonly used for NPs size determination since it provides a simple and fast estimate of particle size. DLS can measure the association of the time depend fluctuations in the light scattered by a suspension of nanoparticles (autocorrelation function), which is determined

by their Brownian motion. Giving to their size, NPs and aggregates obtain different mobility, which is referred as hydrodynamic diameter (HD) [44]. Despite of commonly DLS being used to establish the size of NPs in solution [42], there is limitations which are related with signal loss by smaller particles owing to the signal intensity of bigger ones, i.e., the scattering intensity of small particles lean towards to be masked by the larger ones [45].

Other technique widely used for NPs size determination is x-ray diffraction (XRD), which also provides information on surface properties and coatings, crystallographic structure or elemental composition [40]. XRD applies the scherrer method to calculate particle size, but the accuracy of such method is poor [46]. Techniques like UV-vis and Fourier transform infrared spectroscopy (FTIR) are spectroscopic methods usually employed in fullerenes and derivatives characterization particularly in aquatic environments [47]. An innovative system for NPs sizing is nanoparticle tracking analysis (NTA), a single particle tracking technique based on dark field or fluorescence microscopy and automatic imaging analysis. NTA is an advantageous method since it tracks individual NPs and provides a high resolution for multimodal samples and aggregation/agglomeration [48]. Elemental composition and chemical state of NPs can be assessed by X-ray photoelectron spectroscopy [49]. Secondary ion mass spectroscopy is another technique used to verify NPs elemental composition by ionization and sputtering of the surface atoms [50]. It is well known that an appropriate characterization is needed. Since all these techniques depend on different sample preparation and physical principles, the results of NPs characterization in ecotoxicological tests differ according to the method used [51]. In fact, with all current still have tough problems when encountering complex environmental

samples. In addition, considering the complexity of biological systems, the detection of NPs requires that the analytical methods have very high tolerance to biomolecules and very good selectivity. For instance, the detection limits of some of the current techniques are not low enough for determining nanoparticles concentration in natural water and biological samples (in the scale of ng/L or pg/L). They also have difficulties in are not low enough for determining nanoparticles concentration in natural water samples (in the scale of ng/L or pg/L) distinguishing mixture between engineered nanoparticles and natural colloid [52].

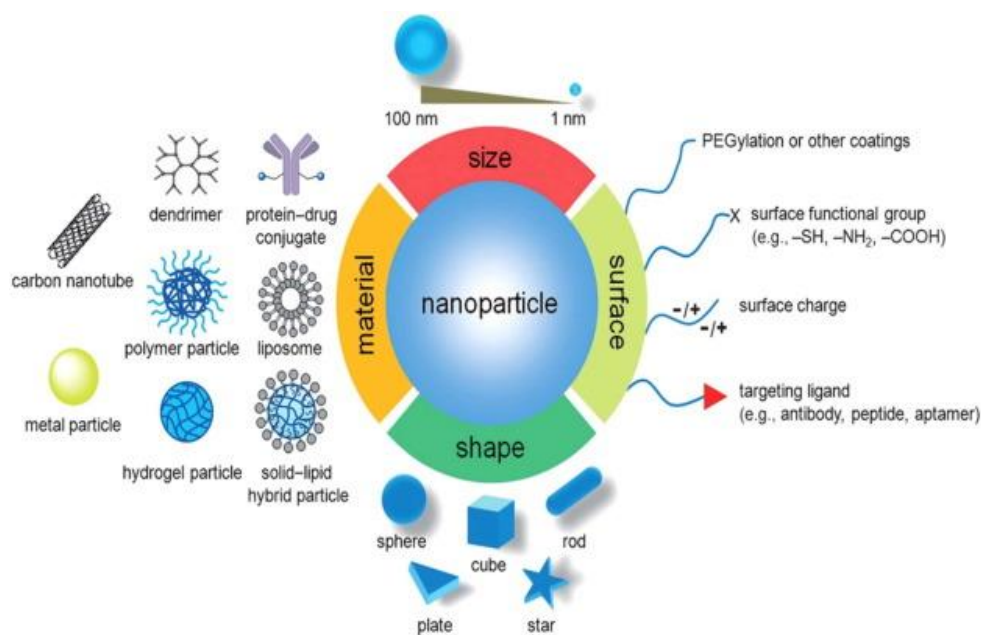


**Figure 1.1** Schematic representation of size controlled silver nanoparticles synthesized employing co-reduction approaches [Shekhar et.al 155]

### 1.2.3 Ag NPs properties and applications

NPs often exhibit unique physio-chemical properties and reactivity's due to their small size, homogenous structure, and surface characteristics that are not present at the larger scale such as electron spatial constraints, large specific surface area and high surface energy [53]. In the nanoscale range, the ratio between the surface area and volume is higher compare with in bulk material [54]. These properties influenced by a small size of NPs, and it determine their behavior, biological effects and consequently their toxicity. AgNPs as knowing widely used in many applications because of its antibacterial and antiviral properties, superior catalytic activity and surface enhanced raman spectroscopy (SERS) [15, 16, 55- 58]. There are multiple factors can affect surface functionalization of NPs e, g .capping agent were used to achieve the required stability [57]. Thus, the particle stability can change when suspended in other aquatic conditions [58]. Surfaces functionalities of AgNPs can be controlling during the synthesis such as surface-enhanced Raman scattering [58], contaminant remediation [59], etc. Toxicity of AgNPs influenced by dissolution rate and its associated with both the characteristics of Ag NPs [60] and the surrounding environmental factors [61]. In addition, these physicochemical properties of Ag NPs can affect their interactions with organisms and environment [52, 62, 63]. The distinctive properties of NPs have been representative of their potential uses in medicine, catalysis, optics, cosmetics, renewable energies, inks, microelectronics, medical imaging, environmental remediation, and biomedical devices [17–21]. Moreover, AgNPs display a broad spectrum of bactericidal and fungicidal activity [22]. Mainly, Ag used as antimicrobial agents in the form of polymer nanocomposites [65], and their effectiveness is due to the release of germicidal silver ions [66]. Consequently, the use of AgNPs turn

out to be exceptionally trendy for the wide range of consumer goods such as soaps, plastics, food packaging, cleaning products, antibacterial wound dressings, cosmetics, personal care products and textiles [19, 21-24]. For instance, AgNPs are became popular in the scientific research and highly commercialized due to their excellent antimicrobial activities.



**Figure 1.2** Properties and applications of NPs. Madannejada R. et al., [158].



## **1.3 AgNPs and the environment**

### **1.3.1 AgNPs release**

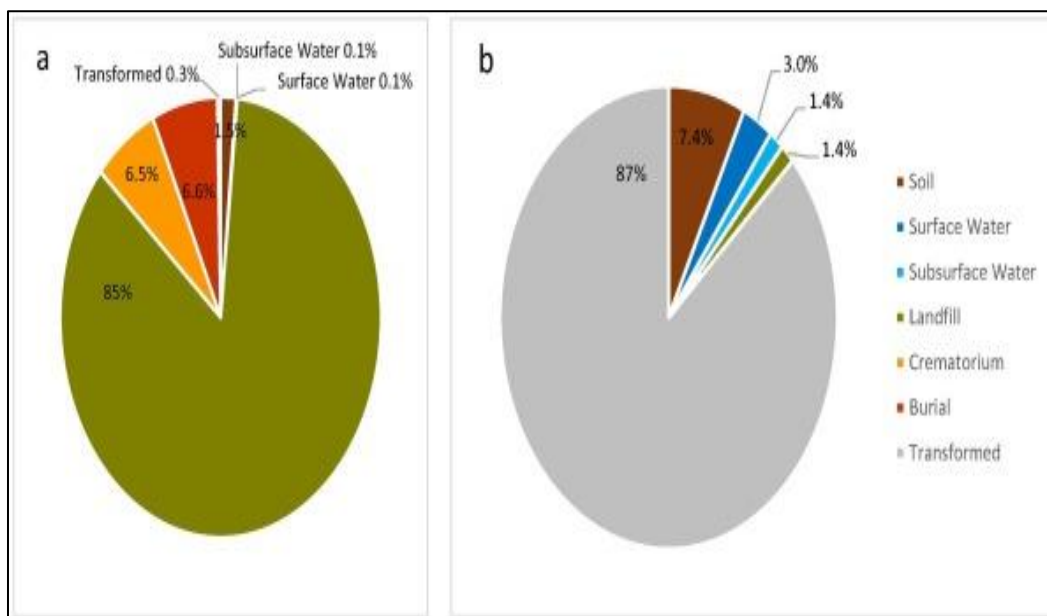
With increase growing the use applications of AgNPs, their release to the environment is a critical [67]. Release of AgNPs to the environment can occur in several ways, including during synthesis, manufacturing and incorporation of the NPs into products, during use and recycling [68]. There are several studies conducting release silver nanoparticles from different products such as, textiles [687, 69], paints [70] as well as some of modelling studies to predict the release of silver in the environment [71-73]. However, a key challenge is in the measurement faced these studies to distinguish between AgNPs and Ag ions in environment or in exposure media and these challenges due to high detection limit in analytical instruments, time consuming, and analysis costly. Geranio et al [74] did an example on studies concern Ag NPs release. In this study a nine fabrics were tested and the result showed that the amount of released silver varies between 1% and 45% of total silver mass and the nanoparticle dissolution rate is rather low. Silver is released mainly as big (>450 nm) particles. The result of modeled environmental concentrations study of NPs for the U.S., Europe and Switzerland that was done by Gottschalk et al.[71] emphasized exposure g NPs to aquatic organisms in sewage treatment effluents and in surface waters cannot excluded. In this study, AgNPs concentration was low in natural water systems due to sedimentation processes compared with the previously study of Mueller and Nowack et al., [75] and the calculated concentrations generally reflect the worldwide production volumes.

The risk AgNPs to freshwater ecosystem incorporated with release textiles and plastics products, and it was investigated by Blaser et al., [76]. The investigators proposed a model of silver mass flow, estimated emission, assessed the fate of silver in a river system, estimated the predicted environmental concentrations (PEC), and evaluated available toxicity data for environmentally relevant forms of silver in order to make an estimation of predicted no-effect concentrations (PNEC) and risk characterization. Silver incorporated into plastics and textiles accounts for up to 15% of total silver released into water systems in the EU (of which Ag NPs is only a fraction). Three different emission scenarios were studied, in which silver release was in the range 110–230 t/yr. The authors made an assumption that only silver ion is released into the environment (no whole particles), which is contrary to some available experimental data [72, 73]. Based on available data, Blaser et al. [76]. The majority of silver released into water is incorporated into sewage sludge (which is in agreement with more recent experimental data [73] and then deposited on landfills or used as a fertilizer, soil and groundwater contamination should also be taken into account. The hazard of Ag NPs on soil organisms investigated by Shoults-Wilson et al. [77]. A large proportion of the annually produced sewage sludge is used as a fertilizer in agricultural soil in countries such as USA and UK. [77], even though other countries burn up such waste. The concentration of AgNPs in each parts of environments received affected by the exact disposal route. Increase the use AgNPs from consumer products lead to increase release AgNPs and thus pose potential risk to environment and human health [78]. A probabilistic flow modeling data from wastewater treatment plants (WTP) employing activated sludge for treatment suggest that NPs will partition into the sludge phase. The small fraction might remain in aqueous phase after treatment and will be

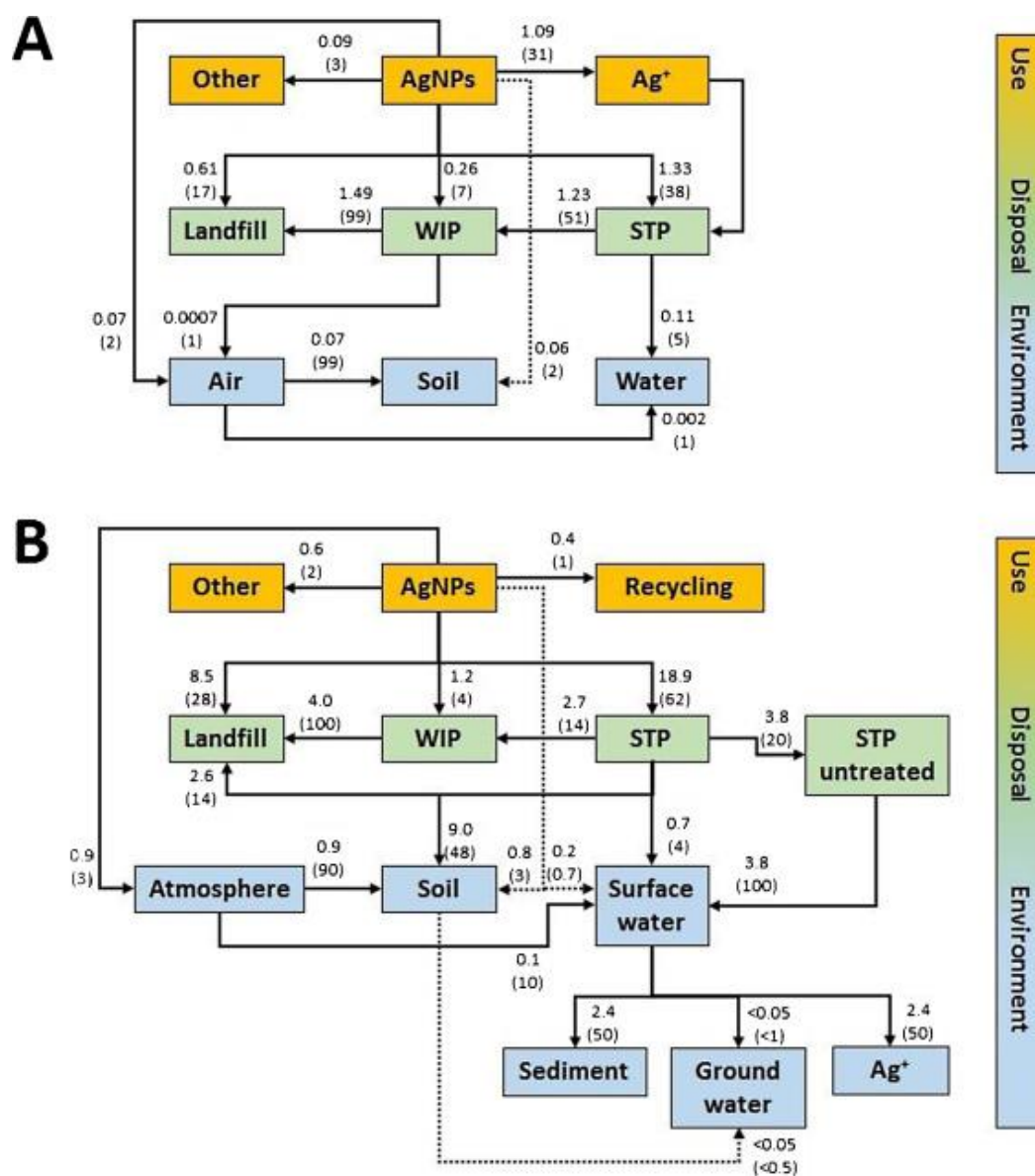
released in WTP effluent [76, 79]. The range AgNPs in between 0.01 ng L<sup>-1</sup> in surface water and 1.6 mg Kg<sup>-1</sup> in sludge [72, 73]. Despite the low PEC (ng L<sup>-1</sup> to µg L<sup>-1</sup>) of Ag NPs in different environmental compartments and as AgNPs highly discharged the environment loading increased. To predict the starting point if the life cycle of AgNP-containing products and how release by using modelling approaches and they also can be provide information on how accumulation of Ag compounds in the environment may occur and at what concentrations. The probabilistic methods that used for determining PECs in Europe and in the US. These methods mainly based on the life cycle perspective of products that are containing NMs depend on the life cycle. The perspective of products containing NPs demonstrate current predicted environmental concentrations in Europe with lower and upper quartiles (Q0.15 and Q0.85) of 0.5–2 ng L<sup>-1</sup> in surface waters, 32–111 ng L<sup>-1</sup> in sewage treatment plant effluents, and 1.3–4.4 mg kg<sup>-1</sup> in sewage sludge [71]. With predicted exponential yearly increase of Ag NP in most environmental compartments [72, 73]. Again, there is a lack of data to validate such model predictions.

### **1.3.2 Ag NPs fate and behavior in the environment**

Ag NPs are the most commonly used and present in commercial products, such as medical products, textiles, personal care products, optical materials, and antibacterial agents [80]. AgNPs will without doubt enter the environment without treatment, airs great possible risks to the environment and human health [81, 82]. Thus, investigation AgNPs fate and behavior in aquatic environment is crucial in natural water system. The fate of AgNPs might take in the possible pathways of domestic sewage, municipal wastewater treatment plant, landfill, etc., ultimately ending up in the aquatic environment [83-85].



**Figure 1.3** Distribution of nano-Ag (realistic and probable applications) (a) and PLGA (b) to final sinks. Total amount of nano-Ag 5,650 kg. , and total amount of PLGA 48,000 kg. [Marina. H, and B. Nowack, 338].



There have been several studies confirmed that AgNPs keep on stable in freshwater, while become instable in seawater [86, 87]. This assumed that AgNPs discharged may be transported along rivers and perhaps finish up in the ocean. Studies regarding fate AgNPs the fate in natural waters are very scarce. Thus, investigating the behaviors of AgNPs in natural aquatic environments is highly important. The processes such as surface reactions, stability, mobility, and dissolution play a major role in controlling their fate and behavior in the aqueous environment. The extent of these processes is regulated, among other things, by surface properties of the particles and environmental conditions such as pH value, ionic strength, and natural organic matter [83]. Because of increased AgNPs in consumer products and industrial applications provokes questions regarding appropriate risk assessment strategies for the prediction of their fate and behavior in various environment media or identifying the controlling processes of their fate and behavior in the environment.

#### **1.3.2.1 Transformations Ag NPs in the environment**

Ag NPs are vulnerable to face transformations in environment and biological system. These transformations such as chemical, physical, photo, and biological transformation. (Figure 1.5) Illustrates the critical NPs transformations affecting AgNPs and their interactions. Chemical transformation occurred by the reduction and oxidation processes. These two process define as the transfer of electron between the chemical moieties in the natural system. Principally elemental metal such as silver and iron are vulnerable to undergo reduction and oxidation processes [88, 89]. The transfer of electron between the chemical moieties in the natural systems defines the reduction and oxidation processes. In case AgNP, the oxidation of Ag (0) to Ag (I) is required to dissolve and release bactericidal Ag<sup>+</sup> [22]. As regards the redox state, three different types of environment can be

recognized, the oxidizing environment rich with oxygen such as aerated soils and natural waters, the reductive environment depleted of oxygen such as groundwater and carbon rich sediments and the dynamic redox environments like the tidal zones in which different redox states can take place [90]. As well, photo-oxidation and photo-reduction are sunlight catalyzed redox reactions that affect nanoparticles oxidation state, ROS, persistence and coating. The soft metal that categorized under class B such as Ag, Zn, and Cu are the best example of nanoparticles that ensure dissolution and sulfidation processes. The sulfidation of AgNPs is mainly occurred by their high affinity organic and inorganic sulfide ligands whereas their dissolution leads to the production of partially soluble metal oxides. Hence, dissolution and sulfidation process result in a great effect on NPs surfaces properties. The sulfidation processes in class B metal induce their aggregation due to the formation of an insoluble metal sulfide on their surface, which changes the particle surface charge. On the other hand, the toxicity of class B metals NP is documented to be induced by their dissolution, which cause the release of toxic cations and reduce their persistence [91]. For instance, dissolution and sulfidation processes have a great impact on NP surface properties, persistence and toxicity. Adsorption of inorganic and organic ligands and macromolecules on NPs change significantly the behavior and surface chemistry of NPs. Thus, the stability, charge and dissolution of NPs are greatly affected by the adsorption of organic ligands having thiol groups. Furthermore, polymer coating adsorption to NPs increases their mobility and thus their removal from drinking water [92, 93].

Moving to the possible NPs physical transformations occurred when the form of aggregation and agglomeration may take place at all stages of NPs life cycle. Theoretically, aggregation is an irreversible process that binds particles together through electrostatic

forces or strong chemical bonds, leading to a decrease in surface area. There are two different form of aggregation: Homoaggregation, which occurs between the same kind of NPs and heteroaggregation, which oppositely take place between NP and other surrounding particles in the environment affect in turn ROS generation or dissolution [90]. On the contrary, agglomeration is a reversible process binding particles together by van der Waals forces without affecting the surface area since particles remain as individual entities [94-96]. Agglomeration occurs when the attractive van der Waals forces become stronger than the repulsive electrostatic forces [97, 98]. It is initiated by the presence of divalent ions with high ionic strength, at pH close to a point of zero charge affected by the photocatalytic reactions caused by the sunlight [97, 99]. It is known that the Brownian movement or random movement of nanoparticles is the dominant force enhancing nanoparticles agglomeration whereas the gravity forces induce their sedimentation [100,101].

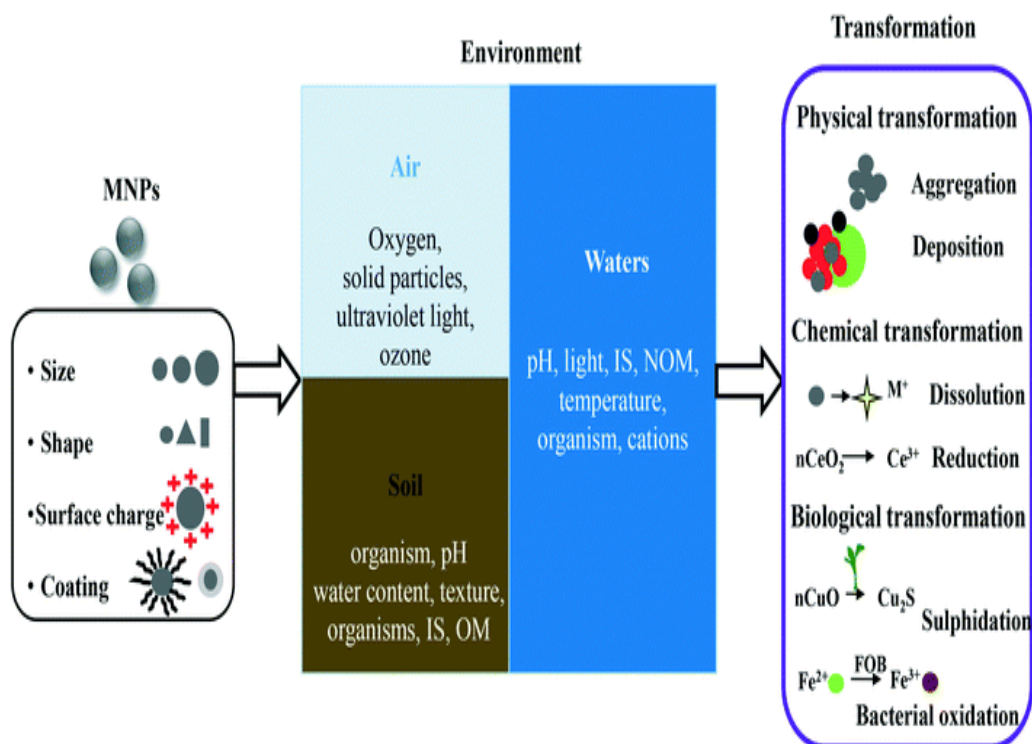
Biological transformations is the last kind of transformations that may affect nanoparticles is the biological transformation. It is inevitable especially when nanoparticles are in contact with living organisms. The uptake of these nanoparticles into living organisms occurs through different processes such as the redox reactions, changes related to core/coating of nanoparticles or by the interactions with other molecules. All these transformations will change the reactivity, toxicity, surface charge, and aggregation state properties of nanoparticles [100]. It was established that the fundamental redox reactions in bacteria such as *Geobacter* and *Shewanella* results in the reduction of Ag<sup>+</sup> from solution and the production of nanoscale silver particles [102]. Furthermore, it was reported that the biotransformation of the bioavailable poly (ethylene glycol) (PEG) coatings on engineered



NPs initiate their aggregation [103]. At this end, NPs transformation processes rely mainly on the chemical environmental characteristics of the receiving water body [44].

#### **1.3.2.2 Ag NPs in freshwaters**

AgNPs reactions is probably short due to their reactions and highly affinity of Ag to link with inorganic and organic molecules particular those containing thiol groups [104]. The fate and behavior of AgNPs can be influenced by different water conditions. The study by Liu et al., [105] demonstrated that the presence natural organic matter (NOM) had a significant effect on AgNPs stability and toxicity but the addition of  $\text{Ca}^{2+}$  in the media, AgNPs stability and toxicity decline significantly may be because of the NPs aggregation. Some of research studies have been explored the impact of particular environmental conditions on the assessment of AgNPs toxicity in aquatic living systems. In addition, Römer and colleagues [107], have been determined in their result the stability of AgNPs in OECD media (used for toxicity tests in *D. magna*). The result showed confirmation that standard OECD media drive aggregation of AgNPs, which is result in altered in organism exposure levels and the nature of the exposed particles compared to exposure to fully dispersed particles. In many microbe's studies toxicity AgNPs was assessed as the percentage of inhibition of microbial activity. The oxidation reactions that release  $\text{Ag}^+$  from AgNPs needs dissolved oxygen and protons. The rate of ion release can be increased through temperature elevation ( $37^\circ\text{C}$ ) and can be decreased by increasing pH or by the addition of Humic or Fulvic acids [106, 107]. Even though in the environment, beneath high concentration of dissolved oxygen, AgNPs cannot persist as individual particles due to the transformation to  $\text{Ag}^+$ ; however, the slow rate of the oxidation process may provide



**Figure 1.5** Transformations of NPs in the environment. NPs may undergo physically, chemically and biologically mediated transformations in the air, soils and waters. The physicochemical properties of MNPs, together with environmental factors, determine the type of transformation processes. Zhang et al., [235].

enough time for the AgNPs to reach biological targets by various pathways such as ingestion or endocytic/phagocytic activity [108].

#### **1.3.2.3 Ag NPs in marine waters**

Altering the physicochemical parameters in marine water such as temperature, salinity, and dissolved oxygen, can change the outline of AgNPs and alter their aggregation state. Thus, perhaps due to the higher ionic strength of saltwater (in comparison to freshwater) AgNPs display several negative impacts and toxicity [109]. Sodium, magnesium, calcium, and potassium cations and chlorides, sulphate, and bicarbonate anions, are the main ionic components of seawater. Among them, the sodium and chloride concentrations are higher. Stuart et al., [110] reported that the complete aggregation of AgNPs in artificial seawater (salinity 31 psu, with 487 mM NaCl) occurred in 47 minutes. Moreover, it was also reported growth of AgNPs of 40 nm in artificial seawater and the formation of aggregates larger than 400 nm [111,112]. A study by Gomes, Araújo, et al., [112] posted an acceleration in the formation of larger AgNPs in seawater (salinity 36.3 psu), particles' size ranging from 97 to 690 nm. Meyer et al. AgNPs aggregation and suspension stability in different salinities –deionized, estuarine (17 psu), and marine (33 psu) waters– during 7 days have displayed increased aggregation at higher salinities [113]. The results of the investigation on the instability of carboxylate–AgNPs in the presence of Na<sup>+</sup> or K<sup>+</sup> revealed that the presence of AgNPs with salt (NaCl or KCl) could change the pH in the aqueous media [114]. Changes in pH values were more pronounced in the case of NaCl, showing different interactions of Na<sup>+</sup> and K<sup>+</sup> with Ag NPs in aqueous media [115]. NOM can increase ENMs stability extending their residence time in the water column and consequently increasing the exposure of aquatic biota, including benthic organisms [116].

NOM can stabilize ENMs by the formation of a coating, which can involve a complex combination of electrostatic forces and steric effects between NOM and ENMs surface [117]. The majority of authors reported that NOM increase ENMs stability even in the presence of high concentrations of salts such as NaCl. However, this often was not observed in the presence of divalent ions at levels exceeding the critical coagulation concentration [118]. Divalent species can assist in the formation of complexes between humic substances and ENMs promoting aggregation/agglomeration [119]. Several authors demonstrated that the presence of NOM resulted in lower toxicity to the majority of the organisms, as shown by Grillo et.al [119].

Despite the numerous reports on the behavior of Ag NPs in saltwater. The data on AgNPs toxicity to marine species are scarce [120]. There is a wide literature record on the negative impacts and toxicity for freshwater species. However, the behavior of AgNPs is proved to differ between freshwater and the marine environment being impossible to use the data reported for freshwater to infer the toxicity in marine species [121]. Even undergoing rapid aggregation and sedimentation in saltwater, Ag NPs might be a threat for biological communities in marine ecosystems. AgNPs aggregate and sediment, but can also be trapped to the organic surface micro layers, thus posing risks to both benthic species and to zooplankton, including the early life stages of many organisms [122]. AgNPs toxicity is particularly challenging for early life stages, since they may not be as resilient as the adults may due to this vulnerability, and as recruitment cohorts are the foundation for any population success, the assessment of the toxicity of AgNPs to marine embryos and larvae is of primary importance [122].

## 1.4 Nanotoxicity

AgNPs are the one of the most extensively used metal nanoparticles. They are encompassed in various consumer products along with in medical applications owing to their antibacterial activities. The toxicity of AgNPs will be in a straight line correlated with their characteristics such as shape, size, concentration, aggregation, chemical coating, surface charge, and the processes used for their synthesis (biological, physical or chemical routes), [122,123]. The toxicity of AgNPs may also vary depend on the test organism or type species which is related to the defense mechanisms that their produced to prevent any threats. In addition, toxicity AgNPs to organism tests will be influenced by the media in which the organisms are exposed [124]. As in terrestrial ecosystems, AgNPs can accumulate and interact with other constituents of the water, inducing significant transformations of the nanoparticle. These processes may drastically change the properties of AgNPs: reactivity, mobility, bioavailability, uptake and ecotoxicity [125]. In aquatic environment, the main exposure routes are by contact or ingestion, due to sorption to phytoplankton/zooplankton, transfer from water to sediment and uptake in benthic organisms, which can then be directly ingested by larger vertebrates such as fish [126]. The mechanisms that are directed the toxicity effects of Ag NPS still under investigation [128-130]. The hypothesis behind NPs toxic in small size because of generate radical oxygen species (ROS) and inflammation compared to larger-size particles [131-133]. Another aspect of nanotoxicity of Ag NPs is associated with formation of a biomolecular layer on their surface, referred to as eco-corona. This is happened through van der Waals, electrostatic, hydrogen bonding and hydrophilic/hydrophobic interactions, biomolecules such as lipids, sugars and especially proteins, will bind to the surface of NPs once in contact

with biological fluids<sup>1</sup>[134,135]. Composition of protein corona highly important because of it might affect agglomeration, toxic kinetics, signaling, and ultimately, toxicity [135]. In such as an example, negatively charged NPs will appeal positively charged biomolecules, which in sequence will increase interactions with the negatively charged cell membrane [136]. In this way, this as well has implications for deducing nanotoxicity results across different organisms' models and exposure scenarios [136].

### **1.5 Risks of ENPs in the aquatic environment**

Risk assessment of NPs is a procedure to predict and to evaluate the probability that potential release of NPs. At present, there is a lot of consideration have pave the way to potential impacts of such NPs on humans health and the environment. The risk of NPs is determined by both their effect and their exposure. In another ward, the risk is just the product of NPs hazard and exposure [137]. Hazards are the biological, chemical or physical stressor that can have an adverse effect on organisms or environment [138]. When a hazard is determine the exposure, which is the magnitude of contact that a receptor has with a hazard [138], need to be determine. Besides the exposure, also the effect, which is the biological response of an organism, population, or ecological system to a stressor, needs to be determined [138]. In order to facilitate quantitative environmental risk assessment procedures need the development of techniques to measure and characterize NPs in aquatic and terrestrial environments is an important. Therefore, the predicted environmental concentration (PEC) is based on modal. Consequently, the environmental risk, can be quantify by the PEC/PNEC ratios [139]. This is general pattern of risk assessment has been identified as equally applicable to ENPs as to conventional categories of environmental contaminants [140]. However, classic approaches used in aquatic ecological risk

assessments may be less applicable to NPs [141], since exposure assessments have been depended on predicting the soluble portion of the contaminant. Moreover, it has been assumed that the predominant bioavailable portion of the total contaminant was the soluble form [142]. These assumptions and approaches must be taken with caution and modified to deal with the issues of particle fate and behavior, bioavailability, and toxicity that are central to quantitative ecological risk assessment of NPs. In fact, environmental chemists, toxicologists, and risk assessors might be well served to preface research on NPs with a primer on colloid fate, behavior, and toxicity (for a comprehensive review of colloids and other particles, see Wilkinson and Lead [142]). As noted previously, characterization of NPs fate and behavior in the environment is needed to quantify exposure scenarios. Related to this, differences in speciation (between dissolved, colloidal, and particulate phases) due to dissolution and aggregation of NPs under environmental conditions are also important. It is worth noting here that these are characteristics usually unfamiliar to most environmental toxicologists, chemists, and risk assessors, although they have now been progressively addressed in the context of toxicology and ecotoxicology of nanomaterials [143]. Clearly, the need for interdisciplinary collaboration among biologists, chemists, physicists, and material scientists is essential.

## **1.6 Importance of bivalve molluscs as target organisms for toxicological and environmental studies**

Bivalve's molluscs a group belong of the one largest invertebrate phyla, the Mollusca [144,145]. They are soft bodied and, in the main, have a prominent shell to protect the inner body mass. Bivalves are mostly aquatic and their habitats range from the deep ocean through to the water surface. A significant ecological impact of bivalves because of

multiple impacts such as ecological significant role of bivalves, biogeochemical cycling, bivalves as filter feeders, Environmental remediation, and Bivalves as indicators of metal pollution, many of toxicology studies used bivalves as “biomonitoring tools”. Bivalves are useful “biomonitor” and ecotoxicology study species due to their propensity to bioaccumulate environmental contaminants [146,147]. As filter feeders, they process large volumes of water and thus exert a considerable impact on their environment [148]. Their filter-feeding ability adds greatly to their ecological significance in that bivalves are important calcium and carbon accumulators, they link primary producers (bacteria and phytoplankton) with higher organisms in aquatic food chains and are responsible in tidal zones for filtration of the water body [149,150,152]. The process of filtering particles from the environment is initiated when water passes across the gills. It has been estimated that one kilogram of bivalves (multiple sizes) will filter 180 liters of water per hour [152]. Filter feeding affects both the water column from which food and other suspended particles are removed, and the sediment to which faeces and pseudofaeces are deposited. Pseudofaeces are undigested particles that are rejected by the bivalves. The filtering activity of bivalves is influenced by the size of the organism, phytoplankton concentration, and the size and quality of the food/suspended particles. Abiotic factors such as temperature, salinity and water flow can also affect filtration rates [153].

### **1.7 Research objectives and hypothesis**

The objectives of the study focusing on the following:

1. To synthesize and well characterize AgNPs in environmental relevant concentrations.
2. To investigate AgNPs fate and behavior in the prescne clams and without in synthetic seawater and natural seawater.



3. To quantify uptake, accumulation, AgNPs and ionic Ag in clams.
4. To emphasize that AgNPs to be toxic to juvenile bivalve hard clam *M. mercenaria* in both natural filtered seawater (NFSW) and synthetic seawater (SSW).

By investigating these objectives, the following research hypotheses will testing:

Hypothesis 1: Size of AgNPs will significantly increase in both synthetic seawater and natural seawater.

Hypothesis 2: Transformation and aggregation NPs will drive the fate and behavior AgNPs in both seawater types.

Hypothesis 3: Clam will uptake ionic Ag dissolved from NPs in both types of seawater.

Hypothesis 4: Toxicity of Cit. AgNPs and PVP will be enhanced in (SSW) more than (NFSW).

## **1.8 Dissertation overview**

### **Chapter 1: General introduction**

This first Chapter reviews the subject of NPs as a new chemical threat, including its main properties, a brief description of the AgNPs, synthesis characterization, and applications, then describe the challenges of emissions and behavior of AgNPs in the environment. In addition, the importance use clam *M. mercenaria* as excellent bivalve species in ecotoxicological studies.

### **Chapter 2: Secondary data about physio-chemo characteristics, and toxicity AgNPs**

Review studies of AgNPs in the bivalve mollusk hard clam *M. mercenaria* to explore the way of uptake, target tissues and toxicity of AgNPs in clam species. Secondary data

analysis will use from review literature studies to explain the potential impact Ag NPs on Marine Bivalve Mollusks to assess the risk of Ag NPs to organism and human health.

### **Chapter 3: Characterization AgNPs in exposure media: Comparison study**

In this chapter, the synthesis and characterization of Ag NPs are reported in ultrapure water and in natural and synthetic seawater by using multiple characterization techniques mainly using transmission electron microscope (TEM) to characterize the NPs and relate their behavior in the aquatic environment with the biological effects observed in acute exposure and chronic exposure.

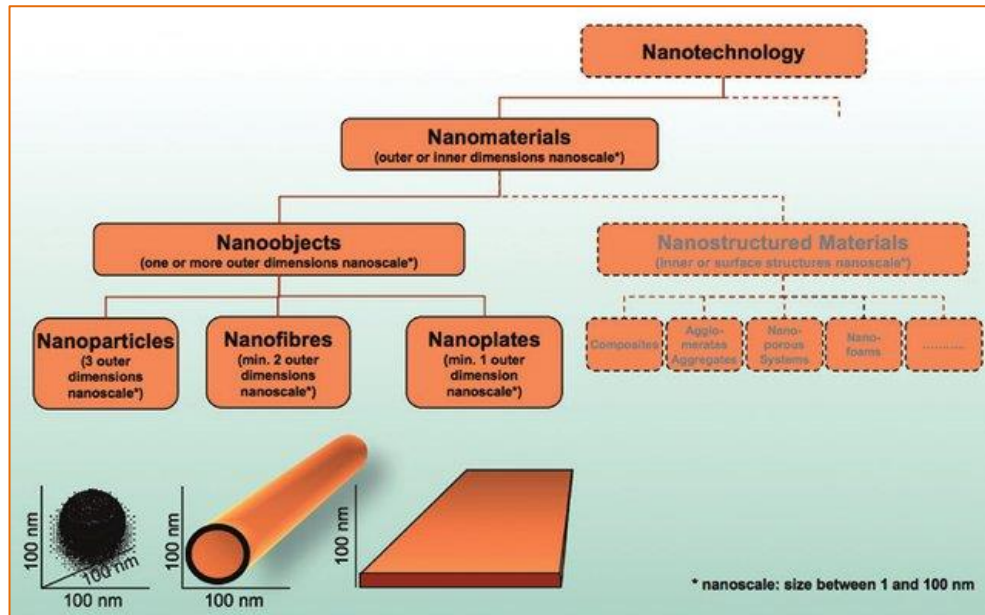
### **Chapter 4: Uptake, accumulation, and toxicity of AgNPs on marine bivalve molluscs hard clam *M.mercenaria***

The aim of this Chapter was to address the bioavailability, uptake, accumulation and effects of AgNPs in clam *M. Mercenaria* and to identify concentration range of AgNPs toxicity. For this purpose, the effects of AgNPs were studied of clam exposed to an environmental realistic concentration (1-100  $\mu\text{g. L}^{-1}$ ) of AgNPs for 24h and 7 days and compared to that of Ag<sup>+</sup>. The result were evaluated with complete characterization of AgNPs by using transmission electron microscopy and dynamic light scattering were also used to characterize the NPs and relate their behaviour in the aquatic environment with the biological effects observed in acute exposure and chronic exposure.

### **Chapter 5: Overall conclusion**

In this chapter will summarize the research work and conclude the data with diction the future research work that will need to solve the challenges counter nanotoxicological

studies practically in environmental relevant conditions and discuss likely impact increase used nanoparticles in varies of consumer products.



**Figure 1.6** Nanostructured materials in nanoscale: a size of between 1 and 100 nm. Harald F. Krug, [149].

## CHAPTER 2

### HOW DO SILVER NANOPARTICLES INFLUENCE UPTAKE, BIOACCUMULATION, AND TOXICITY IN MARINE BIVALVE MOLLUSCS CLAM SPECIES AS A MODEL ORGANISM?

#### **2.1. Introduction**

Bivalves such as (e.g., mussels, clams, oysters, and scallops). They are marine and freshwater molluscs with two hinged shells. Bivalves are a main constituent of the benthic organisms of many marine and estuarine areas. They represent an important food supplier to other aquatic species and human [155]. Bivalve species widely distribution around the globe in a variety of environments [156, 157]. There are numerous potential ecological drivers of bivalve distributions; salinity [158], sediment composition [159], temperature [160], water flow [161], larval transport [162], and chemical pollutants [163,164] have earlier been linked to bivalve occurrence, abundance, and growth. Bivalves are largely filter feeders, although some species, feed on detritus (i.e., deposit or pedal feeding). Most bivalve species are mostly sedentary as adults, with movement recognized for foraging, reproduction, and predator escape. Bivalves be able to combine benthic and pelagic systems by filtering particles and by excreting and depositing nutrients. [155, 164].

Understanding bivalve feeding physiology is a requirement to quantifying their ecosystem impacts. Bivalves feed on suspended particles (i.e., filtration), sediment (i.e., pedal or deposit feeding), or a mixture. Consumption occurs via an aperture, siphon, or proboscides, then cilia and mucus move particles toward the labial palps and stomach [155]. Bivalves display alteration in filtration rate, preferential filtration, and pre-ingestion sorting based on factors such as particle size, shape, surface chemistry, and filtrate composition. In mucus, rejected particles encapsulated and expelled as pseudofeces before ingestion. Post-ingestion sorting also happens. Less nutritious substances are moved to the intestine for quick egestion as feces, and higher quality nourishing material goes to the diverticula for complete digestion [155]. Collectively, feces, and pseudofeces repackage nutrients and are termed biodeposits. Therefore, even if bivalves are mainly stationary, their selective filtration, ingestion, and digestion choices are equivalent to foraging by mobile animals, with significant ecological consequences [165]. Bivalves are filter feeder, which cleared suspended particles such as organic matter and fine particles from the water column in the regular time cycle [166]. As a result, the quantity of energy available for bivalve's growth based greatly on filtration rate and suspended organic matter concentration [167]. The wide range of filtration rate (2–3552 ml/h) is recorded between bivalve's species [166, 168]. The scientific literature documented that bivalve able to consume more than 95% of the phytoplankton within 24 h [169]. The evaluation of filtration rate is useful to detect optimal conditions of bivalve's culture [170]. Salinity is an important abiotic parameter that able to affect the filtration rate in bivalves. The initial response of bivalves is valve closure when exposed to near their salinity tolerance limits

and reduced filtration of surrounding water column [171]. Furthermore, a decline in energy input was also observed when filtration rate reduced [172].

Bivalves as bio-filters for chemicals, and pathogens, and because of they are sessile and widely distributed, there is growing interest in using them in programs for monitoring [173] and removal of contaminants (i.e., bio- extraction) [174]. In this manner, animals are cultured then, removed to harvest contaminants, similar to the process of nutrient removal via shellfish harvest. As example, Asian clams (*Corbicula fluminea*) accumulated metals in water from acid mine drainage effluent [175]. Bioaccumulated contaminants, which are harvested from bivalves, have been considered is the one component in the toolbox of ecosystem managers for sustainable water remediation and merits further study.

### **2.1.2 Bivalve's molluscus hard clam *M. mercenaria* as a module test study**

The hard clam, *M. mercenaria*, burrows shallowly in sediments of either mud or sand [176]. It is the one of the most commercially important species of invertebrate. Similar to other bivalve's species, it is a filter feeder. The quahog, or hard clam, are found along the entire eastern coast of the U.S.A and into the Gulf of Mexico. Salinity is a critical factor for surviving hard clams, the ranges of salinity from 12.8 to 35 psu [177-179]. The limitations in hard clam's survival depend on the correlation between salinity and the length of exposure because of salinity is highly variable extend with the time of exposure. As the result of Burrell et al., [180], the study showed the mortality of less than 5 percent in clams exposed to salinities lower than 10 psu for periods of 2 and 3 weeks. This result of this behavior makes challenges to allocate the hard clam with a set lethal salinity level. Therefore, salinity tolerances should be deal by way of a range not a specific point. The

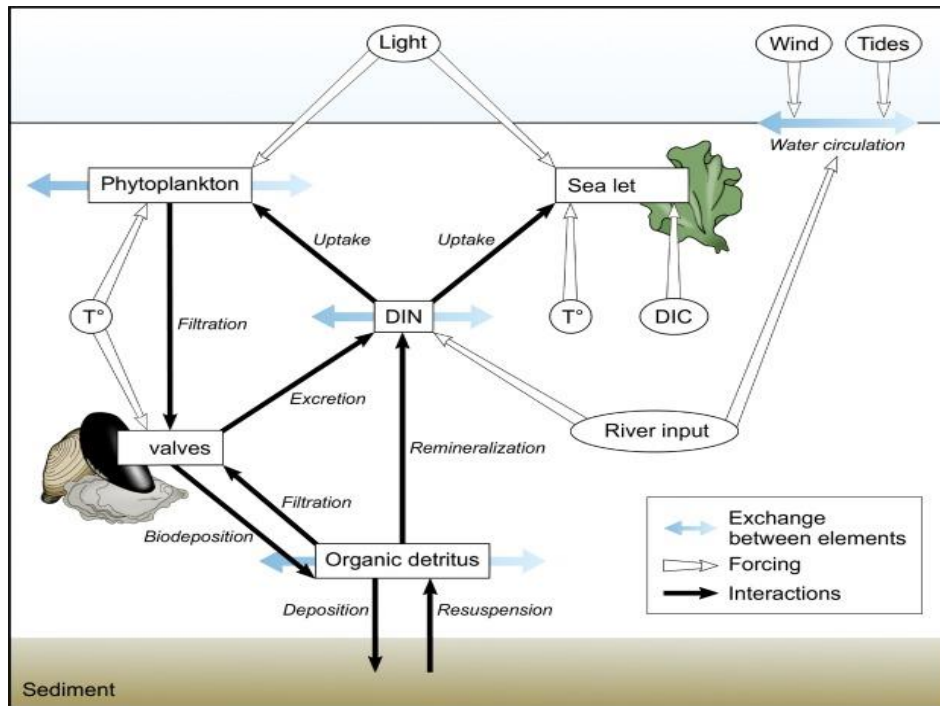
best development founded for clam larvae at salinity 20 psu or above [181] Lowering salinities to 17.5 psu or less will affect larvae development and lead to death occurs. Hard clams feed primarily on single celled algae and diatoms, which are taken in by the inhalant siphon, filtered over the gills, and eventually passed to the mouth via ciliary tracts. Hard clam *M. Mercenaria* has limited locomotion in that it is able to burrow via use of its muscular foot; however, they are generally sedentary if left undisturbed. In terms of aquaculture, hard clams account for a large percentage of total aquaculture production in Florida, ranking third in dollar value behind tropical fishes and aquatic plants.

### **2.1.3 Bivalve molluscs as a suitable group for NP toxicity**

Bivalve molluscs in particular, may represent an appropriate group for NPs toxicology. These organisms have very much advanced processes for the cellular internalization of NPs and micro-scale particles, endocytosis and phagocytosis, respectively, that are essential to key physiological functions such as intracellular digestion and cellular immunity. These organisms have been long recognized as valuable indicators of pollution, and extensive background information is now available on their biological responses to a wide range of both inorganic and organic chemicals [182, 183]. In particular, the mussel *Mytilus* spp., abundant in coastal marine and estuarine environments, can represent a suitable model organism for characterizing the potential impact of NPs. In *Mytilus*, food particles trapped by the gill sieve are moved towards the labial palps and the mouth thus entering the gut, and reaching the digestive gland, where digestion occurs. Digestive cells have an extremely developed lysosomal system for intra-cellular digestion and nutrient accumulation for gametogenesis [184]. Actually, the first indication of interactions of NPs with bivalve cells was the observation of endosomal and lysosomal accumulation and oxy radical production

following endocytosis of Nile red labeled sucrose polyester nanoparticles in isolated digestive gland cells [185]. In purpose to accomplish wealth knowledge, will explorer the toxicological effects of Ag NPs on the clam species and other bivalves by using data from the scientific literature studies to explore the toxic effects of Ag NPs particularly on bivalve mullscus clam's species. In order to use these data will help to fill the gap surrounding the broader aspects of bivalve filter feeding interaction with AgNPs. The toxic effect of nanoparticle depends on its physical state, solubility, and bioavailability to the organism. It has been shown that nanoparticle in contact with aqueous medium undergo a variety of modifications like agglomeration, state of surface oxidation, dissolution and each state of the particle may have different toxicological reactions. It requires excessive efforts by using the expensive methodology to understand the phenomenon of each state of modification [183]. To do more investigation on how the NPs proprties influencing uptake and toxicity bivalves group particularly clams species will expolore the effect NPs physical and chmical properties AgNPs on their behavior and toxicity to clam species to undersdand their impact on uptake, bioaccumulation, and to toxicity AgNPs to aquatic organisms.





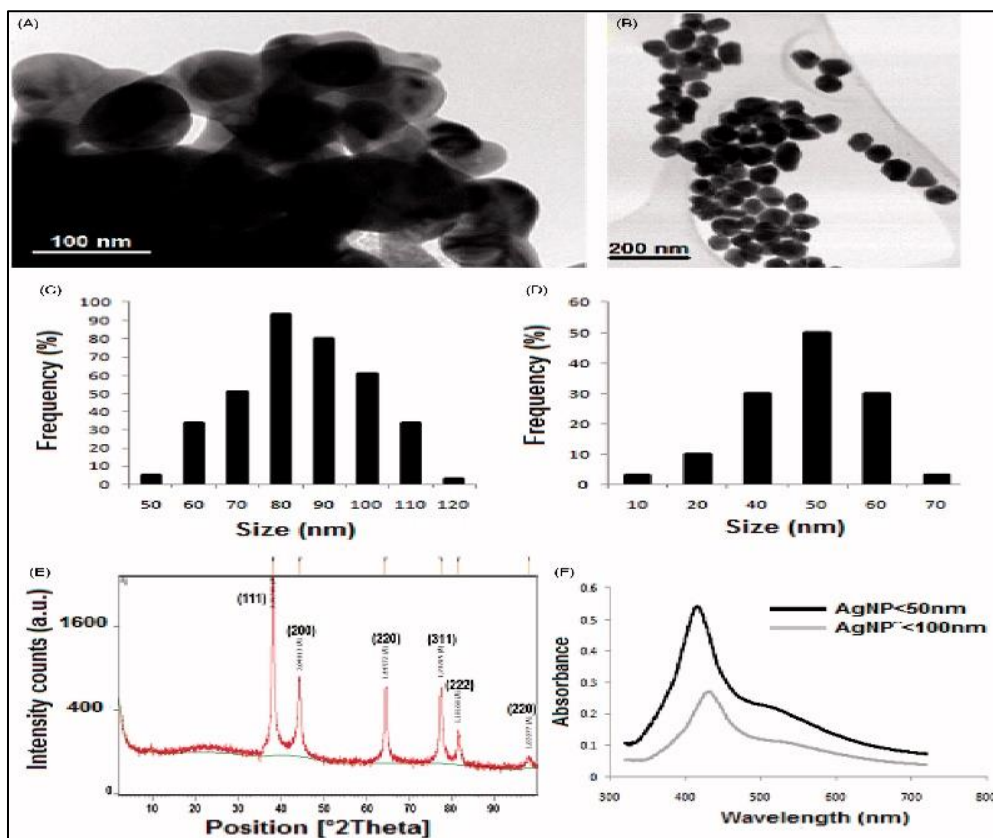
**Figure 2.1** Conceptual diagram of the major processes governing sub-model interactions in the ecosystem model. Lavaud et al. [172].

## **2.2 Ag NPs properties and toxicity to clams species**

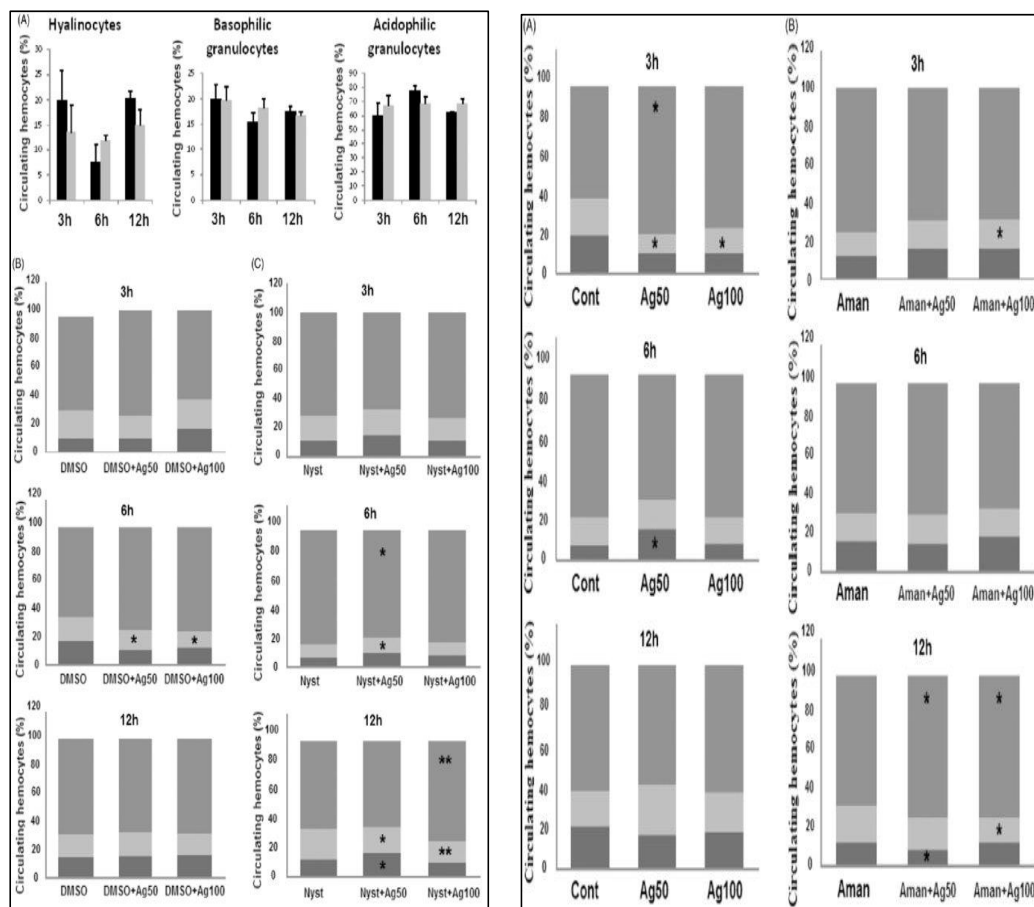
### **2.2.1 NPs size effects**

Toxicity of NPs might be affected by different factors including NPs size, which is highly important to be measured to insure the stability of NPs. Depend on NPs size, the level of cellular uptake or interaction with biological cells will be determine [185]. The smaller size of the particle has more biological influences and toxicity due to their ability to reach and translocate easier than larger particles in biological systems [186]. With decrease, size NPs resulted in increased surface area of the NPs, which is another important factor, leads to the number of particles per mass unit that are exposed to tissues or cells [187]. A few studies were published in purpose to assess the effect nano scale of Ag on bivalve's species in past decades. The study by Bouallegui et al. [188], which is investigated effects from two size types of silver nanoparticles (Ag NP: 50 and 100 nm) on the frequency of hemocytes subpopulations as immunomodulation biomarkers exposed in a mollusk host. This study was performed using exposures prior to and after inhibition of potential NP uptake pathways (i.e. clathrin- and caveolae-mediated endocytosis) and over different durations of exposure (3, 6 and 12 h). The result expressed existing differential hemocyte counts (DHC) revealed significant variations in frequency of different immune cells in mussels exposed for 3 hr. to either Ag NP size. However, as exposure duration progressed cell levels were subsequently differentially altered depending on particle size (i.e. no significant effects after 3 h with larger Ag NPs). The results also indicated significant decreases in basophil, which is a type of white blood cell levels with host exposures for 3 h to either size AgNPs (but no significant variations with 6- and 12-h exposures) and a significant increase in hyalinocytes levels only with AgNP50

for 6 h. In this study, the reasonable choice to have used Ag NP with sizes of <50 and <100 nm was based on the literature on potential uptake pathways for each size particle. Typical clathrin-coated pits vessels for clathrin-mediated endocytosis) have diameters in the range 120 nm; conversely, internalization via caveolae-mediated endocytosis is considered the predominant mechanism of entry for structures of 40–50 nm (and below in diameter. Thus, while effects on clathrin-mediated endocytosis would reflect how the cells interacted with both size AgNPs here, any impact of exposure on caveolae-mediated endocytosis would then be more directly impactful upon the AgNPs <50 nm only [186-187,189]. Obviously, keeping with this assumption, an Ag NP size-dependent effect variation in the percentages of cell categories was in fact observed in the study. Other studies also reported size-dependent toxicity of AgNPs, that is, with maltose-stabilized AgNPs [188]. In that study, small NP (Ag20-Mal) were significantly more toxic than larger NP (Ag40-Mal and Ag100-Mal). Such outcomes were expected based on the concept proposed by Hine [190], that is posited differences in phagocytosis between granulocytes and hyalinocytes were related to characteristics of the involved particles (i.e. differences in size properties here) rather than differences in immune cell ability to phagocytize/process the particles.



**Figure 2.2** (A) TEM image of AgNP shows homogenous distribution in size (average size  $\approx 50$  nm). (B) Histogram of size (diameter) distribution for AgNP < 50 nm. (C) XRD pattern of AgNP powder. (D) Size UV-Vis absorption spectra of PVP-coated AgNP dissolved in MiliQ water. Narrow peak confirms the size of the particles. Bouallegui et al., [188].



**Figure 2.3** Variations in circulating hemocyte sub-populations (%) as marker of immunomodulation from AgNP. (A) Exposure to only AgNP. (B) Exposure to AgNP and Amantadine. Data shown are percentages. Hyalinocytes (dark grey), basophilic granulocytes (light grey), acidophilic granulocytes (medium grey), Cont: untreated, Aman: amantadine, Ag50: AgNP < 50 nm, Ag100: AgNP < 100 nm for 3, 6 or 12 h. N  $\frac{1}{4}$  10/group. Value significantly different from negative control [ $\tilde{A}$  p < 0.05]. Bouallegui et al., [188].

### 2.2.2 NPs shape effects

Different synthesis processes result diverse shapes of Ag-NPs e.g. spherical, triangular, square, cubic, rectangular, rod, oval and flower. From the nano-toxicological point of view, it is unknown whether particle shape has any significant effect on the biological system. This might depend on multiple factors rather than one. There are gaps in data regarding effects Ag NPs shape on its toxicity particularly their influences on bivalve clams' species. Therefore, will use other types of nanoparticles to explore impact shapes of NPs on organism. The study by Abtahi et al., [191], the result demonstrated increase clearance rate with increase size. The AR 8 of gold nanoparticles (Au NPs) had the highest clearance rate ( $=0.064 \text{ h}^{-1}$ ) among all tested nanoparticles despite having a smaller equivalent spherical diameter than the 45 nm AuNPs. The AR4 AuNRs followed the same trend with a 25% higher clearance rate compared to 19 nm Au NPs. A clear pattern relating clearance rates and shape-related nanoparticle size was observed if these rates are sorted based on the longest axis (nanoscale feature) of these nanoparticles rather than the apparent spherical size. Accordingly, these results suggest that not only size, but also shape-related features such as AR play an important role in the filtration of nanoparticles by *C. fluminea*. *C. fluminea* is generally known as a non-selective filter-feeder with a filtration mechanism that is mainly based on the quantity and the size of particulates rather than quality [192, 193]. Filter feeders such as *C. fluminea* take in and expel water through siphons for feeding and respiration. The water is pushed through the organism by the collective movement of millions of hairs like fibers called cilia on the gills. Simultaneously, cilia strain food (mainly particles) from the influent water and transport it into the clam [193].

### 2.2.3 NPs concentration effects

The mass concentration of NPs is another important factor affecting toxicity. It is critical to determine the minimum concentration level of NPs that induces toxicity and its variation in different subjects. Mostly, Ag NPs showed cytotoxicity in a concentration dependent manner. The concentration range of NPs that can induce toxicity depends on the particle size, type of medium, temperature, surface functionalization, and particle crystallinity [194]. In the study Cleveland et al. [196], the concentration of Ag in hard clams were determined by ICP-MS, clams in the three CP-treated, 20 nm Ag NP-treated, and 80 nm Ag NP-treated systems. The result showed that the concentrations of silver in the clam tissues reached maxima between the 13 d and 30 d sampling times, and remained elevated compared to the blank clams for the duration of the study, and no significant amounts of silver relative to the blank were found in those clams at any sampling time. This result was unexpected because the Ag<sup>+</sup>-treated clams began unexpectedly coming to the surface of the sand after 7 d of exposure. It was estimated that the Ag<sup>+</sup> treated clam mortality rate was approximately 90% after 13 d and 100% after 60 d also as if these effects were not observed for any of the other compartments, or treatments, except for the 20 nm Ag NP tank. In that tank, only a few clams had come to the surface of the sand after 60 d. These results support the fact that hard clams feed by filtering suspended particulate matter like algae and detritus in the water through their gills [195]. Zhuo et al., [197] who is performed study to determine metal nanoparticles and concentration in clam and oyster tissues. In this work, the concentrations and particle size distributions of MNPs and concentrations of associated metal ionic species in shellfish seafood (clams and oysters) were investigated using single particle inductively coupled plasma mass spectrometry (SP-

ICP-MS) and inductively coupled plasma mass spectrometry (ICP-MS). The MNPs in the clam and oyster tissues were extracted via an alkaline digestion method with a recovery rate of 95.9% for gold nanoparticles (AuNPs). Then total concentrations of 41 metal elements were measured in the two types of seafood, of which 20 were selected for (SP-ICP-MS) analysis. In the three-clam sample tested, nanoparticles were detected in two. A detailed distribution of metal nanoparticles, including particle mass, proportion and concentration size are shown in (Table 2.1) The results showed that 5 types of MNPs were detectable in clams (Y, La, Ce, Pr, Gd) and 5 types of MNPs were detectable in oysters (Y, La, Ce, Pr, Nd). Size distributions of MNPs in clams and oysters were in the range of 35–55 nm and 30–65 nm, respectively. Nanoparticle concentrations in clams and oysters ranged from 0.6 to 37.7 ng/g and 4.2–19.7 ng/g and accounted for 3.4%–50% and 5.5%–46% of the total metal content, respectively. Based on this analysis, the health risks of metals in the two kinds of seafood were evaluated by comparing the Provisional Tolerable Weekly Intake (PTWI) with limits recommended by the World Health Organization (WHO)/Food and Agriculture Organization (FAO). These results provide important information about the presence of metal nanoparticles in seafood and, to the best of our knowledge, this is the first time that the nanoparticles of rare earth elements have been detected and reported in bivalve molluscs tissues. In the study, the total concentrations of 41 varies of elements were categories as the following: (1) the common metals (Al, Ti, V, Mn, Fe, Co, Ni, Cu, Zn, Mo). The toxic elements (Cr, Ga, Ge, As, Se, Sr, Cd, Sn, Sb, Ba, Hg). (2) The rare earth elements (Y, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu). (3) Group of others (Ag, W, Pt, Au, Bi) were measured in the clam and oyster samples using ICP-MS. The ten types of common metal elements (ranging from 347.2 to



53709.3 ng/g and 84.0–101681.7 ng/g). The nine types of toxic elements (ranging from 21.2 to 12802.3 ng/g and 5.0–5385.1 ng/g). Others the fifteen types of rare earth elements (ranging from 1.1 to 284.4 ng/g and 1.7–127.3 ng/g) and three types of other elements (ranging from 17.3 to 181.0 ng/g and 3.7–293.2 ng/g) were detected in clam and oyster samples, respectively. Sm was only detected in clams (11.8 ng/g). Another seven elements (Sn, Sb, Tb, Lu, Pt, Hg and Bi) were below the detection limits in both types of seafood samples.

**Table 2.1** Mass concentration, particle mass ratio and particle size distribution in clams' samples by SP-ICPMS. Zhuo et al., [197]

Metal particles	Sample 1			Sample 2			Sample 3		
	Mass ng/g	Mass ratio 100	Size (nm)	Mass (ng/g)	Mass ratio 100	Size (nm)	Mass (ng/g)	Mass ratio 100%	Size (nm)
Y	ND	ND	ND	2.4	3.4	42 ± 5	9.7	6.1	38 ± 5
La	ND	ND	ND	21.6	7.0	40 ± 4	34.6	27.1	41 ± 4
Ce	ND	ND	ND	5.9	8.8	46 ± 4	37.7	11.0	51 ± 4
Pr	ND	ND	ND	0.6	4.0	40 ± 5	4.1	9.2	44 ± 5
Gd	ND	ND	ND	ND	ND	ND	2.1	5.6	44 ± 2

## 2.2.4 Coating effects

Surface modifications of AgNPs are introduced in the attempt to provide additional properties such as optimized persistence, toxicity and interaction with biological targets such as microorganisms. Hence, the coatings could also influence the bioavailability and

toxicity of Ag NPs in addition to the size and form. Coating AgNPs by addition capping agents to produce electrostatic as well as electrostatic repulsions between particles, which further helps to stabilize the NPs. The type of coating depends on the capping agent properties such as organic capping agents (polysaccharides, citrates, polymers, proteins, NOM, etc.) and inorganic capping agents (sulfide, chloride, borate, and carbonate). Since the capping material plays a role in maintaining the surface chemistry of Ag NPs by stabilizing. Thus, in this section, we discuss the possible effects of Ag NP coatings on their toxicological phenomena. Ag NPs-induced cytotoxicity may vary depending on several factors including the type of coating materials. Usually the processes involved in toxicity induction involve ROS generation, depletion of antioxidant defense systems, and loss of mitochondrial membrane potential. Surface coating of Ag NPs can affect shape, aggregation, and dissolution ratio. However, the method and extent of Ag NPs toxicity varies based on the coating materials. For example, chitosan-derived polysaccharide-coated Ag NPs showed antimicrobial activity with no toxicity to eukaryotic cells [204]. An investigation on the influence of surface coatings of silver NPs on the bioavailability and toxicity to *Elliptio complanata* mussels by (Auclair et al., [199]. The purpose of this study was to determine if the selected coatings of silver nanoparticles (AgNPs) could influence the fate, bioavailability, and toxicity toward suspension feeding freshwater mussels, *Elliptio complanata*. Mussels were exposed for 96 h to 50 µg/L of Ag NP with the following surface coatings: citrate, silicate (Si), polyvinylpyrrolidone (PVP), and branched polyethylenimine (bPEI). The bioavailability of AgNP was examined in mussel digestive gland and gill tissues (Figures 2.4]. After the exposure period, mussels were analyzed for total Ag, resistance to air emersion, oxidative stress, genotoxicity, and autophagosome

protein uptake (protein ubiquitinylation) in gills and digestive glands. The data revealed that citrate- and PVP-coated Ag NP were 2 times more abundant in the digestive gland compared to bPEI- and Sicoated Ag NP with estimated bioaccumulation factors between 5 and 10. The data revealed that tissue Ag levels were closely associated with air survival time, weight loss during air exposure, DNA strand breaks, LPO, and protein-ubiquitin levels in the digestive gland. The data supports the hypothesis that the coatings could influence bioavailability and toxicity in freshwater mussels.

### **2.2.6 Transformation**

Once NPs enter the environment, NPs undertake more than one transformation process that might dictate their biotic interaction and toxicological effects. A highly complexity of these physical and chemical processes that would be made difficult to estimate the fate NPs in environment. For example, the affinity of nanoparticles (NPs) to biological surface can be altered as a function of the conditions in the surrounding medium, such as pH, ionic strength or presence of colloids. In general, the fate and transformation of Ag NPs in environmental circumstances were influenced by their inherent properties, environmental factors (pH, dissolved oxygen, natural organic matter, and sulfide) etc., [200]. Several of current studies showed that Ag NPs likely experienced oxidative dissolution to release  $\text{Ag}^+$ , adsorption of NOM, reactions with sulfur species or chloride, or aggregation [201].

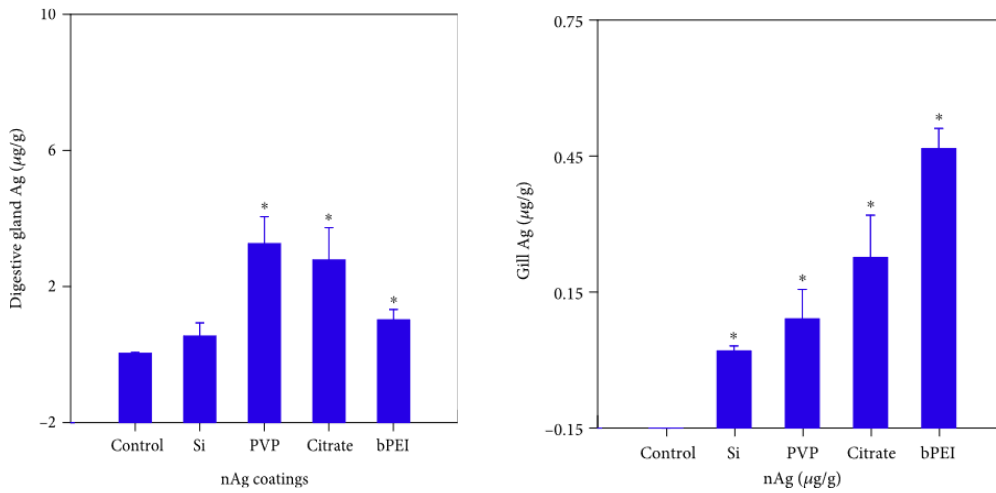
In this study, a sharp decline in Ag amount and zeta potential value happened in the 3rd day in both control and experimental groups, which means probability of settling Ag NPs into sediment. One of the artificial ecosystem experiments documented that Ag NPs go through rapid oxidative dissolution at initial 12 h, and then dissolved silver contents

reached a highest concentration [202]. Hence, it could be indicated that Ag NPs possibly occurred to aggregation in the form of Ag<sup>+</sup> complexes or Ag NPs aggregates [203]. The second sharp drop of Ag concentrations and the increase in absolute value of zeta potential in experimental groups suggest that Ag NPs might be settle into sediment again. Zeta potential gradually decrease and Ag amount no longer decreased in control groups, which is means that the main transformation form of Ag NPs could be oxidative dissolution, thus amounts of released Ag<sup>+</sup> existed in the water phase with an increase of cations. Thus, can be conclude from the above contrasting result that the *C. fluminea* affected the fate and transformation of Ag NPs and promoted the sedimentation of Ag NPs. After bivalves overcoming the adaptive phase, behaviors of bivalves probably promoted the movement of Ag NPs to sediment.

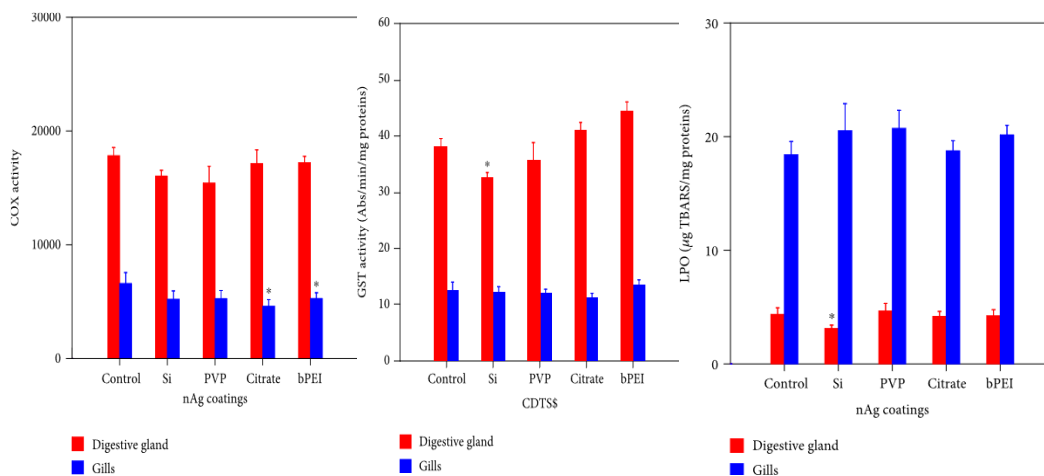
### **2.2.5 Dissolution**

Dissolution Ag NPs is also critical in uptake and toxicity to aquatic organisms, it found that transformation of NPs occurred in the environment and inside organism [294]. His study concludes that Ag<sup>+</sup> release from Ag NPs is a cooperative oxidation process requiring both protons and dissolved O<sub>2</sub>. The primary particle size, shape, surface coating and concentration can control NPs dissolution [202-295]. Furthermore, the effects of environmental factors such as dissolved oxygen, pH, ionic strength, chloride and dissolved organic carbon on the dissolution of NPs were interpreted [206, 207]. In addition, physical processes such as aggregation were found to become the potential factor controlling silver release in the natural surface water [207]. Not like Ag NPs, gold NPs are resistant to oxidative dissolution and release of dissolved Au ions, whereas another characteristic is that they are present at low natural background concentrations [207-212]. The effect

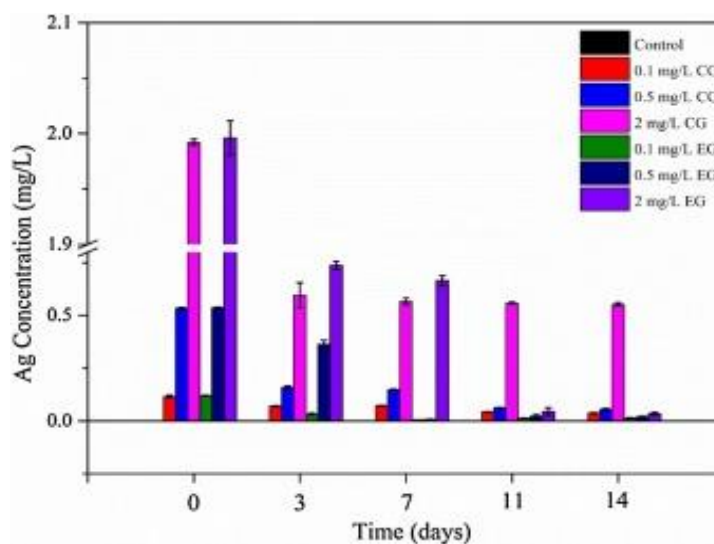
dissolution Ag NPs on marine bivalve the clam *Ruditapes philippinarum* has been studied by Aouini et.al [322]. The result showed that Ag dissolution rates over 48h from Ag NP are shown in (Fig.2.7), values increased over the time from 20.67% at  $t = 0h$  to 29.09% at  $t = 48h$ . In several studies, it has been demonstrated an important mechanism of AgNPs toxicity in marine waters is related to Ag dissolution from AgNPs, which has a relationship with the size and exposed surface of the NPs [213, 214].



**Figure 2.4** Bioavailability of AgNP of different coatings in the digestive gland and gills of freshwater mussels. Mussels were exposed to four coatings of nAg for 96 h at 15°C. Total Ag was determined in the digestive gland and gills indicates significance from controls. Auclair et al., [199].



**Figure 2.5** Oxidative stress and damage of coated Ag NPs in mussels. Mussels were exposed to 50  $\mu$ g/L Ag NPs with different coatings for 96 h. The activities in COX (a) and GST (b) and LPO levels (c) were determined in the digestive gland (DG) and gills. indicates a significant effect between controls in the digestive gland and gills. Auclair et al., [199].

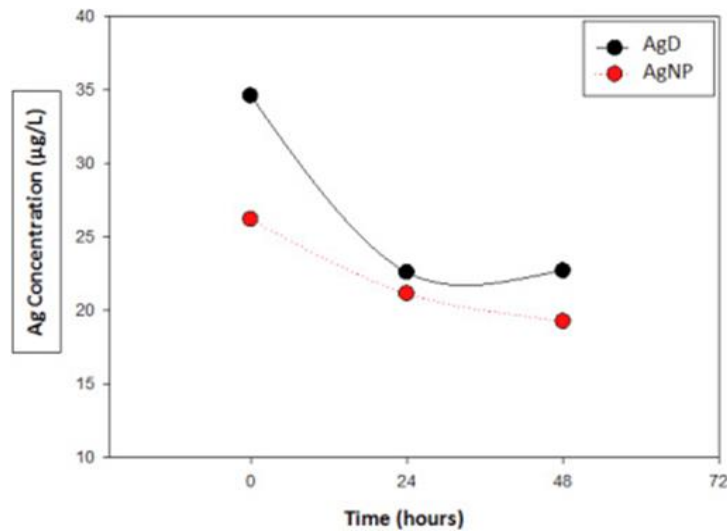


**Figure 2.6** Concentration of Ag in the upper water after exposure to the four experimental conditions (0.0, 0.1, 0.5, and 2  $\text{mg}\cdot\text{L}^{-1}$  Ag NPs). CG, EG represent control groups, experimental groups respectively. Liu et al., [212].

### 2.2.7 Aggregation NPs

Agglomeration NPs impacts have high potential to aggregate or agglomerate in solution. Dependent on diffusion, gravitation, and convection forces, the interaction potentiality of NPs with cells [215, 216]. Several factors might affect the agglomeration process that there are the pH, electrolyte or salt content, and protein composition in the culture medium [217]. Number of studies showed that the binding capability of NPs with protein is diverse depend on the composition of both the NPs and protein [218-220]. Agglomeration states of Ag NPs in medium depend on treatment preparation. A study by Lankoff et al., [221], which is illustrated that 20 nm and 200 nm-sized Ag-NPs aggregated in culture medium, and the aggregation range changed depending on the NPs suspension preparation. The hydrodynamic diameter of Ag-NPs could be larger than the nominal size of the particles depends on the suspension preparation [222]. As a final point, more aggregated particles revealed fewer effects on the cellular level [222]. In addition, cellular localization of NPs might rely on the agglomeration states of the NPs [223]. For instance, under the same conditions, AgNPs look as if to aggregate very loosely compared with TiO<sub>2</sub> NPs. Therefore, AgNPs were observed in the cytoplasm, nucleus, and mitochondria with a slight agglomeration whereas clusters of agglomerated TiO<sub>2</sub> were mainly distributed in the vacuole [224]. This occurs because intracellular localization of AgNPs and TiO<sub>2</sub> NPs depends on the interaction of the particles with protein and DNA inside the cell, which also initiates toxicity [225]. AgNPs have a high agglomeration tendency in culture medium because of their high surface area [226]. This agglomeration may induce toxicity rather than the ionic metal-induced toxicity. Sometimes, aggregation plays a role in the various types of intracellular responses. Hence, from the point of view of toxicological interest, it

is very important to know how agglomeration or aggregation states of NPs affect different biological responses [225, 226]. Like other NPs, agglomeration is a common phenomenon observed for AgNPs. As agglomeration and aggregation are barriers to cytotoxicity measurement, usually a different surface coating is used on the NP surface. However, the surface coating materials, such as organic (citrate, PVP) and inorganic coatings (sulfide, chloride), potentially interfere with cytotoxicity measurements [226]. In addition, easy penetration of agglomerated Ag-NPs into mesenchymal stem cells and the nuclei made evident by several studies [227, 228].



**Figure. 2.7** Ag concentration in tanks exposed to dissolved silver (AgD) and silver nanoparticles (AgNPs) for 48 h. Aouini et.al [322].



## 2.3 Uptake and bioaccumulation Ag NPs by clam sp.

### 2.3.1 Uptake Ag NPs

Many studies have been investigated probability of those filter-feeding mollusk species are likely ingest ENPs released to the environment, particularly if the nanoparticles associate with natural particles. Bivalves filter feeding may accumulate contaminants absorb to suspended particles and sediment [229, 230]. Bivalves such as clams filter food from water that passing over their gills moving food particles to the mouth. NPs might be trapped and ingest with suspended detritus and compromised the food [59]. Benthic marine bivalves such as the blue mussel (*Mytilus edulis*) might absorb ingested NPs by endocytosis [230]. Uptake Ag NPs by filter feeding bivalves mollusks has not fully investigated and the rate of uptake likely is depend on the amount of water passing over gills in which animal and the proportion of the Ag NPs trapped by the gill lamella. Juvenile clams (*M. mercenaria*) managed to accumulate over 5% of the Au NPs despite only accounting for 0.01% by mass, again underlining filter feeders as organisms linked to potential high uptake of NPs and subsequent biotransformation [196]. In this study, filter-feeding juvenile hard clams (*M. mercenaria*) in NP and CP treatments maintained high tissue concentrations of Ag, however clams exposed to ionic Ag showed no body concentration, yet after only thirteen days 90% of these had surfaced and died. Adsorption of silver from the sand, rather than direct uptake, was believed to be responsible for the mortality. The sand itself contained no significantly higher burden of silver compared to the controls; the highest burdens recorded were associated with CPs, most likely through constant leaching (82–99% over sixty days). This study highlights the variation of effects on different species not only by different delivery methods of silver (ionic, NP, CP), but

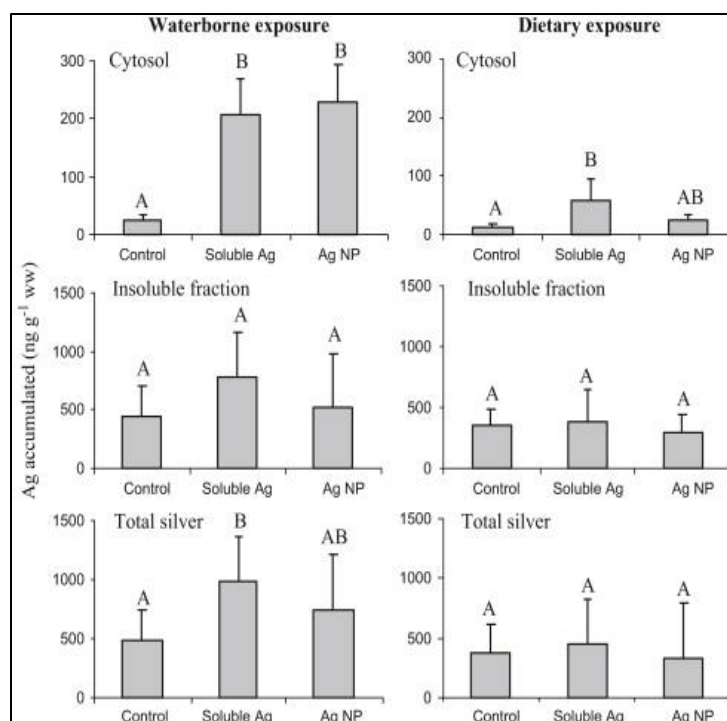
also that size of the NP, dissolution, and the manufacturing process of CPs will make a difference to the availability and effects of NPs in marine systems.

### **2.3.2 Bioaccumulation of Ag NPs nanoparticles by clam's species**

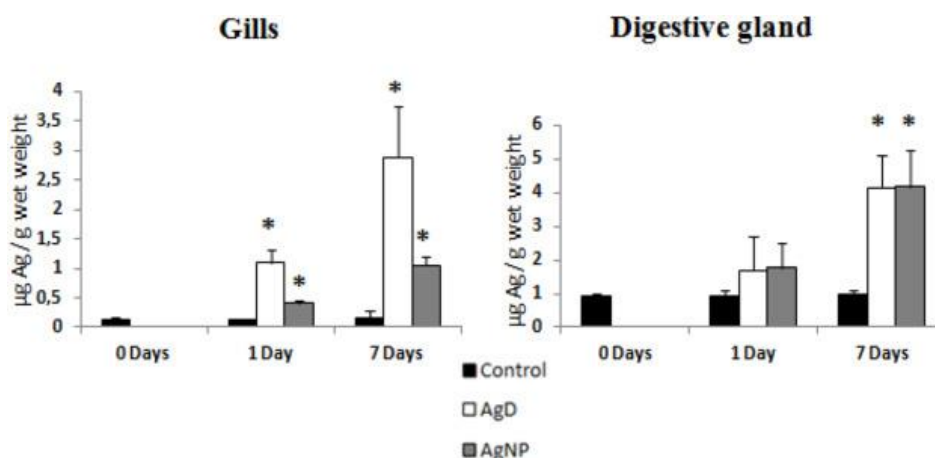
Bioaccumulation is an important process that links exposure and toxicity of contaminants to organism [231]. It is potential to assess risk exposure from Ag NPs. Bioaccumulation and is precursor of Ag NPs toxicity [17]. Bioavailability is that Ag ions possible animal can take it up from media or food [227]. The most basic considerations of bioaccumulation and bioavailability is whether Ag NP penetrate the cell or remain on the cell surfaces can cause damage. All living cells organisms are surrounding with a lipid plasma membrane. Plasma membrane is a complex structure that limited enter materials into cells. However, it has structures and mechanisms promote entry molecules such as the pores and protein carriers. Endocytosis is the one translocator process [23]. As we describe above by formation of vesicles that enclose the material and the transport of the vesicle into cell [23]. Besides endocytosis a second possible routes is association Ag NP with the surface of the membrane and the release of free metal ions within the surface layers. Ag NPs can continuously provide Ag<sup>+</sup> directly to cells with association on the cell surface. This may result in nano-environment of high Ag<sup>+</sup> content on the cells surface and finally cause toxicity [235]. Ag NPs can also act as carriers to transport Ag<sup>+</sup> or other metal ions in the medium to targeted cells since AgNPs have strong adsorption properties. This can be used by ionic delivery systems for drugs or for measurement of metal concentrations [196, 226]. So far, these mechanisms have not examined in organisms yet, and more studies are still required. The bioaccumulation of AgNPs in bivalves may be influenced by combination of several factors, including the concentration, NPs nature, exposure routes,

the nature of the environment, and other biological and ecological functional involved Buffet et al., [287].

In the study were conducted by Aouini et al., [322], accumulation of Ag was observed significantly in the gill and gland tissues of exposed clams as explained in Figure [2.9]. The result showed that Ag accumulated significantly ( $p < 0.05$ ) in the gill and the digestive gland tissues of exposed clams (Fig. 2.9). The clams that are exposed to AgD accumulated in their gills higher Ag contents compared to those exposed to Ag NPs (2.6 and 2.7-fold higher at day 1 and day 7 respectively). Consequently, the Ag increased with the time of exposure with both Ag forms in the exposed groups. Therefore, increasing in the Ag contents with the time of exposure with both Ag forms in the exposed groups. Where results from previous studies, founded like this finding. These studies suggested that a higher bioavailability of Ag correlated to incorporate potential Ag dissolution, possibly associated to exist of Ag chloro-complexes and it play an important role in Ag accumulation in organisms [229-211]. In addition, gills in bivalves play critical role in Ag accumulation [59]. Because of they may act as a filter for nanoparticles due to the presence of aggregates and accordingly a lower accumulation of Ag from the AgNPs form with respect to the AgD form. In contrast, in the digestive gland were no significant difference in the Ag bioavailability between AgD and AgNPs of the exposed groups founded. It could be related to the existence of some similarities between Ag forms [322], which lead to their accumulation in the same way in tissues. Additionally, the digestive gland accumulated Ag rate in both forms (1.4–4.3) fold higher than the gills. The higher uptake rate of digestive gland considered a key role in bioaccumulation and detoxification under Ag exposur [322].



**Figure. 2.8** Bioaccumulated concentrations of Ag in the completely soft tissues of clams after 14 days of waterborne or dietary exposure to soluble or nanoparticle forms. Bars with different superscripts correspond to significant differences (Mann and Whitney,  $p < 0.05$ ).



**Figure 2.9** Bioaccumulation Ag in *Ruditapes philippinarum* exposed to dissolved silver (AgD) and silver nanoparticles (AgNPs) for 7 days. Data are given as mean  $\pm$  standard deviation. Asterisks mean a significant difference ( $p < 0.05$ ) between exposure and respective control in each sampling time as measured by one-way ANOVA. Aouini et al., [322].

Tough the study by Gomes et al., [284]. , which is referred that the differences the origin of differential uptake between tissues to the tissue-specific functions, to the redox requirements and to the different modes. In addition, it has been suggested that NPs are preferentially accumulated in the organisms via capture and ingestion, which makes the digestive gland the main tissue for NPs uptake [243]. Similar findings were reported with a higher uptake of contaminants in the digestive glands of marine invertebrates, not only exposed to AgNPs but also to CuO NPs [112] and Au NPs [241], favored by the presence of aggregates [250].

### **2.3.3 Toxicity Ag NPs in clam sp.**

Studies with various exposure scenario were conducting to evaluate toxicity Ag NPs for example, clam *Scrobicularia plana* tested by Buffet et al., [287]. He examined the uptake and effect of Ag ions or as lactate-coated Ag NPs of 40 nm) at the concentration of  $10 \mu\text{g L}^{-1}$  in the organisms exposed to the contaminants directly (water) or via the diet (microalgae). The authors showed that for both forms of Ag, bioaccumulation was much more relevant for waterborne than for dietary exposure. The response of oxidative stress biomarkers (catalase, glutathione S-transferase, superoxide dismutase) was significantly high after dietary than waterborne exposure to Ag (soluble and NPs). The result also showed that was no effect for burrowing in bivalves exposed directly or through the diet to both Ag forms but feeding behavior was impaired. Since no differences of responses to Ag either soluble or nanoparticulate were observed, it seemed that labile Ag released from Ag NPs was mainly responsible for toxicity. The same authors Buffet et al., [287], exposed the same bivalves to the same concentration of Ag NPs. This study demonstrated a bioaccumulation of either Ag NP or their ionic forms. With concerning a biomarker

response, both soluble and nanoparticle Ag forms, induced defenses against oxidative stress, detoxification, apoptosis, genotoxicity and immunomodulation. Nevertheless, DNA damages in the digestive gland of *S. plana*, and Phenoloxidase were higher in the presence of Ag NPs compared to soluble Ag suggesting a specific nano effect. Another clam species (*Sphaerium corneum*) was used to investigate the chronic effects of Ag NPs [223]. On the other hand, not always the toxic effect was detected when bivalves were exposed to AgNPs. This is the case of deposit-feeder clam, *Macoma balthica*, which was reared in sediments spiked with Ag NPs in different forms (aqueous ions, nanoparticles, and micrometer-sized particles) at 150–200  $\mu\text{g g}^{-1}$  concentrations. Authors evidenced that seawater reduced Ag bioavailability, mainly complexed by chlorides. Dai et al., [230] exposed the clam *Macoma balthica* to Ag spiked sediment, at 200  $\mu\text{g/g}$  for 35d, observing burrowing activity delays and condition index general decrease. A form dependent uptake of Ag was also observed, decreasing with increasing particle size. Effect of Ag NP on *Scrobicularia plana* was investigated by Buffet et al., [287] after the waterborne exposure at 0.01 mg/L of Ag NP for 21 d. Ag accumulation was detected in soft tissues (250 ng/g) like changes in stress related biomarkers. Reduced burrowing kinetics were observed as significant DNA damage in gill and DG. Toxicity effects of silver nanoparticles (AgNPs) on the freshwater bivalve *Corbicula fluminea* (*C. fluminea*) were investigated through experiments. In this study, *C. fluminea* promoted the sedimentation of AgNPs and affected the fate and transformation of AgNPs. A series of biomarkers of *C. fluminea in vivo* were evaluated after 14 days exposure to various doses (0–2  $\text{mg L}^{-1}$ ) of polyvinyl pyrrolidone (PVP) coated AgNPs. The levels of antioxidants increased obviously in 2  $\text{mg L}^{-1}$  AgNPs treatments to protect *C. fluminea* from oxidative damage. Glutathione peroxidase (GPx) and glutathione (GSH)

played important roles in tissues detoxification in 0.1 and 0.5 mg L<sup>-1</sup> exposure, respectively. The biological behaviors (feeding rate, ammonia excretion rate) were inhibited at 0.1 mg L<sup>-1</sup>, induced at 0.5 mg L<sup>-1</sup>, and inhibited again at 2 mg L<sup>-1</sup> AgNPs, which indicated that AgNPs influenced the physiological metabolism of *C. fluminea*. Ag contents in tissue and shell of *C. fluminea* were much higher than blank groups. In addition, *in vivo* tissues were more sensitive to low AgNPs concentration compared with shells, indicating that *C. fluminea* could be used as a good indicator for AgNPs freshwater pollution. No Ag was detected in feces probably implying that nanoparticles had long gut retention time in *C. fluminea*. Overall, this study reveals the interactions between AgNPs and *C. fluminea* and provides important implications about the fate and toxicity of AgNPs in natural aquatic environment. In order to use multiple biochemical biomarkers (superoxide dismutase, catalase and glutathione reductase activity, lipid peroxidation and metallothionein to assess the impact dissolved silver (AgD) and AgNPs in *R. philippinarum*, the organisms were exposed to 20 µg L<sup>-1</sup> of AgD and AgNPs (15 nm) over 7 days. The study results for biochemical biomarkers provoked a general increase in the integrated biomarker response index (IBR) values) indicating the induction of oxidative stress in the clams exposed to both Ag treatments. Therefore, the presence of Ag forms at the tested concentration in the aquatic medium represent a risk for *R. philippinarum*. Aouini et al. [322] aquatic medium represent a risk for *R. philippinarum*. Aouini et al., [322].

## 2.4 Environmental Impact on nontoxicity Ag to calm Species.

When NPs entering aquatic systems, the extent of aggregation/agglomeration, stabilization and settling of ENPs will determine their environmental impact, as well as the characteristics of the environmental matrix itself [231-233]. As we mention in chapter one the rate of NPs aggregation/agglomeration and sedimentation depend upon concentration, surface area and forces involved in collision, but variations in NOM, pH, ionic strength and surfactant present in fresh and marine waters will have a substantial influence on these phenomena [232]. In the case of NPs sedimentation, horizontal transport in the water column is reduced while local exposure to NPs can increase. After sedimentation, benthic bivalve species are important targets for accumulation and toxicity of NPs, as observed for the clam *Scrobicularia plana* exposed to AgNPs (40, 50 nm; 10 mg L<sup>-1</sup>) in sediment for short exposure time. Furthermore, bioturbation and resuspension in the sediments can lead to an increase of NPs concentration in the sediment-water interface, promoting particle exchange between the sediment and water column, potentially enhancing the bioaccumulation and impact of NPs. Furthermore, NPs and climate change as noted earlier, pH, ionic strength and composition, NOM, temperature, and nanoparticle concentration all interact to affect aggregation or stabilization of Ag NPs [234]. Although the advance on knowledge regarding the impacts of climate change and Ag NPs to aquatic organisms, still significant scientific uncertainties remain in understanding and ultimately predicting the long-term consequences arising from sustained modifications of climate change related factors together with pollution from contaminants of emerging concern. The understanding on the chemical nature of the exposure medium is fundamental in determining bioavailability and a consequent toxicity in exposed organisms. In this



perspective, the influence of salinity (15 vs 30) in the fate and toxicity of AgNPs towards the estuarine bivalve *S. plana* has been recently investigated [204]. The authors showed that at lower salinity Ag was more available for the organisms. At lower salinity, the biological effects of Ag were enhanced inducing apoptosis and oxidative stress and reducing energetic reserves and finally burrowing activities. The study by Dia, et al., [230], which is investigated effects, uptake, and Depuration Kinetics of silver oxide and copper oxide nanoparticles in a Marine Deposit Feeder, clam *Macoma balthica*. The result illustrated that Cu uptake and depuration kinetics were studied in more detail yielding net uptake rates ( $\mu\text{g Cu/g dw soft tissue/d}$ ) in soft tissue of 0.640, 0.464, and 0.091 for sediment spiked with aqueous Cu ions, CuO nanoparticle,s and micrometer-sized CuO particles, respectively, supporting that net uptake was dependent on form. Depuration rate constants ( $d^{-1}$ ) from soft tissue were  $-0.074$ ,  $-0.030$ , and  $0.019$  for Cu added to sediment as aqueous Cu ions, CuO nanoparticles, and micrometer-sized CuO particles, respectively. Ensuring sustainable use of nanotechnology requires the development of better methods for detecting and quantifying ENPs, particularly in sediment.

**Table 2.2:** Representative Studies using toxicity and bioaccumulation ends points of ionic silver and AgNPs.

Clam species	NPs	Size (nm)	Exposure	Con.	Effects	Ref.
Juvenile hard clams <i>Mercenaria mercenaria</i> .	CPs Ag NPs,	20 nm, 80 nm	Mesocosm for 60 d.	620 $\mu\text{g. L}^{-1}$	Uptake Ag NPs leaching from CPs .Ag accumulation	Cleveland et.al. [196].
<i>Macoma balthica</i> clam	Ag, CuO	20, 80 nm Ag 2–3.5 $\mu\text{m}$	Sediment spiked with Ag and Cu in for 35d	150 - 200 $\mu\text{g/g}$	Mortality, behavioral index.No genotoxicity observed. Bioaccumulation of both Ag and Cu.	Dai et al. [230].
<i>Scrobicularia. Plana</i> clam	Ag	40-45 nm	Mesocosm in natural seawater and sediment. 21 d	10 $\mu\text{gL}^{-1}$	Bioaccumulation Ag Nps and genotoxicity	Buffet et al. [287].
<i>Sphaerium corneum</i> clam	Ag, $\text{AgNO}_3$	15 nm	Fresh water 28 d	0–500 $\mu\text{g.L}^{-1}$	Oxidative stress and effects on reproduction	Volker, et al. [223].
<i>Ruditapes decussatus</i> clam	Ag	25 nm	Exposed clam in seawater for 7 days.	1, 2.5, and 5 $\text{mg/L}^{-1}$	Deterioration of the gills due to oxidative stress	Hidouri et al., [224].
<i>Ruditapes philippinarum</i> clam	Ag	9.19 nm	Humic acid (HA), Ag NPs toxicity.	10 $\mu\text{g.L}^{-1}$	Acetylcholines terase activity, oxidative stress response.	Tingwan, et al., [223].
<i>Corbicula fluminea</i> clam	Ag	27.66 $\pm$ 0.80nm	Natural sediments exposed for14 d	0-2 $\text{mg.L}^{-1}$	Biological effects	Liu, et al., [215]
<i>Ruditapes philippinarum</i> clam	Ag D and AgNPs	15nm	Exposure clam for 7 d	20 $\mu\text{gL}^{-1}$	Ag accumulated and oxidative stress	Aouini et al., [225].

## CHAPTER 3

### CHARACTERAZATION AND PROPERTIES OF CITRATE AND PVP COATED SILVER NANOPARTICLES IN NATURAL AND SYNTHETIC WATER

#### 3.1 Abstract

The wide use of silver nanoparticles (AgNPs) has created concerns about their potential impacts on the natural aquatic environment. In this study, the physicochemical properties of AgNPs and implication for this on their toxicity on Juvenile hard clam *Mercenaria mercenaria* (Chapter 4) were investigated in natural filtered seawater (NFSW) and synthetic seawater (SSW). The characterization data showed that AgNPs had some transformation in both seawater types and the average size of AgNPs in NFSW and SSW was significant different from their primary size in ultrapure water (UPHW). The result by TEM images showed significant change in size of Cit. AgNPs in SSW. The median of sizes was (28 nm, interquartile range (IQR) =14.48) at 0.0 h, and (43.92 nm, IQR = 39.73) at 24 h, the  $p < 0.05$ . While PVP in the same seawater type showed significant increase in sizes between 0.0 h and 24 h time. PVP- AgNPs showed median in sizes in (range 22.23 nm, IQR= 6.37 at 0.0 h to 30.27 nm, IQR = 13.36 at 24 h), the  $p > 0.05$ . Which is indicated a

non-significant change in sizes compared with PVP-AgNPs sizes in UTPW media. In NFSW media, both Cit. AgNPs and PVP- AgNPs exhibit changes in size distributions compared with their sizes in UTPW. Cit. AgNPs median sizes in NFSW showed significant change from (20.74 nm, IQR = 4.55 at time 0.0 h to 35.46 nm, IQR = 28.15 at 24h), the  $p < 0.05$  in both times. Whereas, PVP-AgNPs showed increase in size between (21.17 nm, IQR= 4.86 at time 0.0 h to 32.9 nm, IQR= 23.49 nm, IQR= 5.91 at time 24 h). This result indicated that AgNPs transformed in both seawaters were occurred and the highly ionic strength of both seawaters affected Cit. AgNPs behavior in media.

### **3.2 Introduction**

AgNPs is the one of the common used in industrial and scientific applications due to their strong antimicrobial properties [248-250], their products including include fabrics, cleaning products, paints, and food packaging [251, 252]. The frequent increasing use of AgNPs and their release into the environment makes it important to determine in what quantitates they are occurred, and to understand their fate and behavior in aquatic system. AgNPs have a special form of metallic silver having less than 100 nm size in at least one dimension. The nanoscale offers silver nanoparticles a high surface area to volume ratio [242, 91]. In narrowing size, AgNPs is became highly stable, monodispersed, and not aggregated. However, possible alteration might occurred to the novel physiochemical properties when they subjected to environmental matrices. Therefore, characterization NPs is essential to monitor possible transformations of NPs in environment [243]. It is well understood that analyzing the physicochemical properties of NPs gives rise to substantial challenges, and this difficult is exacerbated by complex media such as high ionic strength and natural organic matter (NOM) [244, 245]. Due to these challenges in the detection NPs

in complex media, with environmentally low concentrations. This study aimed to synthesis AgNPs in-house and use a sophisticated instrument such as transmission electron microscopy (TEM) to define AgNPs with the commonly used analytical tool ultraviolet visible spectrometry (UV-vis). TEM uses high resolution and a beam of electrons to penetrate through a sample (e.g. NPs) preserved on a carbon fiber grid. When the beam passes through the grid and scatters, it changes wavelength and as a result, the size of the particle is determined based of the interference pattern [246]. The (UV-vis) measures the absorption of visible wavelength (400-700 nm), and will help determine the stability and distribution of NPs over time. The distinct optical properties of plasmonic NPs (e.g. Ag and Au) can be used for their detection and quantification in environmental systems [247, 248]. Analysis of NP extinction spectra can provide valuable information about NP size, structure, concentration and aggregation properties [249].

With the recent advances in analytical methods, recently many toxicological and environmental experiments have been performed at relevant environmental concentrations [220, 250-251]. In addition, the ability to assess NP fate in environmentally concentrations in complex media is important since these measurements would reflect the real exposure conditions of NPs and organism. To complete understanding the environmental impact of NPs it is critical to investigate the fate and behavior of these particles (i.e. any changes in their physicochemical characteristics) within relevant environmental concentrations. Gathering knowledge regarding release NP either where and amounts will possibly help to determine NP concentration in medium and inside aquatic organisms. However, with the advanced nanotechnologies still there is lacking of knowledge regarding fate and behavior of Ag NPs in environmentally relevant scenarios in addition, concentrations. These

limitations knowledge are due to lacking in availability of advanced instruments and techniques detected and differentiate NPs such as complex environment matrices. For example, DLS, which is the instrument, is only applicable in simple homogeneous systems at particle concentrations typically exceeding ( $1 \text{ mg L}^{-1}$ ); this is likely orders of magnitude higher than those environmentally relevant [252]. There are three major factors highlighted in the prediction Ag NP behavior in the environment, their likely fate after release, dissolution and basic ligand affinities dictated by their surrounding environment [114, 253-254]. For example, adsorption, which is a combination of physical, chemical, and electrostatic interactions, dictated by fate and environmental ligand affinities [255].

Understanding behavior NP such as transformation NP in relevant environmental concentration and after biological interaction becomes important as varying processes such as agglomeration and/ or aggregation, complexation and dissolution may dramatically change the eventual impact upon the environment and organism/ human health [253, 255]. Additionally, many of these parameters are closely related to physicochemical characteristics such as size and charge. Ultimately, these transformations will affect the NPs fate, transport and toxic properties within the organism. These transformations have been shown to be particle, media, environment and organism dependent. Thus, changes in release scenarios can change NP behavior [250, 256-259]. By studying these properties in relation to toxicity or environmental impact, it may be possible to derive certain NP physicochemical properties, which are the main drivers of the seen response. Deriving these common themes or predictors of NP toxicity, will help to understand the key pathways of exposure and toxicity and, as such, will aid the development of appropriate regulatory and risk mitigation frameworks. By focusing on relevant environmental

physicochemical characteristics, some advances are being made. Studies have previously noted that within freshwater systems organic matter and sulphides have a chemical affinity with silver and most likely dominate Ag speciation within this compartment. Studies that are more recent have confirmed this transformation in a study of Ag NPs within sewage sludge and the washing of varying nanotextiles [114, 253, 249-260]. Several studies have already conducting with ionic strength environments [260, 261]. Although these investigations have not clarified what occurs at environmentally relevant concentrations. Therefore, it is important to study AgNPs behavior when dispersed in aqueous matrices. Despite of recent advanced in this investigation; there is still a lack of available information regarding fate and behavior of silver nanoparticles in aqueous environmental and biologically relevant matrices. The major objective of this study was to determine the importance of well characterization of AgNPs in understanding effects high ionic strength of exposure media on the uptake and accumulation, and toxicity AgNPs to juvenile hard Clam *M. mercenaria*. To simulate two different surface characteristics onto homemade AgNPs, two gapping agents were used during synthesise AgNPs by using citrate as major surface capping agent and PVP. The transformation, aggregation behavior of two types of AgNPs with different matrices of seawater were used in biologically and environmentally relevant concentrations.

### **3.3 Methods and materials**

#### **3.3.2 Synthesis of AgNPs**

All Chemical were used in synthesis Citrate AgNPs (Cit. AgNPs) will be explained with details in Chapter 4. Cit. AgNPs were synthesized by using the standard reduction

method. The method is described in Tejamaya et.al. [254]. Briefly, silver nitrate ( $\text{AgNO}_3$ ) was reduced in trisodium citrate as it described in [272- 264]. Three separate solutions were prepared, the amount of 16.79 mg of  $\text{AgNO}_3$  added to 1.69 mL highly purity water UHPW. A trisodium citrate solution were prepared by adding 20.84 mg into 2.92 mL UHPW. The reducing agent solution of sodium borohydride ( $\text{NaBH}_4$ ) were prepared by adding 35.84 mg in 10 mL UHPW. Then, a dilute solution of  $\text{NaBH}_4$  was prepared by taking 1 mL of  $\text{NaBH}_4$  into 10 mL of (UHPW). The synthesis AgNPs were done by taking the two solutions of  $\text{AgNO}_3$  (16.79 mg in 1.69 mL) and trisodium citrate (20.84 mg into 2.92 mL) were mixed together while stirring vigorously and, the (0.5 mL) of  $\text{NaBH}_4$  (dilute solution) was added in a dropwise manner. This solution was added to 400 mL boiling water (UHPW). Then, the solution were keeping boiling at  $200\text{ }^\circ\text{C}$  for a further 30 minutes, and then left overnight to cool down in the dark at room temperature. To emphasize that AgNPs formed in solution, the peak absorbance at 400 nm were measured by UV-vis. Where the mean hydrodynamic size (z-average), polydispersity index (pdi), and zeta potential of the stock solutions were quantified by dynamic light scattering (DLS). The yield of this protocol in the range between (20-25 nm). AgNPs were washed to remove excess reactants, water were used in washing PVP coated AgNPs while the citrate solution were used to wash citrate coated AgNPs (Cit.AgNPs). Washing process done by using diafiltration technique, the ultrafiltration (3kDa cellulose membrane EMD Millipore) were used in filtration unit. Careful use of the washing technique is important to avoid NP changes due to drying and consequently, aggregation in the case of Cit.AgNPs were washed, as well as to remove any excess and unreacted  $\text{AgNO}_3$  ions or  $\text{NaBH}_4$ . When Cit. AgNPs washed, the cleaning process should be repeated at least three times by making a new solution of



trisodium citrate and added in each washing time to replace the amount of sodium citrate that is removed during each wash and to back to the original volume. Therefore, a citrate solution was used to reduce NPs aggregation due to re-equilibration and loss of surface citrate [268]. A ligand exchange approach [210, 262] was used to prepare PVP- AgNPs. A solution of polyvinylpyrrolidone (PVP) were prepared by adding a specific weight to a volume of highly purity water (UHPW). A (PVP10 (MW 10000, sigma Aldrich) were used. It well known that PVP is a non-toxic polymer [262, 265] used to sterically stabilize particles by strongly binding to the AgNPs core [262, 210 ] and protect them from dissolution and aggregation in complex media [264, 265 ]. Briefly, 200 mL cit. AgNPs were converted into PVP-AgNPs by adding 1 mL of 0.94 M PVP10 solution and vigorously stirring (e.g. 700 rpm) for at least 1 hour. This amount of PVP was required to obtain full surface coverage of AgNPs by PVP molecules to impart full steric stabilization.

### **3.3.3 AgNP stocks preparations**

All stocks were kept at 4°C in the dark. Silver nitrate (Ag NO<sub>3</sub> Sigma Aldrich, USA) was kept in the dark in powder form at room temperature; stock solutions were freshly prepared in ultrapure water. AgNPs stock suspension concentrations were checked using ICPMS to quantify mass concentrations. Original stocks were diluted with ultrapure water in 20mL borosilicate vials (1 mL stock to 9 mL UTPW) before ICPMS analysis. Working stock suspensions were prepared by the addition of the UTPW-NP suspension or AgNO<sub>3</sub>-UTPW solution, to natural seawater and to synthetic seawater of the correct strength immediately prior to testing. Particle addition followed by gentle agitation (~15 seconds by hand) in all instances, to avoid any unwanted ablation or interactions that may occur with more intensive mixing processes (e.g. sonication and vortexing). AgNO<sub>3</sub> addition to

any aqueous phase was followed by vortexing. Expected nominal concentrations did not vary in medium or UTPW by more than 10%, as such nominal concentrations are reported throughout for testing concentrations.

#### **3.3.4 Ag NPs characterization**

Ag NPs characterization was done in three suspensions in ultrahigh purity water (UHPW, 18.2 M $\Omega$ .cm), natural seawater (NSW), and synthetic seawater (SSW) medium. NP samples were taken at 1, 24, hours, and 7 days after suspension, to reproduce laboratory exposure scenarios. Z-average hydrodynamic diameter (Z-avg.) and zeta potential in each media were analyzed using dynamic light scattering (DLS) (Zetasizer Nano, Malvern Instruments, Malvern USA). At each time point, at least five repeated measurements per replicate were recorded to calculate z-average and zeta potential; three replicates were measured. To perform each replicate required a 1 mL medium-NP suspension at 0.02 mg L<sup>-1</sup> Ag contained in a 15 mL borosilicate glass vial. To conduct zeta measurement, the AgNP suspensions were removed using a syringe and placed into a disposable capillary zeta-cell ((Malvern Instruments, MA, U.S.A. Ultraviolet and visible light (UV-vis) spectra were measured in triplicate over the 31 wavelength range of 200-800 nm using a UV-Vis spectrophotometer (UV-2600, Shimadzu Co., Kyoto, Japan). Transmission electron microscopy (TEM) a Hitachi HT7800 instrument images and energy dispersive x-ray spectra (EDX) were acquired at CEM, School Arts & Science at University of South Carolina. The TEM images and (EDX) were acquired at electron microscopy center facility. TEM samples were prepared using an ultracentrifugation-based method in UHPW and NSW, and SSW media, respectively. Briefly, a carbon coated copper grid (300 mesh; Agar scientific) was placed into a clear plastic centrifuge tube and 4 mL of sample was

added into the tube very slowly. The tube was covered by parafilm and was centrifuged in Sorvall MTX 150 micro-ultracentrifuge (Thermo Scientific) with an S52-ST rotor for 1 hour at 50,000 rpm. The grid was rinsed thoroughly with high-purity water and left overnight to fully dry. A Hitachi HT8700 then imaged the grids. The PVP-AgNP batch was checked for purity using Energy-dispersive X-ray spectrometry. Size and particles size distribution (PSDs) were analyzed with the National Institutes of Health (NIH) Image J Version 1.46r software (<https://imagej.nih.gov/ij/>) package.

### **3.3.5 Statistical analysis**

The statistical analysis software package (SPSS 22) was used to identify significant treatment effects. A non-parametric Mann Whitney test were used to compare the effect type of seawater, coating, and time of exposure. The change of size of Cit. AgNPs, PVP-AgNPs in NFSW and SSW between initial exposure at 0.0h and the time 24 h were compared with their sizes in UTPW at 0.0h and 24h. Significance level on a  $p \leq 0.05$  as the value to identify statistically significant media effects and time of exposure.

## **3.4 Results**

### **3.4.1 AgNPs characterization**

The synthesized suspension of AgNPs was light yellow in color. The (Table 3.1) shows the pristine particle physicochemical properties of the Ag NPs studied within this thesis, which were manufactured as following the procedure by Tejamaya et al., [274]. Here it can be seen that all Ag NP have hydrodynamic sizes of the (23.5 nm). The increased hydrodynamic sizes obtained by DLS, and the shift in surface plasmon resonance (SPR) to longer wavelengths confirmed the conversion of AgNPs coatings from citrate to PVP

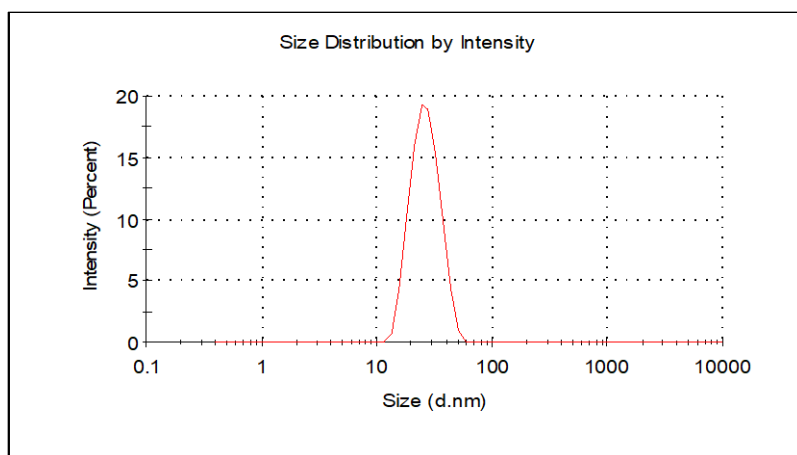
[274]. Images obtained by TEM indicated that Cit. AgNPs in the stock suspension were spherical in shape and the sample had a narrow size distribution ( $18.85 \pm 3.33$  nm) (**Table 3.3**). The total Ag concentration in the stock solution was  $20 \text{ mg L}^{-1}$  as determined by ICPMS measurements. In order to investigate the stability of singly dispersed Ag NPs in natural waters, the pristine synthesized AgNPs were added into different water samples. The maximum absorbance of the full spectra of the identical concentrations of AgNPs over time are shown in (**Table 3.2**) Results showed that the absorbance spectra changed dramatically over time, especially in (UFSW). The maximum absorbance decreased noticeably as soon as the Ag NPs were added. However, the maximum absorbance of AgNPs in the waters increased slightly at high concentration for PVP-AgNPs, then remained stable during the experimental period. In the (SSW), the maximum absorbance decreased markedly. A broader absorbance was observed in both (NFSW) and (SSW), suggesting that much larger agglomerates were probably generated, (**Figure. 3.4 and Figure 3.5**). In exposure ( $1 \text{ } \mu\text{g L}^{-1}$  Cit. AgNP) at 24 h did not show any signal due to detection limit of UV-Vis.

**Table 3.1** Prospective of AgNPs physiochemical characterization data for pristine nanoparticles. Data represented as mean (SD) of at least three sequential measurements.

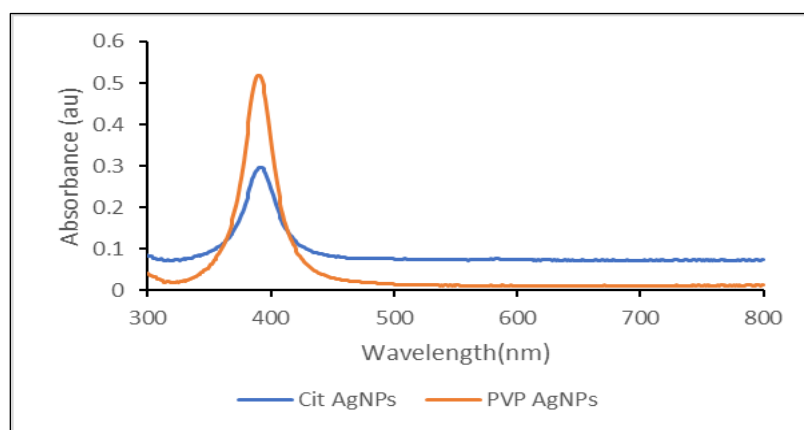
NPs	Size (nm)	PDI	Time (h)	Peak absorbance (nm)
Cit. Ag NPs	22± 3.5	0.08	0.0	389.5
PVP-Ag NPs	34.1± 3.5	0.1	0.0	393.0

**Table 3.2** UV-vis Spectra of Cit. AgNPs and PVP- AgNPs from traditional methods (i.e. UV-vis spectrophotometer; absorbance  $\lambda$ ) at 0 and 24 hours in seawater media at 1 and 24 h.

NPs type & time(h)	Concentrations ( $\mu\text{g/L}^{-1}$ )			
	1 $\mu\text{g/L}^{-1}$	10 $\mu\text{g/L}^{-1}$	50 $\mu\text{g/L}^{-1}$	100 $\mu\text{g/L}^{-1}$
Cit. AgNPs time 0.0 h	0.011	0.045	0.108	0.053
Cit. AgNPs time 24 h	0.008	0.062	0.038	-0.017
PVP-AgNPs time 0.0 h	0.01	0.008	0.005	0.006
PVP-AgNPs time 24 h	0.046	0.013	0.046	0.045

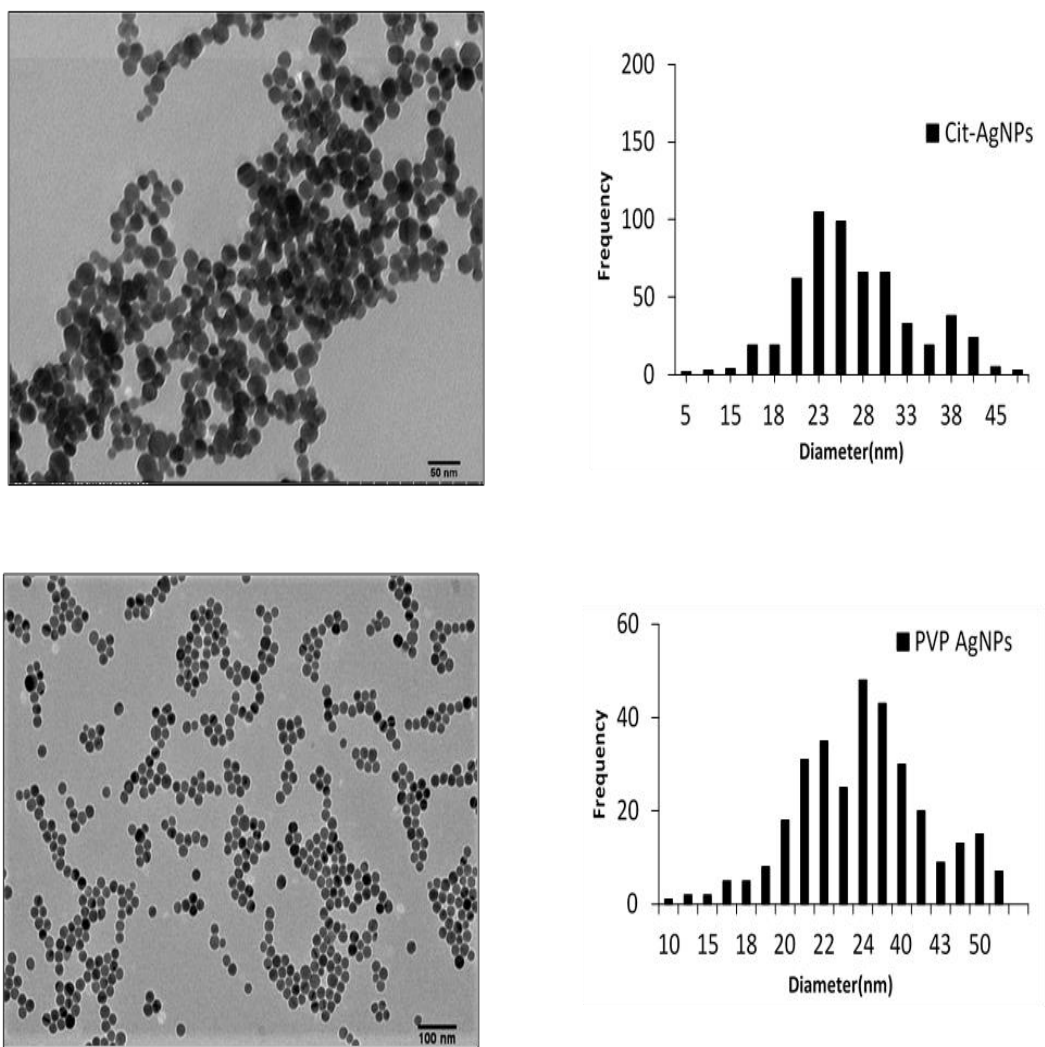


(A)

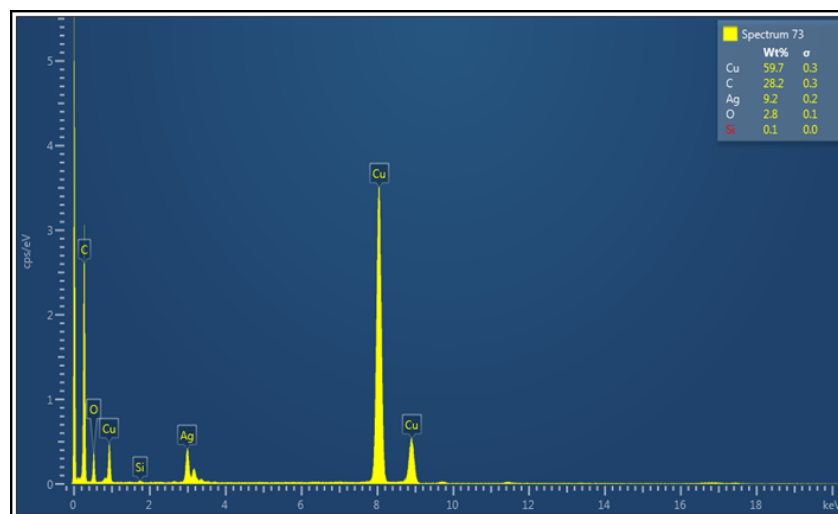


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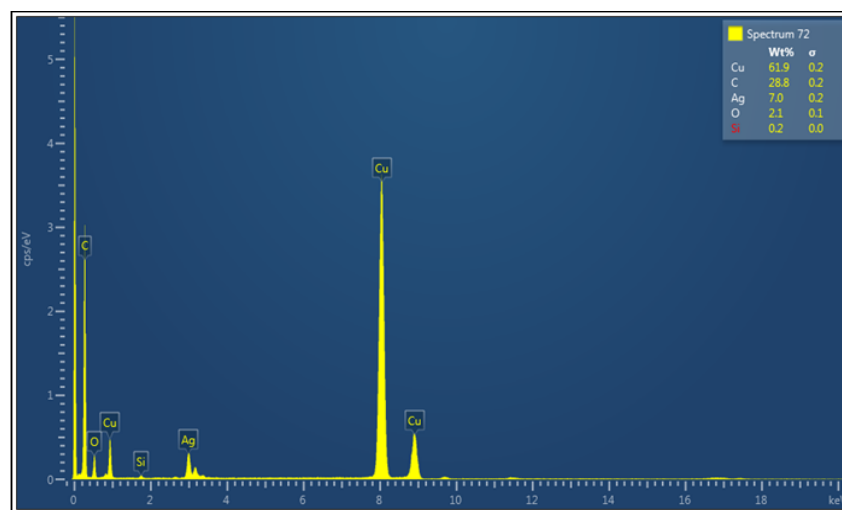
**Figure 3.1** AgNPs characterization using DLS, UV-Vis. **(A)** Dynamic light scattering size distribution graph of the pristine Cit. **(B)** UV. Vis, surface plasmon resonance spectra for Cit. AgNPs and PVP- AgNPs.



**Figure 3.2** Typical transmission electron microscopy (TEM) of size distribution for Cit. AgNPs, PVP-AgNPs. In home synthesized AgNPs placed on formvar-filmed copper grid by ultracentrifugation method. TEM images of  $100 \mu\text{g L}^{-1}$  AgNPs in (UTPW) media (without clams). Size distribution of AgNPs obtained by ImageJ.



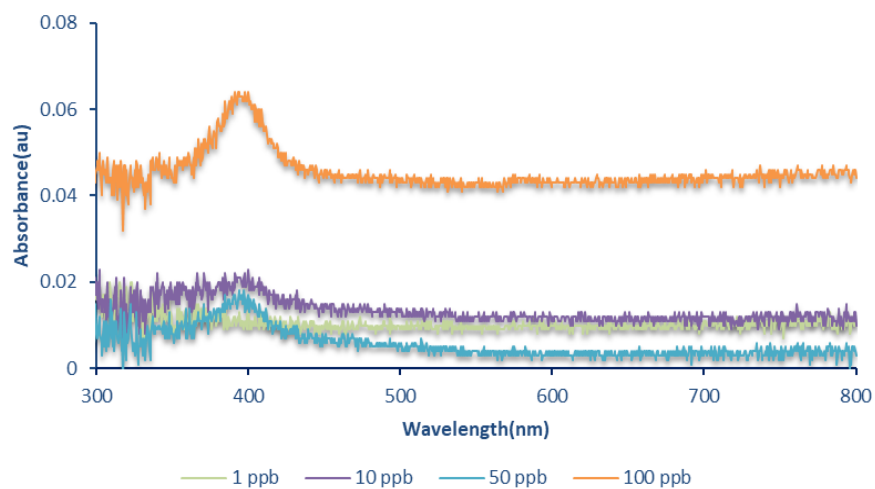
(A)



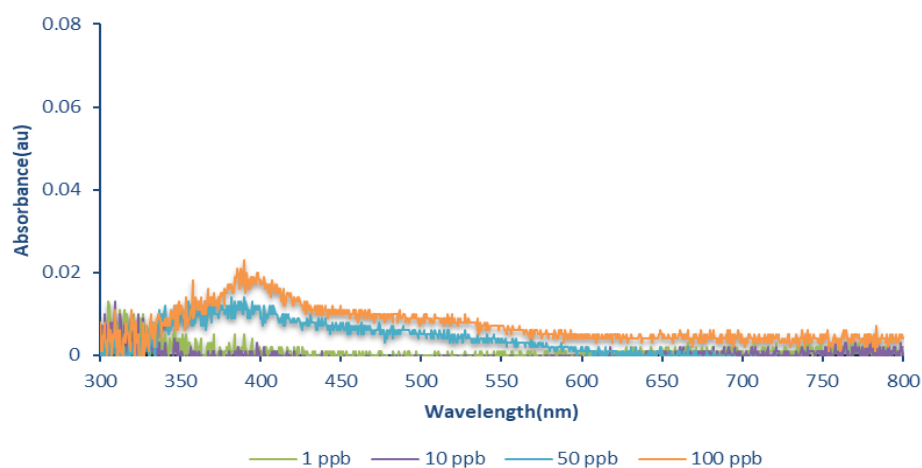
(B)

**Figure 3.3** Characterization 100  $\mu\text{g L}^{-1}$  PVP-AgNPs in (NFSW) EDX images prepared by ultra-centrifugation method by placed on formvar-filmed copper grid by ultracentrifugation, (A) in (UTPW), (B), in (NFSW).



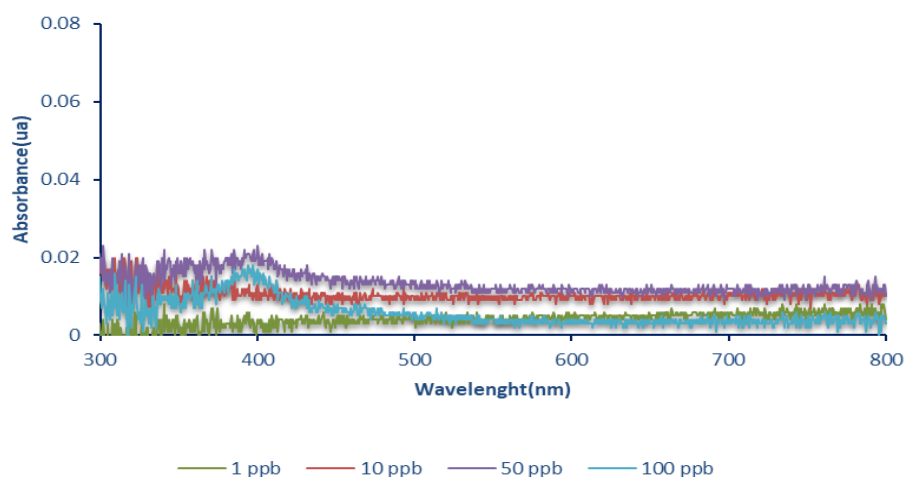


(A)

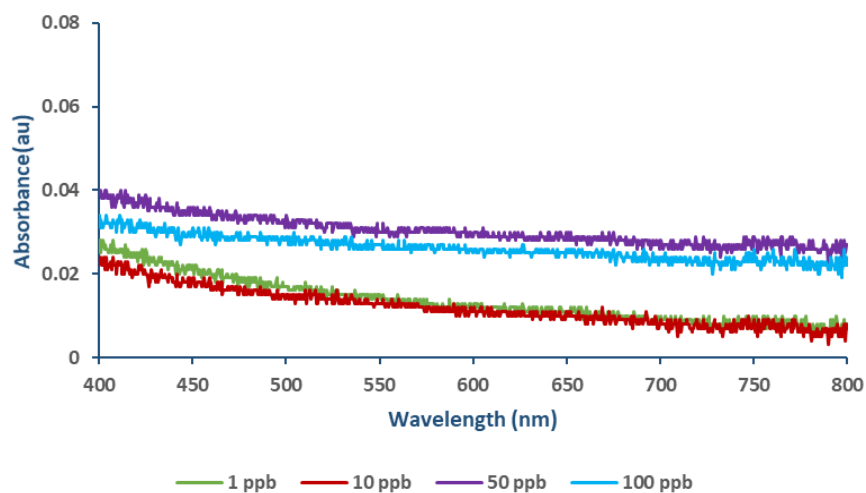


(B)

**Figure 3.4** UV-vis Spectra of Cit. Ag NPs from UV-vis spectrophotometer absorbance  $\lambda$ ) at 0 and 24 hours in seawater medium. Behavior of Cit. AgNPs in NFSW media in (1, 10, 50, and 100  $\mu\text{g L}^{-1}$ ) concentration as a function of time. The lowest concentration of exposure (1  $\mu\text{g L}^{-1}$  PVP-AgNP) at 24 h did not show any signal due to detection limit of UV-Vis.



(A)



(B)

**Figure 3.5** UV-vis Spectra of PVP-. Ag NPs from traditional methods (i.e. UV-vis spectrophotometer; absorbance  $\lambda$ ) A: at 0.0 h and B: at 24 h in seawater medium. Behavior of PVP- AgNPs in NFSW media in (1, 10, 50, and 100  $\mu\text{g L}^{-1}$ ) concentration as a function of time. The lowest concentration of exposure (1  $\mu\text{g L}^{-1}$  PVP-AgNP) at 24 h did not show any signal due to detection limit of UV-Vis.

### 3.4.2 Behavior AgNPs in exposure media

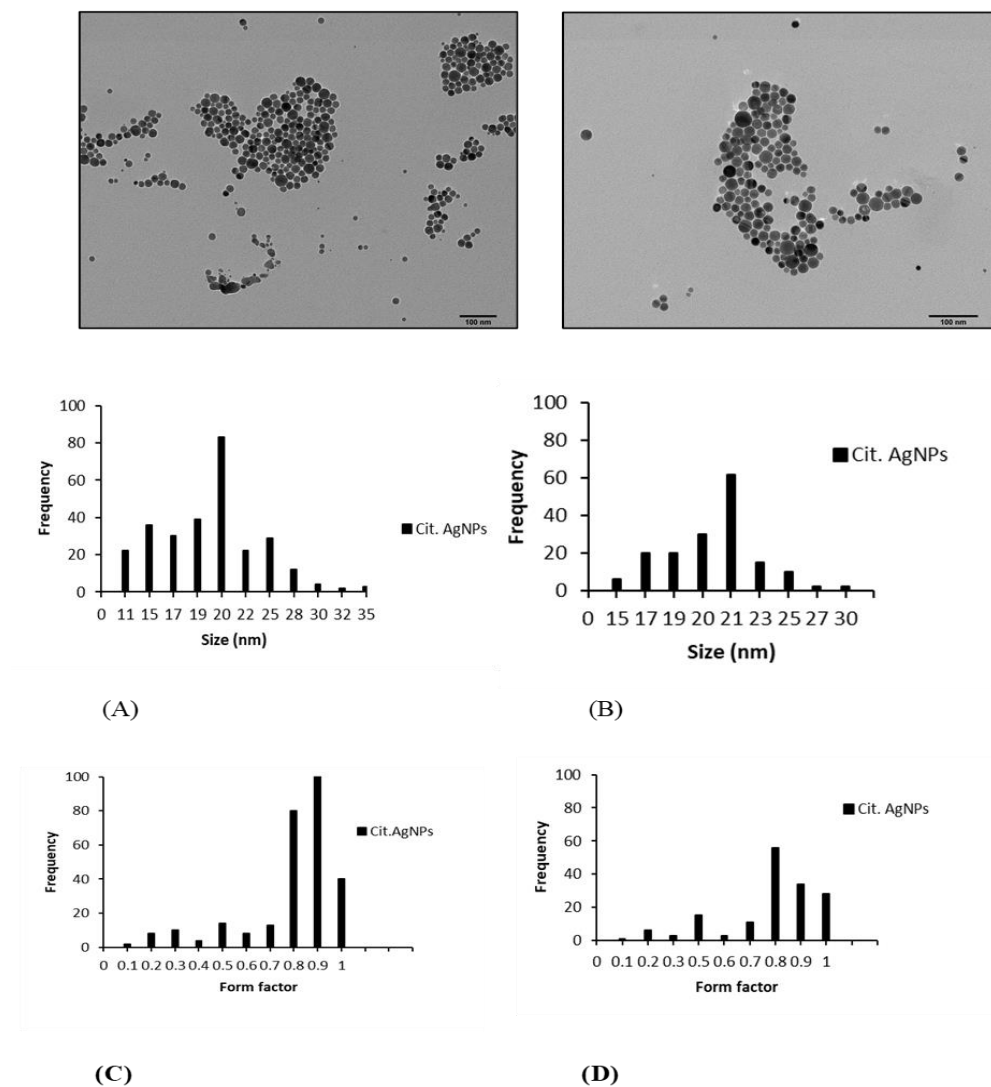
#### 3.4.2.1 AgNPs transformation

Once NP introduced to environment, transformation NP occurred and it is depend on nanoparticle's intrinsic properties (e.g., NP size, shape, surface charge, concentration etc.) [18]. The data from this study showed transformation of AgNPs with both different surface coating (Cit.) and (PVP) in both (NFSW) and (SSW). A direct observation of AgNPs transformation shown in the experiments by visualizing NPs using TEM. TEM micrographs result showed that the changes in sizes and shapes occurred in both Cit. AgNPs and PVP-AgNP in both seawater types. As well as, the TEM images showed significant alteration in morphological of Cit. AgNPs in (SSW) (**Figure 3.8**). The circularity of Cit. AgNPs populated between (0.1) and (0.3). The change of sizes were significant when compared with the size of Cit. Ag NPs in UTPW (**Figure 3. 6**) at the same time, the change in median of sizes was (28 nm, IQR=14.48) at 0.0 h , and (43.92 nm, IQR = 39.73) at 24 h , the  $p < 0.05$ , (**Figure 3.8**). The result is significant at  $p < 0.05$  level based on Mann Whitney test. PVP-AgNPs illustrated slight changes in circularity shape than Cit. AgNPs in SSW (**Figure 3.9**), and the data showed there was significant differences in sizes between time 0h and the 24 h when it was compared with the range in size in UTPW media. PVP AgNPs showed median in sizes in (range 22.23 nm, IQR = 6.37 at 0.0 h to 30.27 nm, IQR= 13.36 at 24 h),  $p < 0.05$ . In the NFSW, Cit.AgNPs media demonstrated changes in their shapes and sizes (**Figure 3.10**). The histogram of PSDs shows that the size of Cit. AgNPs is highly populated at range between (20.74 nm, IQR = 4.55 at time 0.0 h to 35.46 nm, IQR = 28.15 at 24h), and the  $p < 0.05$  in both times , where circularity values more than 0.9 at 0.0 h and in the time 24 h populated at 0.1, (**Figure 3.10**). The result indicated

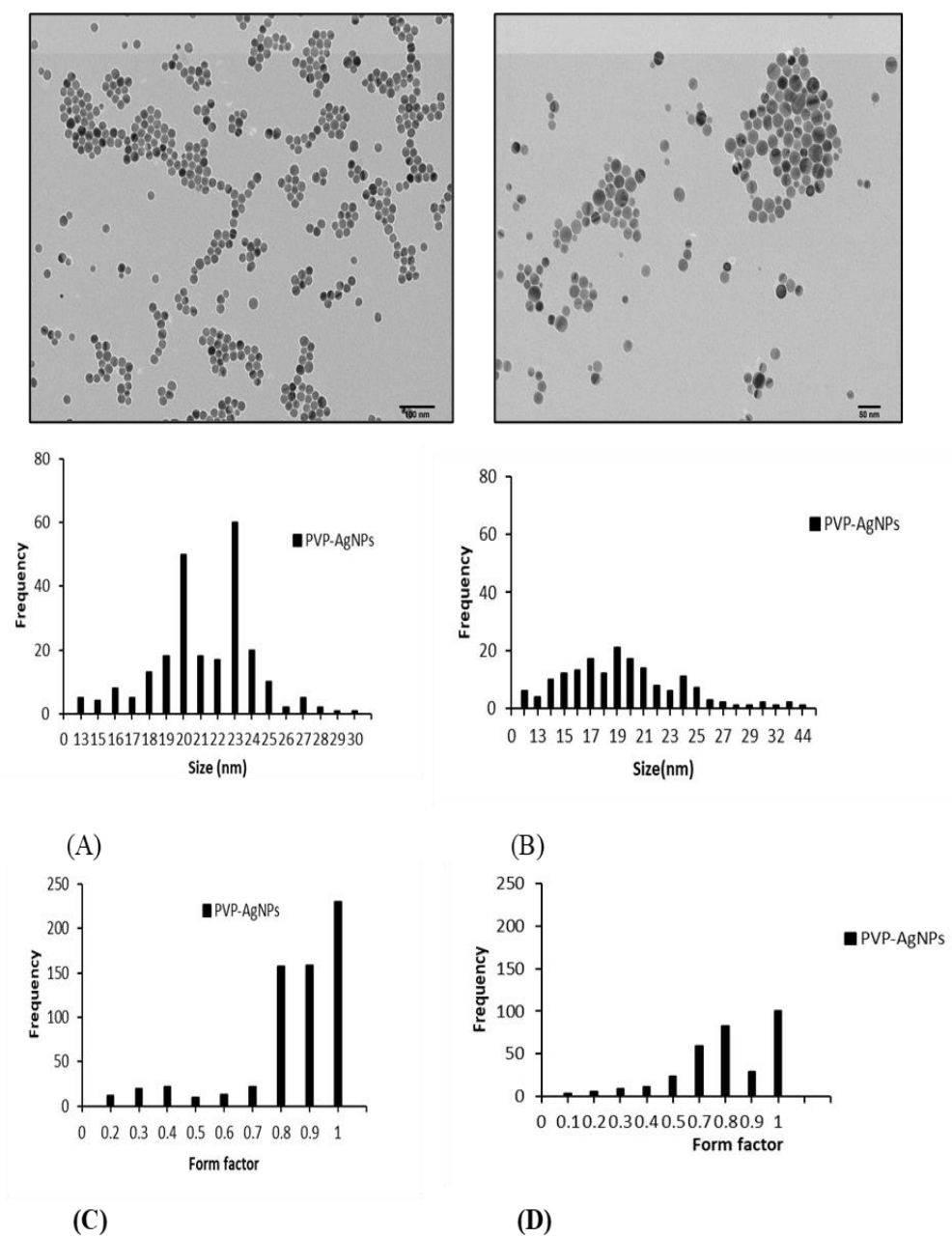
based on Mann Whitney test that there was significant differences between the sizes PSDs in UTPW and PSDs in NFSW. As well as, the data of TEM images showed formation of smaller AgNPs in NFSW means that chemical transformation occurred to AgNPs in (NFSW) media. In the same media, PSDs of PVP-AgNPs range between (21.17 nm, IQR = 4.86 at time 0.0 h to 32.9 nm, IQR = 23.49 nm, IQR = 5.91 at time 24 h) and when compared their sizes with UTPW, and the result showed significant differences between range of sizes NPs in two media. The  $p < 0.05$  based on Mann Whitney test, (**Figure 3.11**).

#### 3.4.3.2 Ag NPs size change

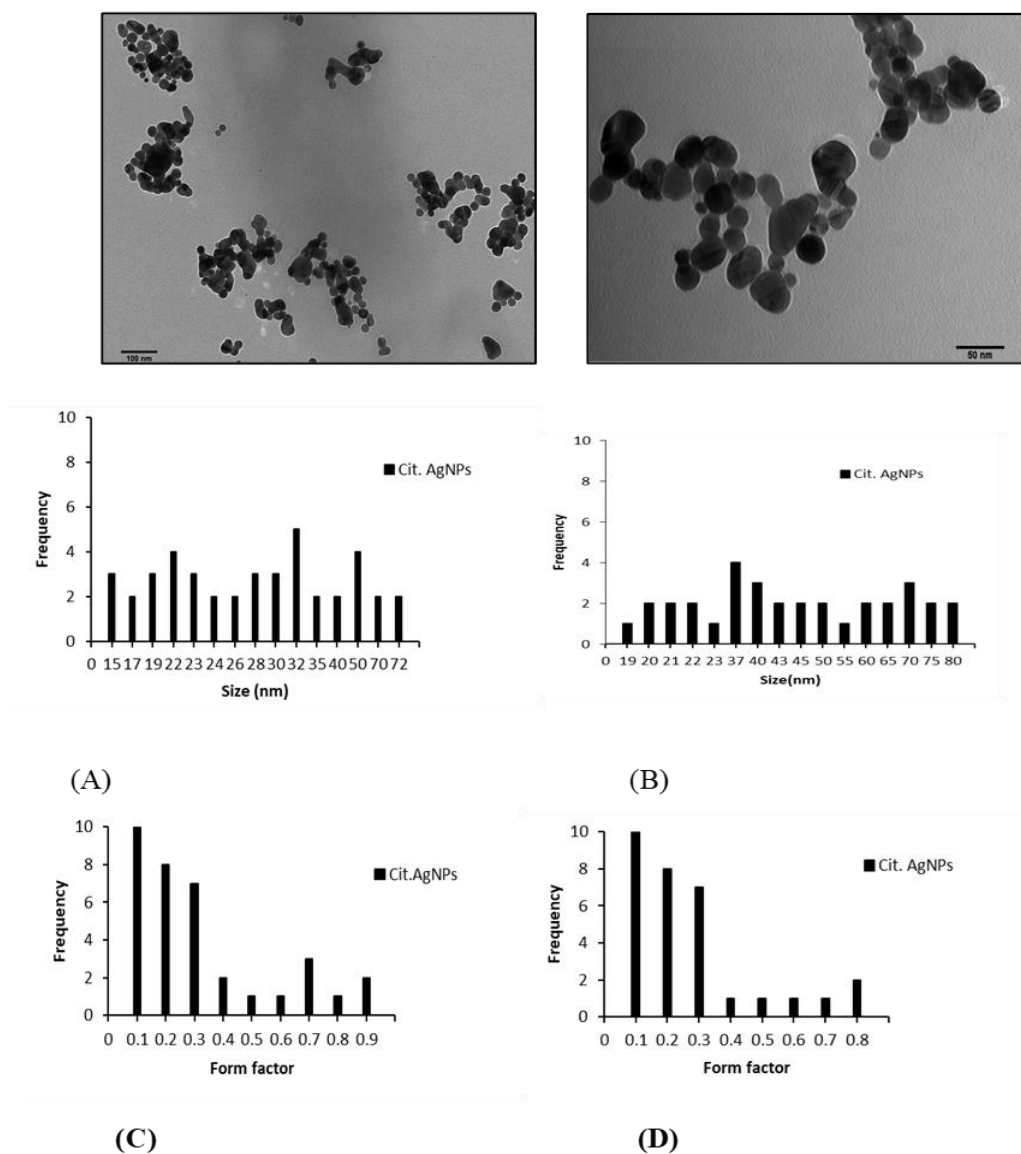
The size change of Cit.AgNPs and PVP-AgNPs in both (NFSW) and (SSW) was measured at concentration ( $100 \mu\text{g L}^{-1}$ ) using TEM. In general, Cit. Ag NPs formed size altered in both (NFSW) and (SSW) more than PVP-AgNPs and PVP AgNPs remain stable during experiment time. From the number of PSDs of Cit. AgNPs and PVP AgNPs in both (NFSW) and (SSW) showed a clear shift towards larger sizes instantaneously after spiking into both seawaters media (0.0 h) correlated to the equivalent PSDs of Cit. AgNP and PVP-AgNPs. (**Figures 3.12, 3.13, 3.14, and 3.15**). At the end of exposure time 24h, the PSDs of Cit. AgNPs and PVP AgNPs moved further toward larger sizes, which is indicated that AgNPs aggregated in both (NFSW) and (SSW). The aggregation of both Cit. AgNPs and PVP-AgNPs in both types of seawater can be related to the higher ionic strength of the (NFSW) and (SSW). In addition, the magnitude of zeta potential for both Cit. AgNPs and PVP-AgNPs (**Table 3.3**) were decrease in (0.0 h) time and further at 24 h exposure time in both NPs were measured by DLS (NFSW and SSW)(data not shown). In the (**Figure. 3.12**), images from (TEM) of  $100 \mu\text{g}$  Cit. Ag NPs in (NFSW) at time 0.0 h. and 24 h. It showed increase in size Cit. AgNPs there were peak size 25 nm at 0.0h of exposure, where



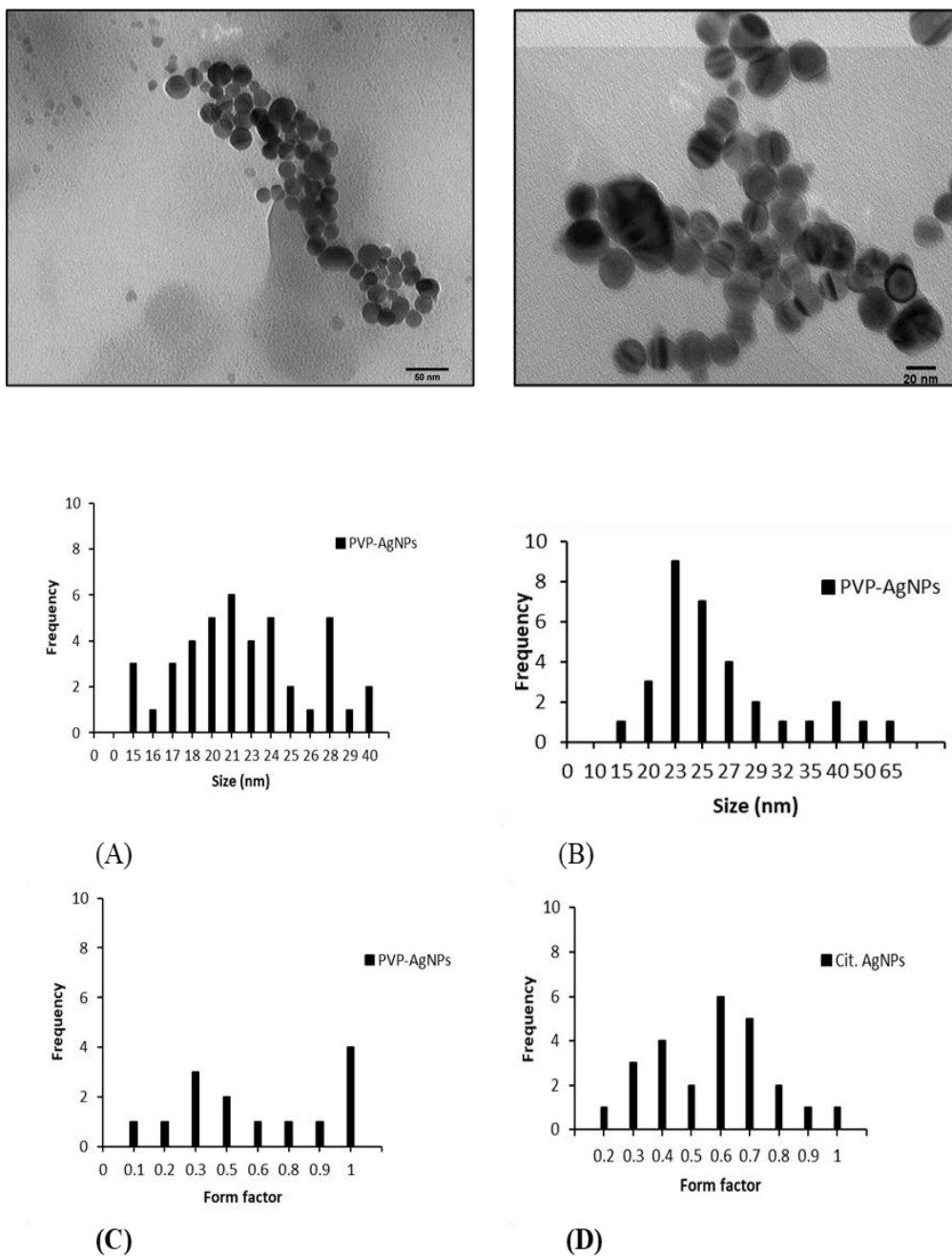
**Figure 3.6** Size distribution and form factor of synthesized AgNPs. (A), Cit. Ag NPs in (UTPW) at time 0.0 h. (B) The 100  $\mu\text{g L}^{-1}$  Cit. AgNPs in (UTPW) at time 24h. (C), Form factor of Cit. AgNPs in UTPW at time 0 h. (D) form factor of Cit. AgNPs in UTPW at time 24h. There is no significant difference.



**Figure 3.7** Size distribution and form factor of synthesized AgNPs. (A), PVP- AgNPs in (UTPW) at time 0.0 h. (B) The  $100 \mu\text{g L}^{-1}$  PVP- AgNPs in (UTPW) at time 24 h. (C), Form factor of PVP- AgNPs in UTPW at time 0 h. (D) form factor of PVP-AgNPs in UTPW at time 24h.

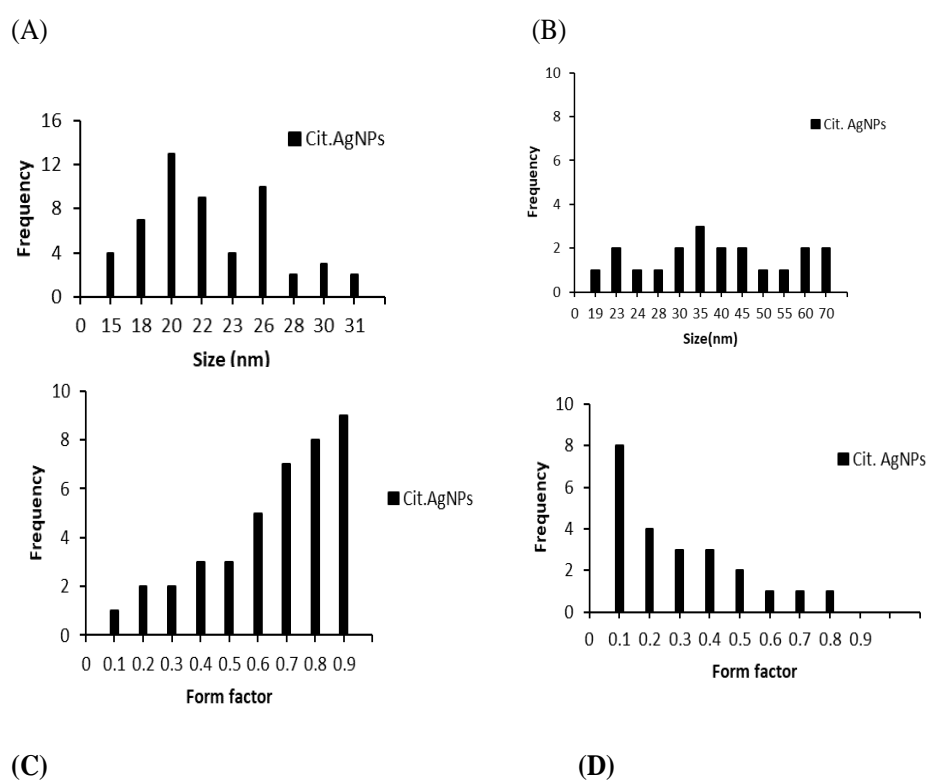
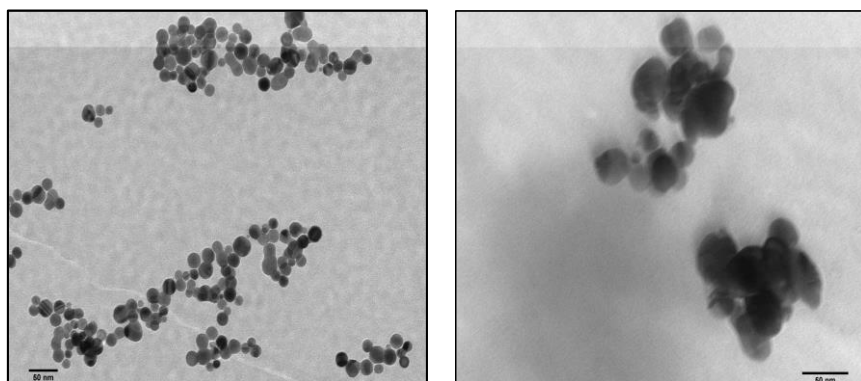


**Figure 3.8** Size distribution and form factor of synthesized AgNPs. (A), Cit. Ag NPs in (SSW) at time 0.0 h. (B) The  $100 \mu\text{g L}^{-1}$  Cit. AgNPs in (SSW) at 24 h. (C), Form factor of Cit. AgNPs time 0.0 h. (D) form factor of Cit. AgNPs at time 24h.

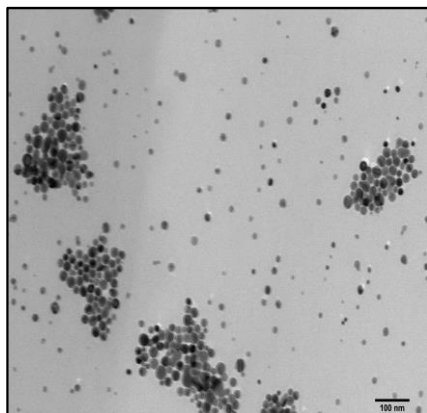


**Figure 3.9** Size distribution and form factor of (A). PVP- AgNPs in (SSW) at time 0.0 h. (B) The  $100 \mu\text{g L}^{-1}$  PVP-AgNPs in (SSW) at 24 h. (C), Form factor of PVP- AgNPs at time 0.0 h. (D) form factor of PVP-AgNPs at time 24h.

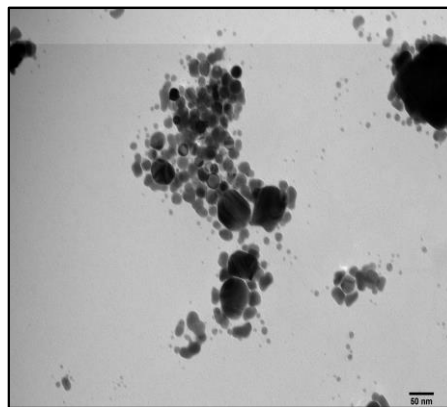
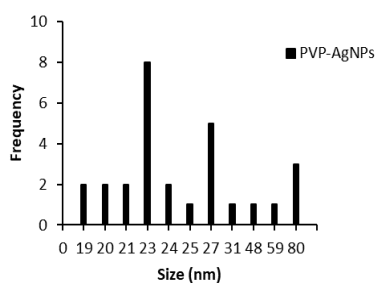




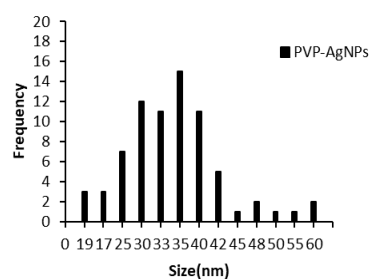
**Figure 3.10** Size distribution and form factor of (A). Cit. AgNPs in (NFSW) at time 0.0 h. (B) The 100  $\mu\text{g L}^{-1}$  Cit. AgNPs in (NFSW) at time 24 h. (C), Form factor of Cit. AgNPs at time 0.0 h. (D) form factor of Cit. AgNPs at time 24h.



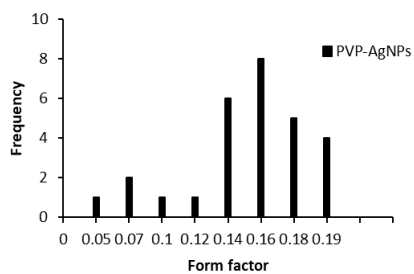
(A)



(B)

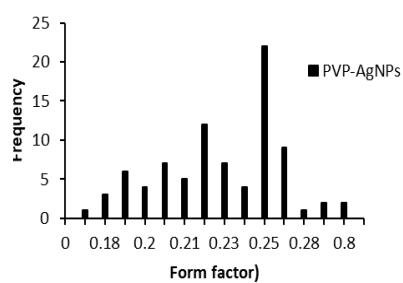


(A)



(C)

(B)



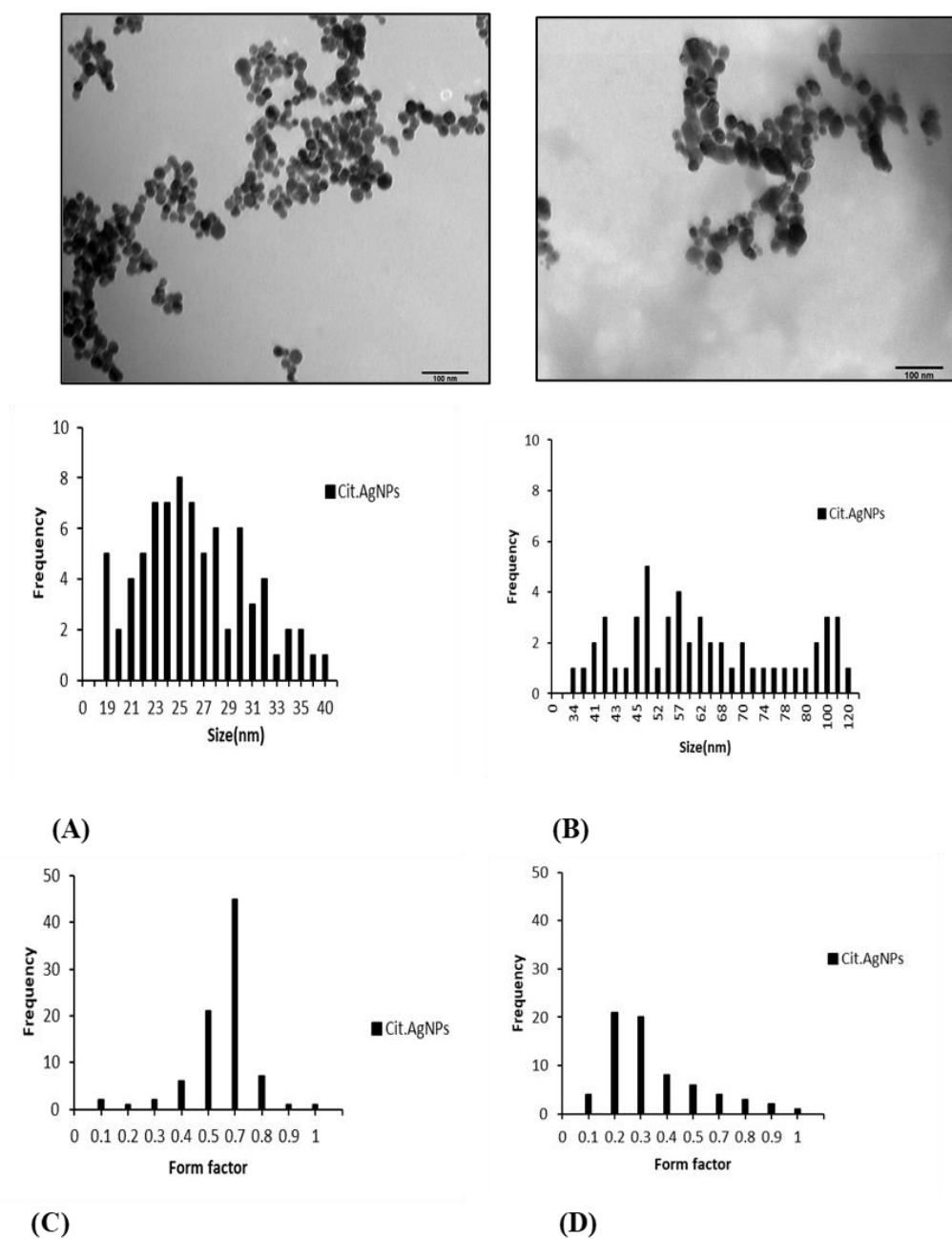
(D)

**Figure 3.11** Size distribution and form factor of (A). PVP- AgNPs in (NFSW) at time 0.0 h. (B) The  $100 \mu\text{g L}^{-1}$  PVP-AgNPs in (NFSW) at time 24 h. (C), Form factor of PVP-AgNPs at time 0.0 h. (D) form factor of PVP-AgNPs at time 24h.

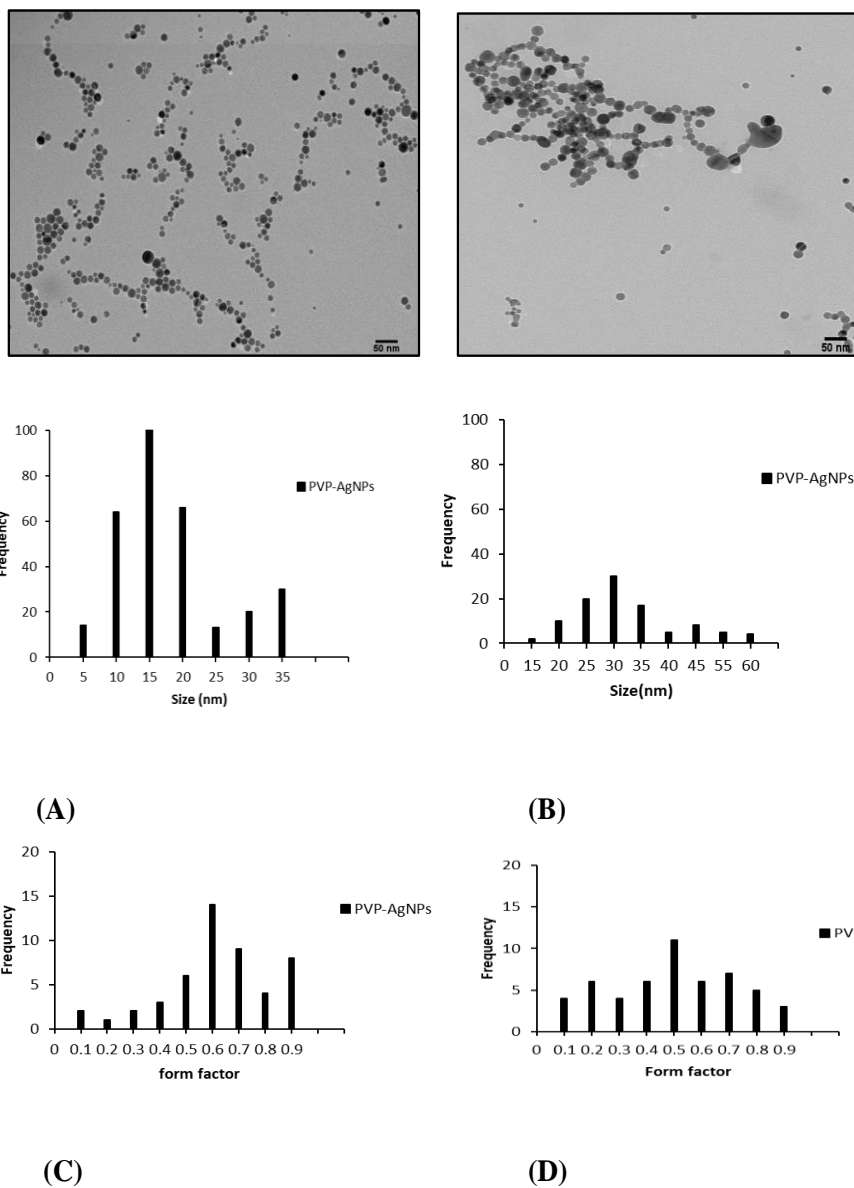
at 24h the sizes of Cit. AgNPs shifted to size 46 nm and total numbers of NPs in size 45 nm and more. When compared this changes in sizes with Cit. AgNPs PSDs in UTPW, the median of sizes was (30.3 nm), and the  $p < 0.05$ . The result is significant at  $p < 0.05$  depend on the Mann Whitney test. In (SSW) Cit. AgNPs showed differences in sizes of NPs between the initial times and the end of exposure. The peak of size distribution was increase from 22 nm to 27 nm, and the median of sizes between time 0.0h and 24 h was 22.5 (nm). **(Table 3.3 and Figure 3.14)**. In (NFSW) AgNPs showed change in size for both Cit. AgNPs and PVP-AgNPs at ( $100 \mu\text{g L}^{-1}$ ), **(Figure. 3.13)**. The result showed changes in the size of AgNPs after 24 h in (NFSW). Cit. AgNPs was more susceptible to highly ionic strength of seawater and the size reached more than 40 nm. Aggregation NPs occur because of NP collision and attachment. PVP-AgNPs seems to be less influenced in seawater media because of the protection of PVP coating layer, and the size of  $100 \mu\text{g L}^{-1}$  PVP AgNPs at (NFSW) were in range between (15-20 nm), **(Figure 3.13)**. Alteration in AgNPs sizes were also occurred in the (SSW) media, the data showed slightly increased NPs sizes after 24h for both Cit. AgNPs and PVP-AgNPs **(Figures 3.12 and 3.13)** However, AgNPs change size in (SSW) less than their alterations size in (NFSW).

**Table 3.3** Characterization and behavior Cit.AgNPs and PVP-AgNPs at 0 h and 24 h in UPHW, NFSW, and SSW.

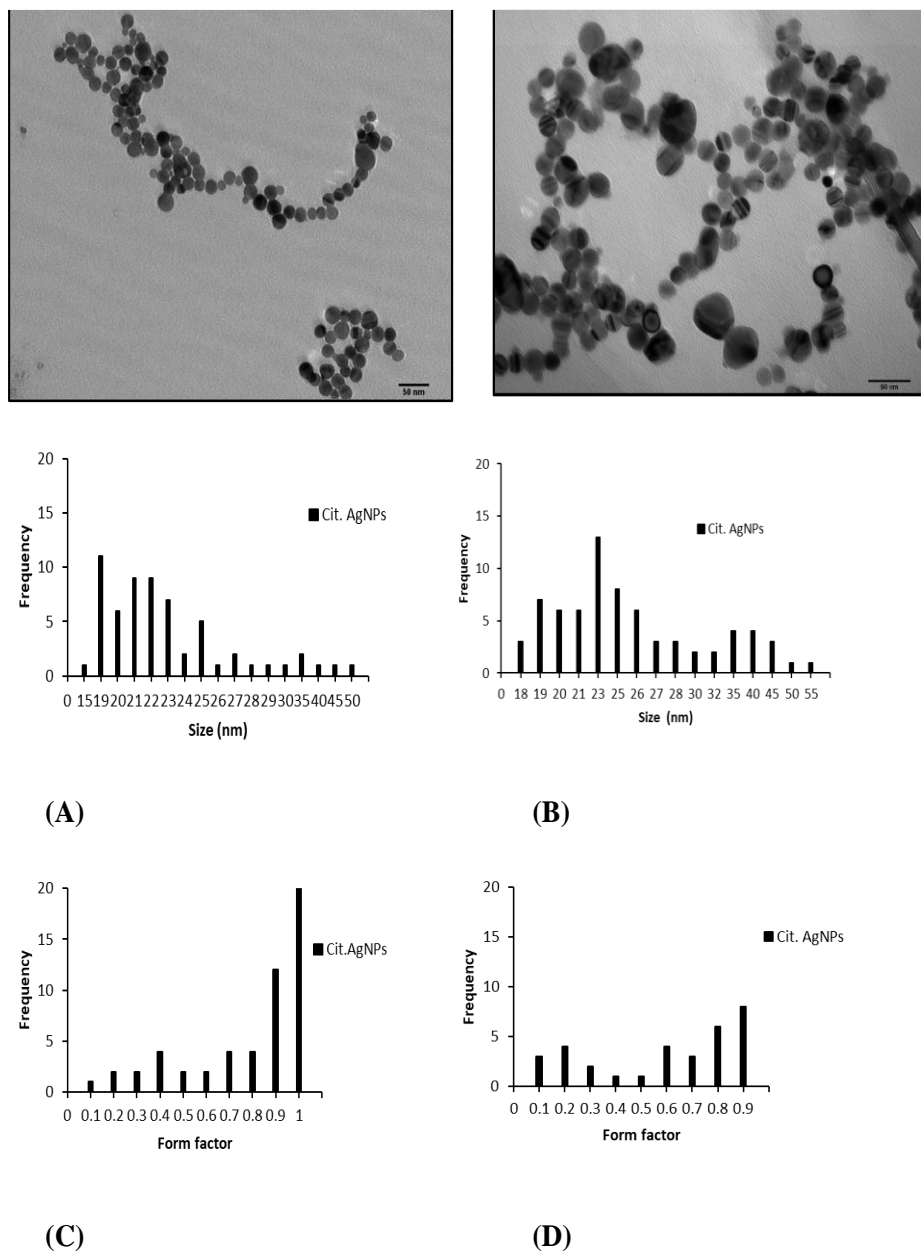
Type of media	NPs	Particles Count at T0 and T24	Mean size T=0	Mean size T=24	Median of sizes	P- value at < 0.05
UHPW	Cit. AgNPs	360	17.6	18.6	16.9	0.3
		360				
	PVP-AgNPs	360	20.6	22.2	20.8	0.3
		360				
NFSW	Cit. AgNPs	100	31.7	33.5	30.2	0.043
		360				
	PVP-AgNPs	360	15.7	37.7	14.1	0.001
		360				
SSW	Cit. AgNPs	360	22	27	22.5	0.001
		360				
	PVP-AgNPs	360	25.9	30.5	27.5	0.001
		360				



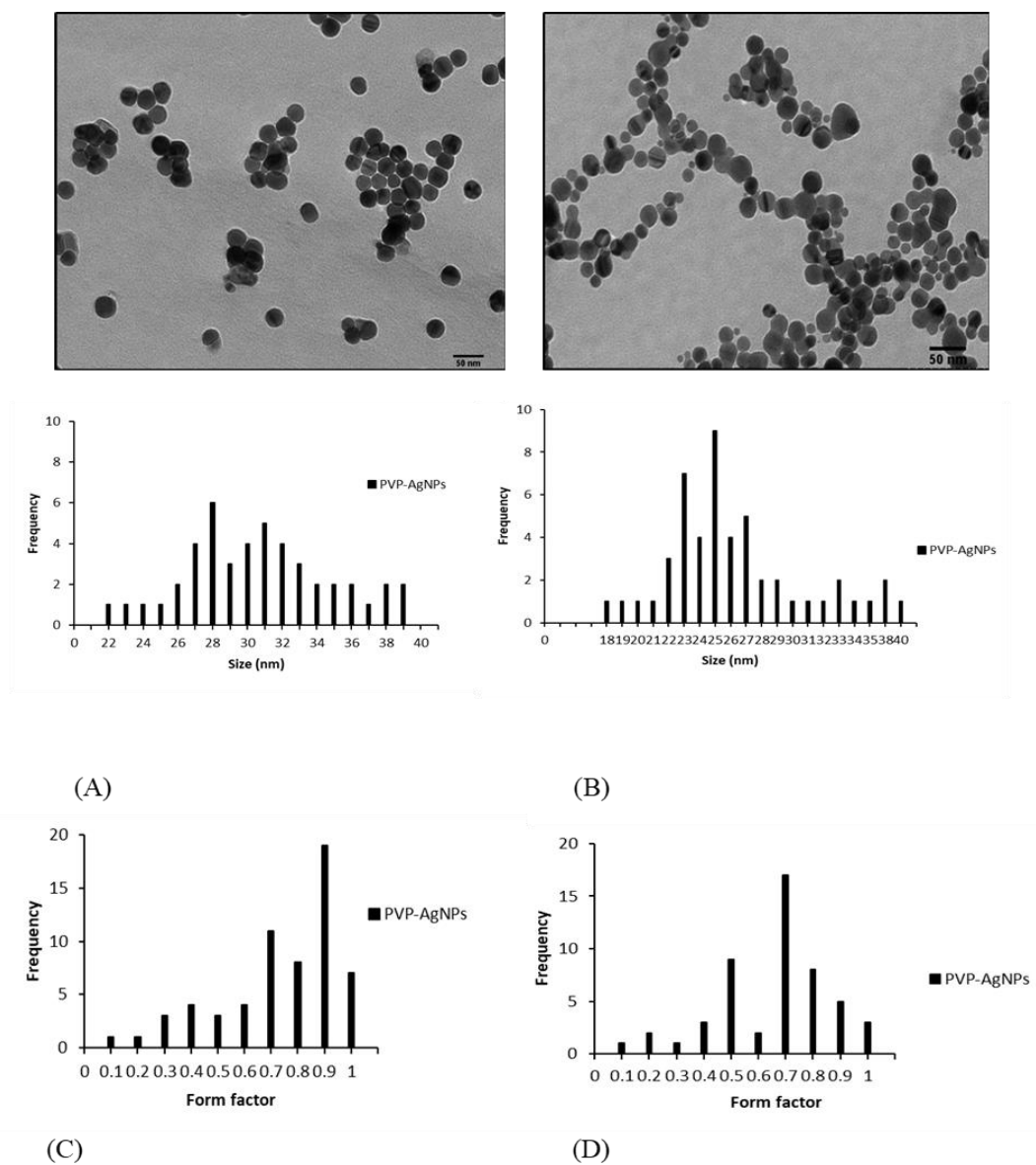
**Figure 3.12** Typical transmission electron microscopy (TEM) of synthesized Cit. Ag NPs in (NFSW) at time 0.0 h. and 24 h., (A). The  $100 \mu\text{g L}^{-1}$  Cit. AgNPs at time 0.0 h, (B).  $100 \mu\text{g L}^{-1}$  Cit. AgNPs at time 24 h. Form factor of  $100 \mu\text{g L}^{-1}$  Cit. AgNPs at time 0.0 h. (D). Form factor of  $100 \mu\text{g L}^{-1}$  Cit. AgNPs at time 24 h.



**Figure 3.13** Typical transmission electron microscopy (TEM) of synthesized PVP- Ag NPs in (NFSW) at time 0.0 h. and 24 h., (A). The  $100 \mu\text{g L}^{-1}$  PVP-AgNPs at time 0.0 h, (B).  $^{109}\text{Ag}$  NPs  $100 \mu\text{g L}^{-1}$  at time 24 h. (C). Form factor of  $100 \mu\text{g L}^{-1}$  PVP-AgNPs at time 0.0 h. (D). Form factor of  $100 \mu\text{g L}^{-1}$  PVP-AgNPs at time 24 h.



**Figure 3.14** Typical transmission electron microscopy (TEM) of synthesized Cit. Ag NPs in (SSW) at time 0.0 h. and 24 h., (A). The  $100 \mu\text{g L}^{-1}$  Cit. Ag NPs at time 0.0 h, (B).  $100 \mu\text{g L}^{-1}$  Cit. Ag NPs at time 24 h. Form factor of  $100 \mu\text{g L}^{-1}$  Cit. Ag NPs at time 0.0 h. (D). Form factor of  $100 \mu\text{g L}^{-1}$  Cit. Ag NPs at time 24 h.



**Figure 3.15** Typical transmission electron microscopy (TEM) of synthesized PVP- Ag NPs in (SSW) at time 0.0 h. and 24 h., (A). PVP-AgNPs  $100 \mu\text{g L}^{-1}$  at time 0.0 h, (B). PVP-AgNPs  $100 \mu\text{g L}^{-1}$  at time 24 h. (C). Form factor of  $100 \mu\text{g L}^{-1}$  PVP-AgNPs at time 0.0 h. (D). Form factor of  $100 \mu\text{g L}^{-1}$  PVP-AgNPs at time 24.



## 3.5 Discussion

### 3.5.1 NPs physicochemical characterization

Physicochemical characteristics and NPs behavior is critical to understanding the impact NPs on environment and organisms and their importance has already highlighted in chapter two. Moreover, several studies have been proposed that these characteristic may be implicated in the subsequent toxicity of the NPs, ranging from dissolution rates to the core material and size of the NP or its agglomerates/ aggregates [267, 268]. The physicochemical characteristics of two differently coated Ag NPs that of (Cit.) and (PVP) coated Ag NPs within differing exposure seawater and exposure time were studied. In this study, the core size of AgNPs was similar as well as the core material (silver), which enabled the examination of the influence of particle coating on the AgNPs subsequent physicochemical characteristics and behavior in seawater medium and pure water (as UTPW). These characteristics are implicated in the toxicological outcomes of the AgNPs within the media, concentrations, and exposure time, particularly in the presences clam *M. mercenaria*, will be presented. From the UV-vis data, AgNPs have an absorbance peak of ~400nm explained by their plasmon resonance for un agglomerated/ un aggregated particles (**Table 3.1**). This shifts to longer wavelengths when agglomeration/ aggregation occurs [269], but this was not measure in this study. Cit. AgNPs altered size immediately upon addition into seawater (**Figure 3.12**). It can be seen that peak absorbance of Cit. AgNPs is not noticeable at 400 nm wavelength which is indicated that AgNPs aggregation occurs in the solution. At lower concentration ( $1 \mu\text{gL}^{-1}$ ), the lack of a peak at 400 nm due to low concentration and lack of sensitivity of the absorbance method [326], but have no

observed it directly. If Cit. AgNPs probably are dissolving in the seawater media and forming silver chloride (AgCl). Once Cit. AgNPs dissolve Cl binds to the free Ag ions, creating AgCl, this new complex does not have a detectable wavelength due to Cl-disguising the Ag signal. However, at (100  $\mu\text{gL}^{-1}$ ) the UV-vis was able to detect the presences of AgNPs in seawater media. In other words, the decrease in the UV-vis absorbance is related to Cit. AgNPs dissolution because of the absence of peak broadening, shoulder formation or the formation of a second peak at higher wavelengths that can be attributed to NP aggregation and/or shape transformation [262, 266]. In figures (3.4 and 3.5), PV- AgNPs peak showed less loss than Cit. AgNPs in seawater samples. This was expected given that PVP has a strong interaction with the AgNPs surface and is known to better stabilize AgNPs than other macromolecules [270], resulting in an average maximum peak loss of 12% after 21 days [254]. This loss of Cit.AgNPs upon addition to environmentally relevant media (OECD) Daphnia media of varying ionic strength of sulfate and nitrate was also observed by Tejamaya et al., [254], where they observed a 100% loss of Cit.AgNP and 40% loss in 10 fold diluted OECD media.

### **3.5.2 Size NPs transformation in the media**

In all cases, when the AgNPs were suspended in seawater medium, there was changed in average size when compared to those suspended in ultrapure water. PVP- AgNPs showed less changes in sizes distributions in both seawater compare with Cit. AgNPs, and this due to steric stabilization of PVP. The Cit. AgNPs had the largest average size, which may be due to the weakly charge bound coating which uses electrostatic stabilization which may be less protective and stable in complex medium [264, 271]. The increased NPs size

is likely due to ripening or slightly aggregation and we did not quantify agglomeration. In the case of PVP-AgNPs, PVP is more strongly bounded and stabilizes via steric mechanisms. Which is providing greater protection from dissolution and ripening. Cit. AgNPs are likely to change to a greater degree in more complex and higher ionic strength media. TEM images for both Cit. AgNPs and PVP-AgNPs show them affected by seawater ionic strength and chemical ligands. In some instances, the presence of Ag could not be confirmed by EDX data of (Cit. AgNPs). Thus, it gives an observable indication of why size characteristics may change in seawater medium between each particle. This would suggest some form of transformation whereby the particles increase in apparent size is due to complexation and interaction with components of the medium other than themselves. PVP-AgNPs, stays well dispersed within the medium and do not seem to interact with medium ligands in the same way, and this has been previously noted within the literature [264, 269]. This may be due to the steric stabilization that PVP creates. In this instance, any increase in size may be due to, likely due to ripening [272]. Agglomeration is far less likely [254].

### **3.6 Conclusion**

The result in this study showed that Cit. AgNPs more influenced by seawater in both types than PVP-AgNPs. It is clear from the result of this study that NP properties, and therefore their fate and behavior of AgNPs in environmentally and biologically relevant concentrations influenced by the ionic strength of seawater. The measurements of sizes showed that AgNPs are monodisperse in UTPW and this is confirmed by UV-vis and TEM images. In general, TEM AgNPs sizes were larger in both types of seawater than UTPW. The changes are likely related to dissolution and re-precipitation of individual NPs. Aggregation may have occurred but was not quantified.

## CHAPTER 4

### UPTAKE, ACCUMULATION, AND TOXICITY OF SILVER NANOPARTICLES IN ENVIRONMENTALLY RELEVANT EXPOSURE TO MARINE BIVALVE CLAM

#### *MERCENARIA MERCENARIA*

#### 4.1 Abstract

Due to the rapid development and production of manufactured nanoparticles, silver nanoparticles (AgNPs), their uptake and toxicity in the aquatic organisms and environment represent a major concern. AgNPs coated with either citrate (cit. AgNPs) or polyvinylpyrrolidone (PVP; PVP-AgNPs) were exposed to bivalve mollusks juvenile hard clam *Mercenaria mercenaria* at a range of concentrations (1–100  $\mu\text{g L}^{-1}$ ). Transformations of the AgNPs, along with their uptake and biological effects were measured. DLS and TEM measurements indicated an average particle size of (23nm) for both particle types in ultrapure water (UTPW). A multi-method approach showed that both particle types underwent rapid and concentration-dependent dissolution and agglomeration. The result of the study confirmed that clams tissues accumulated Ag in all treatments. Acute toxicity tests 24h showed that AgNPs enhanced the toxicity of AgNPs. The mortality data showed that juvenile hard clam *M. mercenaria* are highly sensitive to AgNPs and ionic Ag. Chronic exposure result showed less mortalities percent's among clams. The result of the study

Confirmed that clams tissues accumulated Ag in all treatments. The accumulation Ag effects were most intensification for the AgNPs than Ag ionic. The larger percentage and mass of Ag was most apparent in the ( $1\mu\text{g L}^{-1}$ ) concentration for both Cit. AgNPs and PVP-AgNPs, and ionic Ag, the Ag accumulation was decreased with increased Ag concentrations exposure. This indicate that the higher concentration of AgNPs brought more aggregation, underwent less dissolution, and showed less accumulation, while the lower concentration showed less aggregation, more dissolution and higher accumulation. After the depuration period, Ag accumulation decreased. In the highest concentrations, histological alterations were observed. In general, the significant inductions measured showed that that chronic exposure enhanced sever histological damages than acute treatment.

## 4.2 Introduction

Silver nanoparticles (AgNPs) increasingly used in consumer products. Scientific interest due to their unique optical, catalytic and a broad antimicrobial spectrum [210-265]. At the meantime, they can be found in water filters, paints, cosmetics, detergents, clothing textiles, food packaging, medical devices, and electrical appliances [266]. Meanwhile, AgNPs applications are continue to increase, and there are alarms about their release into aquatic systems, while and their environmental hazards impacts are growing. Modeled AgNPs concentrations in aquatic environments by modeling studies [71, 76, 256, 257] are predicted values to be between 0.002 ng/L AgNPs in European surface waters [257], and 40  $\mu\text{g/L}$  Ag NPs in effluents of Taiwanese rivers [258]. Ag concentration values between 0.7 and 11.1 ng/L Ag NPs were measured in effluents of wastewater treatment plants (WWTP) over the seasons in Germany [259]. In aquatic systems, AgNPs display a strong

tendency toward sedimentation. Consequently, in aquatic habitats the availability of AgNPs is supposed to be higher for benthic organisms. However, there is still challenges in detection and quantification NPs in environmental matrices such as seawater [271]. These challenges may be partially attributed to the difficulties in distinguish between natural and manufactured NPs. Moreover, detection and quantification of NPs in the environment is challenging because of the lack of well-established procedures and technologies [272, 274]. Therefore, modeled studies are useful to provide predicted environmental concentrations (PECs) of NPs [273].

AgNPs is the most toxic metallic nanoparticles towards aquatic species, highly persistent in the environment and with a great aptitude to accumulate in water, sediments and organisms, [210, 272, 265]. AgNPs have not only antibacterial action [273], but also cytotoxic and genotoxic capabilities. ROS-consequent oxidative stress [275, 276], bio-membrane impairment [277], DNA damage [278], bioaccumulation [279-281] were detected in freshwater and marine organisms. Recently, many studies have been investigated AgNPs toxicity to different marine organisms such as microalgae [282], mussels [23, 283-289], clams [111, 287], oysters [288, 289] and fishes [290]. Meanwhile, other studies have been focusing on bivalve mollusks since they have been identified as an important target group for NP toxicity in marine environments [149, 290, 291]. Overall, most of these studies in bivalves focused on understanding the effects of AgNPs on in vivo waterborne exposed adult organisms [292- 310]. In addition, there is not often studies have conducted to investigate AgNPs impact on bivalve embryos [306, 316]. Specifically, in bivalve mussels [292] documented genotoxic effects in hemocytes and different protein expression patterns both in digestive gland and gills after the exposure to AgNPs for 15

days. Observation of the result study, which is investigated AgNPs exposure to mussel during 21 days confirmed that AgNPs caused destabilized the lysosomal membrane, provoked the loss of digestive cells and digestive gland integrity [292]. In addition, edema/hyperplasia in gills and hemocyte infiltration of connective tissues. However, higher-level effects derived from the exposure to AgNPs such as effects on growth, reproduction and embryo development in mussels that could affect mussel's population dynamics have not been explored yet.

Molluscs bivalve's species such as clams, oysters, and mussels are used as bioindicator of health environment in many national and international programming monitoring environmental pollution [111]. Therefore, it highly important obtain information's about bioaccumulation and toxicity of AgNPs in bivalves species, also gathering the data on the behavior of AgNPs in seawater, such as agglomeration, dissolution and deposition onto the sediment surface [115, 306, 310]. This data will help to get a good estimation about the bioavailability of AgNPs and correlate this with the observed toxicity, resulting in better understanding the impact AgNPs on aquatic organisms. Bivalve species are considered extraordinary ecological cleaners owing to their feeding mechanism by filter large volume of water and uptake and bioaccumulate chemical toxicants form water column [20]. Bivalves can be able to concentrate/bioaccumulate small particles in their tissues, which has led to concern as to whether AgNPs may bioaccumulate into the food chain and affect other organisms [303]. The bioaccumulation of AgNPs in bivalves may be influenced by several factors, including the concentration, exposure route, and size of the NPs [111]. *M. mercenaria* ideal bivalves species and highly adjustable to meet the requirements of toxicological experiments of different NPs. The aim of this study is to evaluate the effects

of exposure AgNPs to juvenile hard clam *M. mercenraia* and to assist their behavior in natural seawater (NFSW) and artificial seawater (SSW). Therefore, this study is the one of few studies have been conducted to investigate the effect AgNPs to juvenile bivalve mollusc's hard clams *M. mercenaria* as a model test study exposed to AgNPs at concentrations including environmentally relevant values.

### **4.3 Materials and Methods**

#### **4.3.1 Materials/Chemicals**

AgNPs synthesis performed by used sodium citrate  $C_6H_5O_7 \cdot 2H_2O \cdot 3Na$ ; in the 99% purity, supplied by Sigma Aldrich, (St. Louis, USA), silver nitrate ( $AgNO_3$ ) with 99% purity were supplied by Sigma Aldrich, (St. Louis, USA). Sodium borohydride ( $NaBH_4$ ), 98% pure supplied by Alfa Aesar (Ward Hill, USA), and polyvinylpyrrolidone (PVP) of molecular weight 10,000 (PVP10), 99% pure supplied by Sigma Aldrich (St. Louis, USA). A trace metal grade nitric acid (68-70%  $HNO_3$ ) supplied by Fisher Scientific (Nazareth, USA) was used to acidify samples for ICP-MS analysis. A natural seawater (NFSW) collected near the cost of Charleston in South Carolina, USA. filtered by passed it through sand at marine department at University of South Carolina lab facility. Synthetic seawater (SSW), which is Crystal Seas a bioassay grade sea salts in carbon-scrubbed 18-m $\Omega$  deionized  $H_2O$  were purchased from Pentair.



### **4.3.2 Synthesis and characterization of AgNPs**

A modified chemical reduction method were used to synthesis citrate coated AgNPs (cit. Ag)NPs by reduction of Ag<sup>+</sup> ions using sodium borohydride as a reducing agent and sodium citrate as a capping agent [308, 309, 311]. After synthesis completion, Cit. AgNPs suspension was cleaned with a citrate solution using a diafiltration approach (3 kDa cellulose membrane ultrafiltration disc, EMD Millipore) in order to remove excess reactant species and prevent re-equilibration before use. PVP-AgNPs prepared by using a ligand exchange approach [117, 254]. Half of Cit. AgNPs suspension was recapped by adding a 500 mg L<sup>-1</sup> PVP (10k) solution [254]. This amount of PVP was required to obtain surface coverage of AgNPs by PVP molecules to convey full steric stabilization [254]. Both solutions were stored at 4 °C in dark. All measurements and calculations for AgNP synthesis, exposures, and the data interpretation were based on the mass of Ag, not of AgNO<sub>3</sub>. AgNPs were characterized using a multi-method approach including surface plasmon resonance (SPR), dynamic light scattering (DLS), zeta potential, inductively coupled plasma mass spectroscopy (ICP-MS) and transmission electron microscopy (TEM), [321-323].

### **4.3.3 Exposure**

Prior to exposure, Juvenile hard clam, *M. mercenaria* were acclimated in natural seawater 20 psu, with an air pumping at 24-25 °C and put aquarium under 12 h light and dark cycle. The clams that are used in this study were purchased from commercial aquaculture farm, the sunrayvenus clam, Florida. Once arrival, clam stay under laboratory conditions for acclimation at least one week until mortality was no longer was observed.

In accordance with other toxicological studies [324, 325], which are using bivalves as models organisms. The acclimation period was followed. Subsequently, clams were placed in 300 mL beakers containing seawater of 20 ppt. Two types of coating of AgNPs used as a provider of Ag and compared with Ag source from AgNO<sub>3</sub>. AgNPs and AgNO<sub>3</sub> exposure to *M. mercenaria* were designed for two periods, acute exposure for 24 h without algae feeding and chronic exposure for 7 days with algae feeding. In each aquatic environment exposure clams were exposed to (1, 10, 50, and 100 (µg L<sup>-1</sup>)) of the Cit. AgNPs and PVP Ag-NPs, and AgNO<sub>3</sub> in comparison with the negative control. For chronic exposure, clams were feed with a mixed diet of Shellfish Diet 1800® (Isochrysis sp. (40%), Pavlova sp. (15%), Thalassiosira weissflogii (20%) and Tetraselmis sp. (25%), (Reed Mariculture), were added in amount of (6 x 10<sup>6</sup> cells/mL). Three replicates for each treatment were conducting for both exposure and 10 clams were added per each of treatment. During chronic exposures, the overlaying water was renewed every two days and the algae were supplemented (6 x10<sup>6</sup> cells/mL), afterward, AgNPs were added to the water exposure to maintain the mass of Ag NPs as the same of the exposure were starting.

#### **4.3.4 Measurement of Ag in exposure media**

One the exposure time (24 h and 7 days) done, the clams was taken out, and the survival was scored under light microscopy. The clams that could not move were regarding as dead. For the uptake and depuration of the Ag assay, Alive clams were chosen for depuration test and taken out and put them in clean seawater for further 24 h in the acute exposure only. The remains clams were used for histological analysis and the dead clams were dissecting carefully on 24h and 7 days. The clam's tissues were digested in 5 mL centrifuge tubes, with concentrated trace metal grade nitric acid. Tubes were placed in an incubator shaker

(New Brunswick Scientific Innova 44 Incubator Shaker Series) at 300 rpm, and 20°C, for 24 hours. Digested tissues were made to a total acid concentration of 1%, filtered with a 0.45 µm filter (sterile PTFE membrane) and analyzed for Ag via ICP-MS (Perkin Elmer NexION 350D) to measure total Ag.

#### **4.3.5 Histological observations**

Classic histology methods were used to produce tissue sections for histological analyses of clam's digestive gland and gills. Briefly, clams' tissues were preserved in 4% buffered formalin for 24h, 72 h after which the samples were 50% ethanol. Then, embedding preparation tissues were dehydrated further through 70%, 96% and absolute ethanol and cleared in xylene, and soaked for 24 h in paraffin wax (Paraplast, Fluka, Germany), using Leica-TP 1020 (Leica, Germany) processor. The next step is placed tissues in blocks filed with liquid paraffin at 60 °C. After cooling, sections were cut at 2 µm using a microtome Leica RM 2125 RT (Leica, Germany) and dewaxed overnight in an oven at 60 °C. Dried sections were stained with haematoxylin and eosin stains (Sigma-Aldrich). After staining, sections were dehydrated in increasing concentrations of ethanol (70–100%), cleared in xylene, and mounted in Biomount DPX (Sigma-Aldrich). Tissue sections were examined microscopically using Leica DM 2500 microscope (Leica, Germany). Microphotographs were taken using digital camera (Moticam 352) connected to microscope.

#### 4.3.6 Statistical analysis

The statistical analysis software package (SPSS 22) was used to identify significant treatment effects. Normality of the data and homogeneity of variance were analyzed. A one-way analysis of variance (ANOVA) was performed to compare the effect of concentration, type of Ag used (NP or ionic) and time of exposure. A parametric ANOVA test or T test was used to identify significant differences between the treatments and clean day zero control, followed by a multiple comparison using Dunett's tests and Pearson correlation analysis, applied to assess the correlations between accumulation and concentration of exposure. Trimmed Spearman-Kärber method [303], were used to calculate the median lethal concentrations ( $LC_{50}$ ). This method is accurate to use for calculation the  $LC_{50}$  values and their 95% confidence interval endpoints [303]. Significance level on a  $p \leq 0.05$  as the value to identify statistically significant treatment effects, and it used in the calculation ( $LC_{50}$ ).

### 4.4 Results

#### 4.4.1 AgNPs characterization

Ag NPs stock suspension was monodispersed with the mean value of  $(23.5 \pm 3.5 \text{ nm})$  as shown by dynamic light scattering analysis, which is described previously in chapter 3, (**Figure 3.1**). TEM images of stock NPs confirmed a spherical particle shape for both cit. AgNPs and PVP- AgNPs with an average particle size of  $18.85 \pm 3.33 \text{ nm}$  and  $20.15 \pm 14.08 \text{ nm}$  (mean  $\pm$  1 standard deviation  $n = 100$ ) (**Table 3.3**). In chapter 3 we described Cit. AgNPs and PVP-AgNPs characterization in both SSW and NFSW, and compared their characterization in UHPW. These characterizations are done in all media without clams. In

this chapter will do characterization AgNPs in both seawater types during exposures within addition of clams. In the (Figures. 4.1, 4.2, 4.3, and 4.4) reported the typical transmission electron microscopy (TEM) of synthesized Cit. Ag NPs and PVP-AgNPs in SSW and NFSW at time 0.0 h. and 24 h, in concentrations ( $50 \mu\text{g L}^{-1}$ ) and ( $100 \mu\text{g L}^{-1}$ ). All treatments showed transformed AgNPs and apparent aggregation were observed by TEM images indicated influences both types of media and animals on the fate and behavior AgNPs.

#### **4.4.2 Uptake, accumulation and elimination of Ag**

##### **4.4.2.1 Acute exposure**

Uptake Ag and accumulation of total Ag dissolved from Cit. AgNPs, PVP- AgNPs in 10 juvenile clams in the exposure concentrations (1, 10, 50, and 100)  $\mu\text{g L}^{-1}$  Ag were tested. The trends of Ag accumulation in tissues during the 24h exposure time were shown in (Figure 4.6). To verify Ag accumulation in this study, we need to know in which concentration and which media that have the major NP effect could be waited, due to the Ag locally accumulated. For all concentrations and figures reported the respective background concentration in the not exposed control animals was subtracted ( $0.1 \pm 0.004 \mu\text{g Ag/mg dw. organism}$ ,  $n = 10$ ). This value was determined as the detection limit using the procedure. The uptake experiments results of exposure with Cit. AgNPs, PVP-AgNPs, and  $\text{AgNO}_3$  to juvenile *calm M. mercenraia* showed a rapid increase in animal Ag accumulation from the beginning of exposure reaching Ag accumulation at 24 h in ( $1\mu\text{gL}^{-1}$ ) concentration higher than other exposure concentrations in both SSW and NFSW. In the (Figure 4.6), total uptake percentages of silver in clams. It is represented percentages of

the total silver uptake and depuration in individual clams based on initial addition of Cit. AgNP, PVP-AgNPs, and AgNO<sub>3</sub>. (A and B) represent the uptake and depuration the acute exposure in SSW and (C and D) are uptake and depuration in the acute exposure in NFSW. In the (1 µg L<sup>-1</sup>) concentration, the total uptake percentages showed an average (0.52 ± 1%) uptake of initially added Ag an average of (1.63 ± 1 µg) for PVP-AgNPs treatment in SSW. The (0.38 ± 1 %) uptake of initially added Ag an average of (1.14 µg) for AgNO<sub>3</sub> in SSW, while Cit. AgNPs showed (0.22 ± 1 %) uptake of initially added Ag and an average of (0.67 µg) in SSW media. The total uptake percentages of AgNO<sub>3</sub> in (1 µg L<sup>-1</sup>) in NFSW showed (0.41 ± 1 %) uptake of initially added Ag an average of (1.22 µg). PVP-AgNPs in NFSW at 24h accumulated (0.39 ± 1%) uptake of initially added Ag an average of (1.16 µg), however Cit. AgNPs in this media reached an average (0.17 ± 1 %) uptake of initially added average (0.51 µg), **(Figure 4.6)**. The total uptake percentages in the other concentrations showed decline Ag accumulation reached to levels closed to the blanks values. In the depuration phase, Ag accumulation has a general trend decreasing towards 24h exposure time **(Figure 4.6)**. After transfer to clean seawater medium, a statistically significant decrease Ag accumulation in clams were observed in (1 µg L<sup>-1</sup>) in SSW from starting depuration phase reaching (0.12, 0.32, and 1.06 ng Ag/mg dw) organism in Cit. AgNPs, PVP-AgNPs, and AgNO<sub>3</sub> at 24h respectively. The total Ag percentages in (10, 50, and 100 µg/L) was significantly decline at the end of depuration period after 24h. In NFSW the same trend of Ag depuration from clams tissues were seen in (1 µg L<sup>-1</sup>) with (0.06, 0.31, 0.09) for Cit. AgNPs, PVP-AgNPs, and AgNO<sub>3</sub> respectively. In the other concentration of exposure experiments, Ag depuration percentages flattening towards 24 h was observed **(Figure.4.6)**.

#### 4.4.2.2 Chronic exposure

In the (Figure 4.8) distribution of Ag concentrations between the AgNO<sub>3</sub>, Cit. AgNPs, and PVP- AgNPs of *M.mercenaria* in chronic exposure. The mean masses (n = 3) of Ag accumulated by whole clams tissues (µg/mg dry weight) at all concentrations in SSW and NFSW, which were exposed to the experimental conditions (control, AgNO<sub>3</sub>, Cit. AgNPs, and PVP-Ag NPs) after 7 days. The higher total Ag uptake showed in concentrations (1, 10 µg L<sup>-1</sup>) for AgNO<sub>3</sub> in SSW with (0.2, 0.18 ±1) % uptake of initially added Ag an average of (0.6, 5.5 Ag µg /mg. dw). While in (NFSW) the highest total percentage uptake was (0.165, 0.174, and 0.19 Ag % µg L<sup>-1</sup> )of initially added Ag an average of (0.5, 0.52, and 0.57 µg L<sup>-1</sup> ) for Cit. AgNPs, PVP-AgNPs and AgNO<sub>3</sub> respectively in concentration (1µg/L).

#### 4.4.3 Toxicity of AgNPs

##### 4.4.3.1 Acute exposure

Mortality of juvenile clam's hard clam *M.mercenaria* occurred in all treatments for 24 h and 7 days were reported in (Figure 4.6, A and B). Results generally showed increasing toxicity with increasing Ag concentrations in all treatments in both acute and chronic toxicity tests. Result also showed differences between controls and AgNO<sub>3</sub> and AgNPs treatments in both acute and chronic toxicity tests. Significant mortality generally occurred at higher doses (50,100 µg/L<sup>-1</sup>) in all AgNO<sub>3</sub> and AgNPs treatments, regardless of seawater exposure type [synthetic seawater (SSW) and natural filtered seawater (NFSW)]. Acute Cit. AgNPs exposures resulted in 20% to 60% of mortality in SSW and the LC<sub>50</sub> was 27.51 µg L<sup>-1</sup> (± 1.70 µg L<sup>-1</sup>) [Table 4.1]. Acute exposure to PVP-AgNPs in SSW induced 30%

mortality at the lowest (1  $\mu\text{g/L}$ ) concentration and 70% of mortality at the highest concentration (100  $\mu\text{g/L}$ ) with an  $\text{LC}_{50}$  of 40.1  $\mu\text{g L}^{-1}$  ( $\pm 1.63 \mu\text{g L}^{-1}$ ), respectively. The treatments containing  $\text{AgNO}_3$  in the same media alone caused 10% at the lowest concentration (1  $\mu\text{g/L}$ ) and 80% in the highest concentration (100  $\mu\text{g/L}$ ) tested, with an  $\text{LC}_{50}$  of 25.6  $\mu\text{g L}^{-1}$  ( $\pm 1.54 \mu\text{g L}^{-1}$ ). The NFSW media containing the lowest Cit. AgNPs (1  $\mu\text{g/L}$ ) concentration in acute exposure caused 30% of mortality, and the highest Cit. AgNPs (100  $\mu\text{g/L}$ ) concentration caused more than 60% of mortality, with an  $\text{LC}_{50}$  of 46.56  $\mu\text{g/L}$  ( $\pm 1.38 \mu\text{g L}^{-1}$ ) (**Table 4.1**). Where, in the same media NFSW during acute exposure highest concentrations of PVP-AgNPs induced 50% of mortality. The effects of  $\text{AgNO}_3$  alone in NFSW, after 24h of exposure of *M. mercenaria* are shown in (**Table 4.1 and Figure 4.1**). The survival data indicated that there were some differences in toxicity comparisons between  $\text{AgNO}_3$  and some of the AgNPs in the juvenile hard *M.mercenaria*. Initial statistical comparisons using Trimmed Spearman Karber analysis, suggested there were no significant ( $p > 0.05$ ) differences in acute toxicity among treatments. However, further statistical analysis using multiple comparison tests (Mann Whitney) comparing replicate  $\text{LC}_{50}$  values for each treatment indicated that there were significant ( $p < 0.05$ ) differences among treatments in both the acute and chronic toxicity tests. In the acute toxicity tests,  $\text{AgNO}_3$  in SSW was the most toxic compound, which was only more toxic than in Cit. AgNPs in NFSW and PVP -AgNPs in SSW. All other comparisons in the acute toxicity tests were not significantly different (Toxicity of  $\text{AgNO}_3$  in NFSW =  $\text{AgNO}_3$  in SSW = Cit. AgNPs in SSW = PVP- AgNPs in NFSW).



#### 4.4.3.2 Chronic exposure

Results in the **Table 4.1** and **Figure 4.1 C and D** describe chronic mortality results for the juvenile clam *M. mercenaria* during 7 days exposure to Cit. AgNPs, PVP-AgNPs, and AgNO<sub>3</sub> in NSSW and SSW at concentrations 0, 10, 50 and 100 µg/L. In SSW media, containing Cit. AgNPs mortality ranged from 30% in (1 µg/L) to 60% of mortality in (100 µg/L), with an LC<sub>50</sub> of 35.87 µg L<sup>-1</sup> (± 1.70 µg L<sup>-1</sup>). In the PVP-AgNPs 7 day chronic exposure in SSW, mortality ranged from 10% (1 µg/L) concentration to 60% at the highest concentration (100 µg/L), with an the LC<sub>50</sub> of 66.07 µg L<sup>-1</sup> (± 1.56 µg L<sup>-1</sup>). In AgNO<sub>3</sub> in the same SSW media, mortality ranged from 10% at the lowest concentration (1 µg/L) to 70% in the highest concentration (100 µg/L), with an LC<sub>50</sub> of 46.56 µg L<sup>-1</sup> (± 1.38 µg/L). The NFSW media containing the Cit. AgNPs showed 70% of mortality as the highest mortality percent at the 50 µg/L dose with an LC<sub>50</sub> of 46.56 µg L<sup>-1</sup> (± 1.38 µg L<sup>-1</sup>) (**Table 4.1**). Where, in the same NFSW media, PVP-AgNPs mortality was 60% in both the 50 and 100µg/L doses, with an LC<sub>50</sub> of 69.62 µg L<sup>-1</sup> (± 2.09 µg L<sup>-1</sup>). In the AgNO<sub>3</sub> exposure in NFSW, after 7 days mortality ranged from 10 to 60% mortality in juvenile clams, *M. mercenaria* (**Table 4.1** and **Figure 4.1**). Overall, the survival data indicated that some of the AgNPs were extremely toxic to juvenile hard *M.mercenaria*. Initial statistical comparisons of chronic toxicity of the nanoparticles (Cit AgNPs in NFSW, Cit AgNPs in SSW, PVP-AgNPs in NFSW and PVP- AgNPs in SSW) with conventional silver nitrate (AgNO<sub>3</sub> in NFSW and AgNO<sub>3</sub> in SSW) using Trimmed Spearman Karber analysis, suggested there were no significant (p > 0.05) differences in toxicity among treatments across the range of concentrations tested. However, further statistical analysis using multiple comparison tests (Mann Whitney) comparing triplicate LC<sub>50</sub> values for each

treatment indicated that there were significant ( $p < 0.05$ ) differences among treatments in the chronic toxicity tests. The Cit. AgNPs in NFSW was the most toxic compound, which was more toxic than  $\text{AgNO}_3$  SSW and both of these compounds were more toxic than  $\text{AgNO}_3$  in NFSW, Cit. AgNPs in SSW, PVP- AgNPs in NFSW, and PVP-AgNPs in SSW media. All other comparisons in the acute toxicity tests were not significantly different (Toxicity of  $\text{AgNO}_3$  NFSW = Cit. AgNP s in SSW = PVP- AgNPs in NFSW = PVP-AgNPs in SSW). Similar statistically comparisons of LOEC and NOEC values were calculated for all treatments (**Table 4.1**) and were not statistically different ( $p > 0.05$ ). Thus, it would appear that only differences in the median range ( $\text{LC}_{50}$ ) of toxicity was observed.

#### 4.5 Histological alterations

The microscopy images indicated histopathological changes in the gills, digestive gland, and mantle of clam *M. mercenaria* exposed to AgNPs and compared with control treatment to get information about the histological alterations observed after acute and chronic exposure. The microscopy representative images of haematoxylin and eosin–stained sections of the gills and digestive gland from *M. mercenaria* control group (**Figure.4.9, (A, B)**) and those exposed to AgNPs with concentration (0,1, 10, 50, and 100  $\mu\text{g/L}^{-1}$ ) at 24 h and 7 days are shown in (**Figure 4.9**). Generally, in the control treatment, gills have two plates at each side of the organism and the gill plate has a number of gills filaments. The wall of gill filaments normally surrounded with ciliated columnar epithelial cells and between them; there are a number of mucous secreting cells. The normal structure of the clam's digestive gland consists of digestive tubules (digestive diverticula) formed by a single layer of ciliated epithelial cells, with an almost occluded lumen. The normal intertubular tissue is formed by a few fibrocytes and haemocytes (hyalinocytes). In

specimens from control animals, digestive tubules showed normal round/oval structure, lined by a columnar epithelium; there was no evidence of haemocyte infiltration, necrosis or other damage (**Figure 4.9, A**). In acute exposure treatments, AgNPs exposure cause deterioration in the integrity of the gill structure and resulted in the loss of cilia and contact between gill filaments in the Cit. AgNPs (50, 100 µg/L) (SSW) in (**Figure 4.9, C**). In the same concentrations, necrotic cells were found in the digestive gland tissues and mental (**Figure 4.6, D, E, and F**) in SSW. In chronic exposure PVP-AgNPs treatment in NFSW, gill tissue defects and loss of contact between gill filaments and haemocyte infiltration in the digestive gland were observed; however, the general appearance was close to the control (**Figure 4.9, H, I, and N**). In AgNO<sub>3</sub> treatment, there was a deterioration in the epithelial tissue and haemocyte infiltration in the gills but the general obseravtion of the digestive gland was similar to the control (**Figure 4.9, K, and L**). In the treatment Cit. AgNPs, media SSW, contact loss and haemocyte infiltration were observed between the filaments of the gill tissues, whereas necrotic cells and haemocyte infiltration were found in the digestive gland (Figure 4.9, O, P, Q, and M). It was observed that both goblet cell numbers and AgNPs reaction intensity decreased in Ag NPs and chronic treatments where high Ag accumulation was observed (Figure 4.9. K, and N).

## 4.6 Discussion

### 4.6.1 NP characterization

TEM results, confirm rapid NP transformation in media (In chapter 3). This loss occurred most primarily via dissolution. However, TEM characterization also showed the presence of NPs with a primary NP size of  $20.7 \pm 2.5$  nm, which is significantly larger than the original NPs ( $p < 0.05$ ). Smaller AgNPs at environmentally relevant concentrations (from 0.01 ng/L to 10  $\mu$ g/L in surface water) were shown to dissolve more rapidly and to a greater extent in seawater than in freshwater [312]. It is possible that the remaining AgNPs increased in size due to smaller particles dissolving faster leaving larger particles in suspension. Most images contained agglomerates, rather than individual NPs, and agglomerates were not observed in the original NP sample, indicating aggregation occurred in media. Therefore, it is likely that a fraction of NPs grew in size via dissolution and ripening, and additionally appeared as larger agglomerates rather than dispersed NPs. This is in agreement with previous studies with substantial transformations, which were dependent on NP properties and concentration and media properties [262, 313-314]. Similar losses of AgNPs in SSW have also been observed elsewhere [262]. According to Gomes et al. [285], only 24.5% of the initial amount of AgNPs in seawater remains in the dissolved form, therefore it can be assumed that most of the Ag present in solution is nanoscales. Smaller AgNPs at environmentally relevant concentrations (from 0.01 ng/L to 10  $\mu$ g/L in surface water) were shown to dissolve more rapidly and a greater extent in seawater than in freshwater [312]. On the other hand, due to the complexation with  $\text{Cl}^-$ ,  $\text{Na}^+$ ,  $\text{Ca}^+$ ,  $\text{S}_2\text{O}_3^{2-}$  and NOM, AgNPs might be less bioavailable therefore, less toxic to marine biota [315]. The Ag levels below the LOD in AgNPs solutions in NFSW and SSW

support such hypothesis and confirm similar behaviour of AgNPs present in both seawater types. Results further suggest that, at lower concentrations, behavior is governed by dissolution while, at higher concentrations aggregation is dominant.

#### **4.6.2 Uptake, accumulation and elimination of Ag**

Uptake and accumulation Ag showed small differences despite of the total Ag uptake and ionic Ag concentrations in the exposure seawater were clearly affected by the seawater type and Ag source. The total Ag concentrations in the NFSW and SSW water treatments were clearly influenced by type of seawater and source of Ag dissolved, the **(Figure. 4.5)** distribution of Ag in whole body tissues of clam *M. mercenaria* from the Cit- AgNPs, PVP AgNP , AgNO<sub>3</sub> and control experiment after 24 h and 7 days of Ag accumulation. We observed in all treatments that Ag was accumulated within clam's tissues and in all exposures, the mass of Ag accumulated increased with increasing exposure concentration; as exposure, concentration increased the percentage of total silver accumulated decreased **(Figure 4.5)**. Accumulation shows a concentration dependent trend where higher exposure concentrations accumulated more Ag mass. About more than 50% of the total Ag accumulated within tissues at the (1 µg L<sup>-1</sup>) exposure, which corresponded to a mass of (0.1-1 µg-Ag (wet tissue)). This resulted in a higher percentage of initially added Ag accumulated in the more than (50 µg L<sup>-1</sup>) exposures, but less mass of Ag accumulated. There was (50-100%) of the total Ag accumulated in tissues at the (50 µg L<sup>-1</sup>) exposure, which corresponds to a mass of (50-100 µg-Ag). Ag uptake and bioaccumulation showed only small differences. This trend of Ag uptake, accumulation, and elimination could be explained by the that clams are be able to take up Ag ionic as well as NP form like other bivalves species [296, 301, 317 ]. The evidence come from the result of the study by Canesi

et al.[144] indicated that NP taken up by the gills of mussels are transported to the digestive gland, where intra-cellular uptake of materials in nano scale induces lysosomal perturbations and oxidative stress. A study result that showed NP and ionic Ag in a whole body autoradiography of *Mytilus edulis* had have a similar Ag distribution between animals from both treatments, with maximum concentrations located in the digestive organs in short time exposure [293]. However, modeling studies, which use biodynamic analysis to describe the dissolved metal dynamic inside the water exposure and organisms, the study by Kalman [309], indicated that the dissolved metal fraction as the dominant source of Ag accumulated in the estuarine clam *Scrobicularia plana* under different field exposure conditions. In contrast to bivalves, the toxicity of Ag NPs to other aquatic organisms such as *Daphnia magna* is a function of the dissolved Ag concentration [319]. For both Ag ionic and AgNP the accumulation plateau was reached within a short time after dosing Ag in water exposure. In the result, experiment of the study by Gomes et al. [308], the container water was renewed then re-dosed every 12 h and, the exposure concentration of 10 µg/L-1 was considerably low enough as shown in the present study. This specifies that animals possess 12 regulatory mechanisms to confine the Ag bioaccumulation, which means the Ag concentration increases to a saturation level at which all-binding sites are occupied and excess Ag is eliminated. This hypothesis could also explain the persistent, not decreasing Ag levels in the clam soft tissue during the depuration period. The result of the present study showed that no depuration for 7 days exposure that may be explained by a strong binding of Ag to endogenous binding sites in *M. mercenaria*. Congruently, there was no increase in the aqueous Ag concentration during the depuration. On another word, a clear Ag elimination might be showed after a depuration period of only 72 h for *Mytilus edulis*,

which were previously exposed to radiolabeled Ag [291]. At this point, radiolabeled Ag was found after the depuration in the shells of the mussels [291]. While the concentration was very low, this result may specify Ag elimination from soft tissue into shells. A study by Zuykov et al. [281] presented no Ag concentrations in the beaker water during depuration so that the estimation of the Ag elimination into the ambient water is impossible.

#### **4.6.3 Toxicity of AgNPs**

In this investigation, study result showed 80% mortality observed at the highest concentration (50,100 µg/L) after 24 h of exposure and 70% for 7days chronic exposure indicating that NP can cause severe injuries following the uptake. The results are in agreement with those obtained by Mendonca et al. [208], where a chronic toxicity test in *M. mercenaria* exposed to AgNPs showed highest mortality at concentrations higher than 50 µg/L. The mortality rates observed in the present study can be explained by the fact that these organisms are filter feeders, which capture food by filtering water, and; hence, possibly accumulated high levels of Ag, which in turn impaired their physiological status. NPs, as well as Ag<sup>+</sup> ions concentration in the exposure seawater was far below the nominal exposure concentration. A similar trend was observed for natural water from a eutrophic pond in a plankton exposure study using the same Ag species [310], and in other mussel exposure studies using seawater [124, 291, 296]. The difference between nominal and measured Ag concentrations in the beaker water could be explained by dissolution losses and adsorption processes at surfaces such as the beaker walls and clams' shells in combination with agglomeration/aggregation processes of the nanoparticles. Acute toxicity tests result generally indicate that AgNO<sub>3</sub> SSW was the most toxic compound which was only more toxic than Cit.AgNPs in NFSW and PVP-AgNPs in SSW. All other comparisons

in the acute toxicity tests were not significantly different (Toxicity of AgNO<sub>3</sub> NFSW = AgNO<sub>3</sub> in SSW = Cit. AgNPs in SSW = PVP- AgNPs in NFSW). Chronic toxicity results indicated that Cit. AgNPs in NFSW was more toxic than both AgNO<sub>3</sub> treatments when compared to the acute toxicity results, which indicated AgNO<sub>3</sub> in SSW was more toxic or equivalently toxic to any of the AgNPs treatments. This is suggestive that chronically over time Ag from the AgNPs, may be released and accumulated in clams with increasing levels of toxicity as evidenced by the increased toxicity observed in the Cit. AgNPs in NFSW. Bioaccumulation studies found that the higher rate of Ag uptake in clams was in Cit. AgNPs, which in part explains why the Cit. AgNPs NFSW was the most toxic compound tested in the chronic toxicity tests.

Further experiments with more chronic exposures will be needed to confirm this observed effect further. LOEC and NOEC values were calculated for all treatments (**Table 4.1**), NOEC values for both particle types were not statistically different ( $p > 0.05$ ). PNEC values were obtained by risk assessment procedures using a safety factor value of 1000 (Gottschalk et al. [71]). A Probable Exposure Concentration (PEC) value of 0.166 ng L<sup>-1</sup> ( $1.66 \times 10^{-4}$  µg L<sup>-1</sup>), was divided by PNEC values to calculate PEC/ PNEC risk assessment ratios for each treatment [238]. In all treatments PEC/ PNEC ratios were ( $>1$ ) suggesting that AgNPs may induce risk for juvenile clam *M. mercenaria*, when a 3 fold (1000X) margin of safety standard is applied.



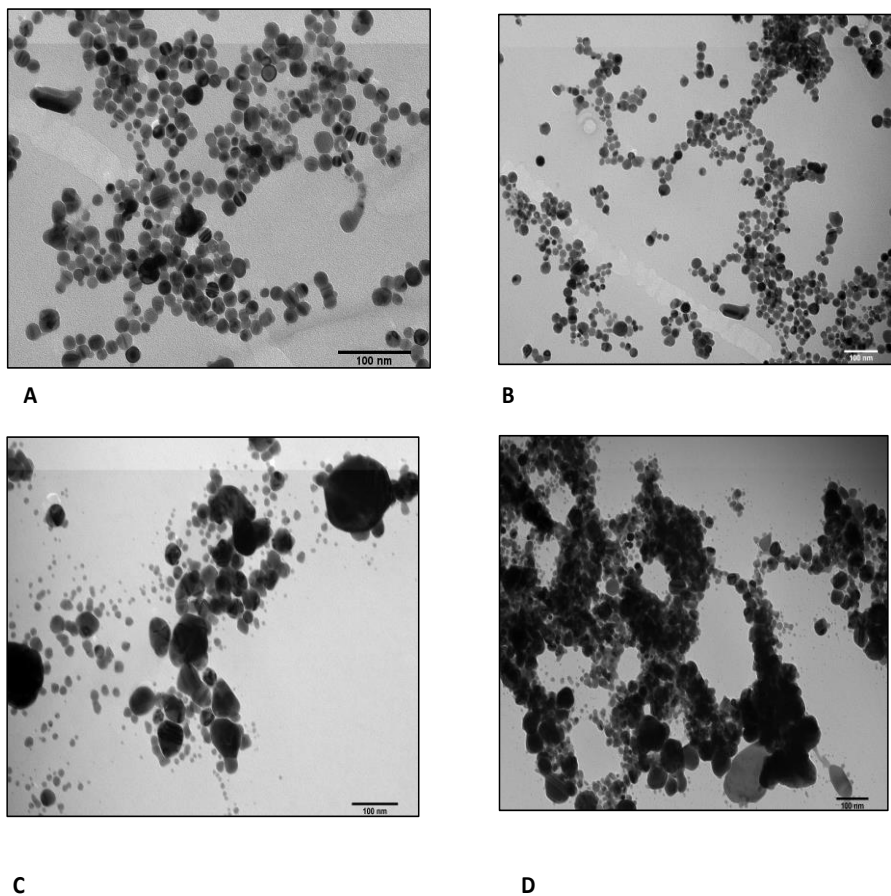
#### 4.6.4 Histological alterations

Histological alterations in gills and digestive glands have been demonstrated to be approachable to numbers of xenobiotic stresses since these organs play a significant role in respiration, food collection and digestive processes [22, 308]. The gills are the most rapidly affected by water pollutants. Bivalves are filter feeder, they use gills for feeding and respiration process. Gills considering with their functioning as a first line of defence against environmental pollutants that includes particle rejection, psuedofaeces formation, and mucous secretion [311, 313]. Gills and digestive gland represent the main sites of particle uptake in bivalves consequently, histopathological effects of nanoparticle exposure were examined in these target tissues. Most common changes in the gills after chronic exposure to AgNPs may be summarized as heavy deposition, epithelial exfoliation, necrosis and epithelial erosion as common occurrences. Comparing these symptoms with chronically exposed *M. mercenaria* revealed that accumulation/deposition of AgNPs also occurred in the gills after acute exposure. In mussels, copper accumulation following exposure to 32 µg/L-1 concentrations for five days caused severe abnormalities in different organs, such as swelling in the adductor muscle, erosion of the gill cilia and necrosis in the digestive tubules [318]. The presence of pigmented brown cells in *M. mercenaria* gills after chronic exposure to AgNPs, wherein brown cell accumulation was also seen in the gills, mantle, digestive tubules in acute exposure. Such pigmentation has been linked to the accumulation of granules thought to affect protein turnover [314, 317]. The higher levels of “dark” residual bodies were also observed within the tissue of species sampled from and mucous secretion [311, 313]. Gills and digestive gland represent the main sites of particle uptake in bivalves consequently, histopathological effects of nanoparticle exposure were

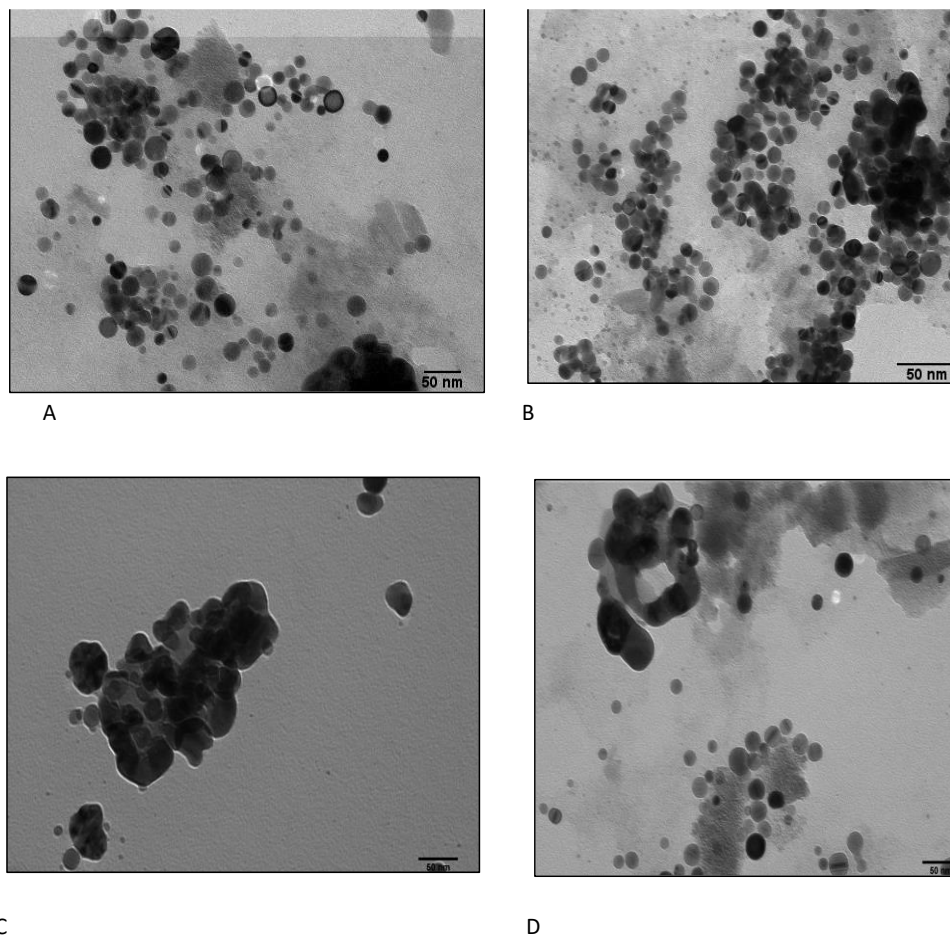
examined in these target tissues. Most common changes in the gills after chronic exposure to AgNPs may be summarized as heavy deposition, epithelial exfoliation, necrosis and epithelial erosion as common occurrences. Comparing these symptoms with chronically exposed *M. mercenaria* revealed that accumulation/deposition of AgNPs also occurred in the gills after acute exposure. In mussels, copper accumulation following exposure to 32 µg/L-1 concentrations for five days caused severe abnormalities in different organs, such as swelling in the adductor muscle, erosion of the gill cilia and necrosis in the digestive tubules [318]. The presence of pigmented brown cells in *M. mercenaria* gills after chronic exposure to AgNPs, wherein brown cell accumulation was also seen in the gills, mantle, digestive tubules in acute exposure. Such pigmentation has been linked to the accumulation of granules thought to affect protein turnover [314, 317]. The higher levels of “dark” residual bodies were also observed within the tissue of species sampled from polluted sites and the authors linked it to heavy metal, and organic pollutant. Increased observation of pigmented cells due to AgNPs exposure could therefore be consistent with altered lysosomal structure and function. Several authors suggest that lysosomes are implicated in non-specific protein turnover and contribute to proteolytic breakdown of ingested foreign matter [319, 320]. The digestive gland is an important organ involved in *M. mercenaria*, the digestive glands acutely exposed to AgNPs displayed degeneration of the epithelium and lumen with cellular damage observed along with widespread intertubular neoplasia and hyperplasty. Necrotic changes were also observed with hypertrophy of the epithelium cells and lumen with cellular debris. Upon chronic exposure to AgNPs the digestive gland exhibited tubule damage and the complete loss of tubule epithelium.

## 4.7 Conclusion

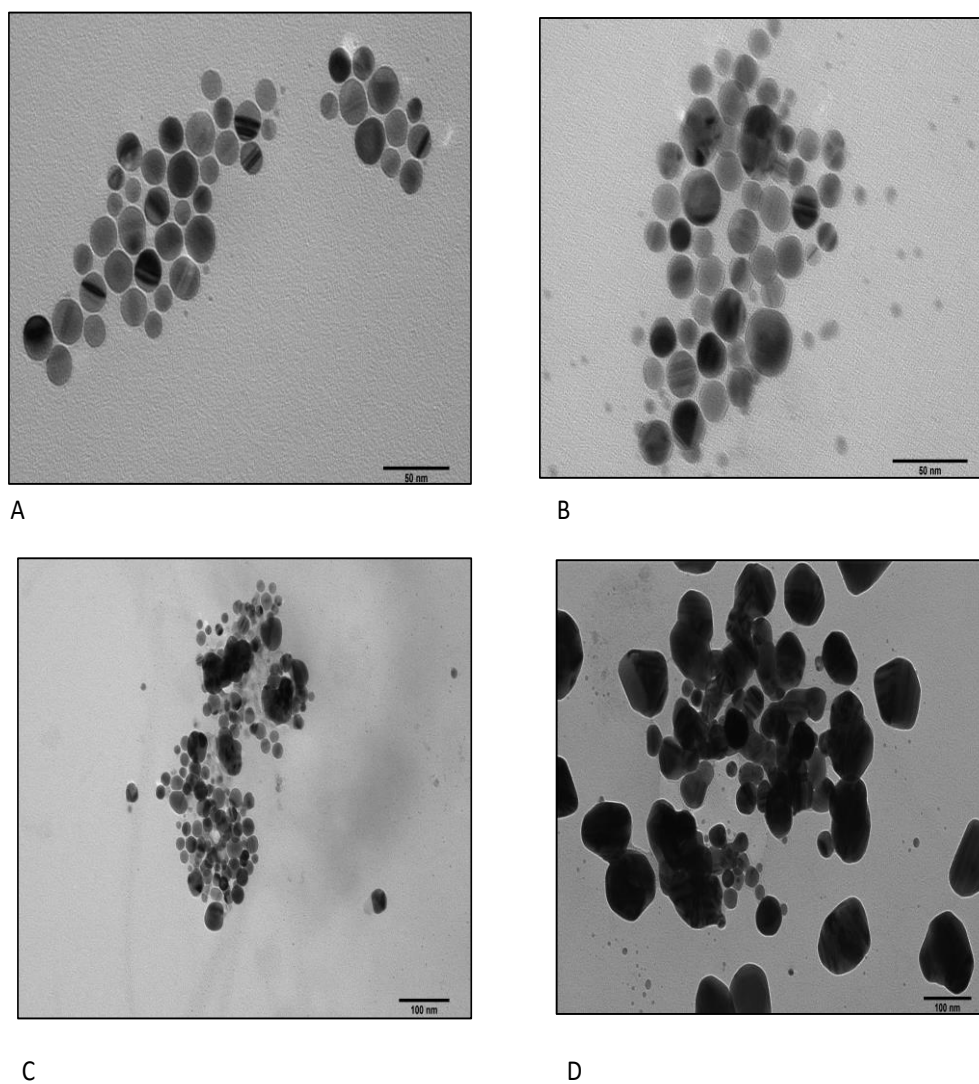
This study was conducted with marine mollusk bivalve clam *M. mercenaria* exposed to Cit AgNPs and PVP AgNPs and the result compared with ionic Ag. In this study, *M. mercenaria* facilitated the taken up AgNPs from water and affected the fate and transformation of AgNPs. Thus, a significant amount of Ag accumulated in clam tissues and posed a threat to organisms. The results revealed that the bioaccumulation of Ag resulted in toxic effects and the intensified of histological alteration in *M. mercenaria*. Ag uptake trend was changed in *M. mercenaria* at varied doses of AgNPs. According to AgNPs accumulation in whole body, tissues are more sensitive to lower concentration. This study furthers the understanding of the interaction between AgNPs and *M. mercenaria*, providing meaningful information on the fate and toxicity of AgNPs occurring in natural aquatic environment. Consequently, the result confirmed that clams could be used as effective accumulation indicators for monitoring of environmental contaminations of ionic Ag and Ag NPs.



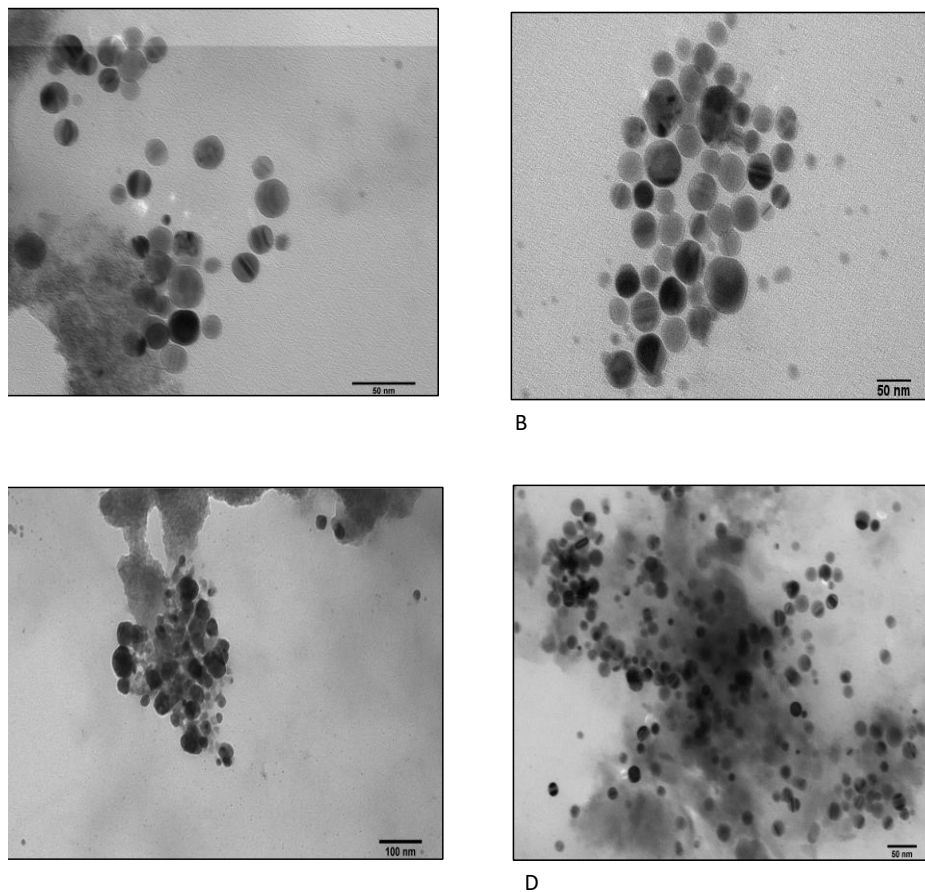
**Figure. 4.1** Typical transmission electron microscopy (TEM) of synthesized Cit- Ag NPs in (SSW) at time 0.0 h. and 24 h., (A).  $^1\text{Cit. AgNPs } 50 \mu\text{g L}^{-1}$  at time 0.0 h, (B).  $^1\text{Cit. AgNPs } 100 \mu\text{g L}^{-1}$  at time 0.0 h, (C).  $50 \mu\text{g L}^{-1}\text{Cit. AgNPs}$  at time 24 h, (D).  $100 \mu\text{g L}^{-1}\text{Cit. AgNPs}$  at time 24



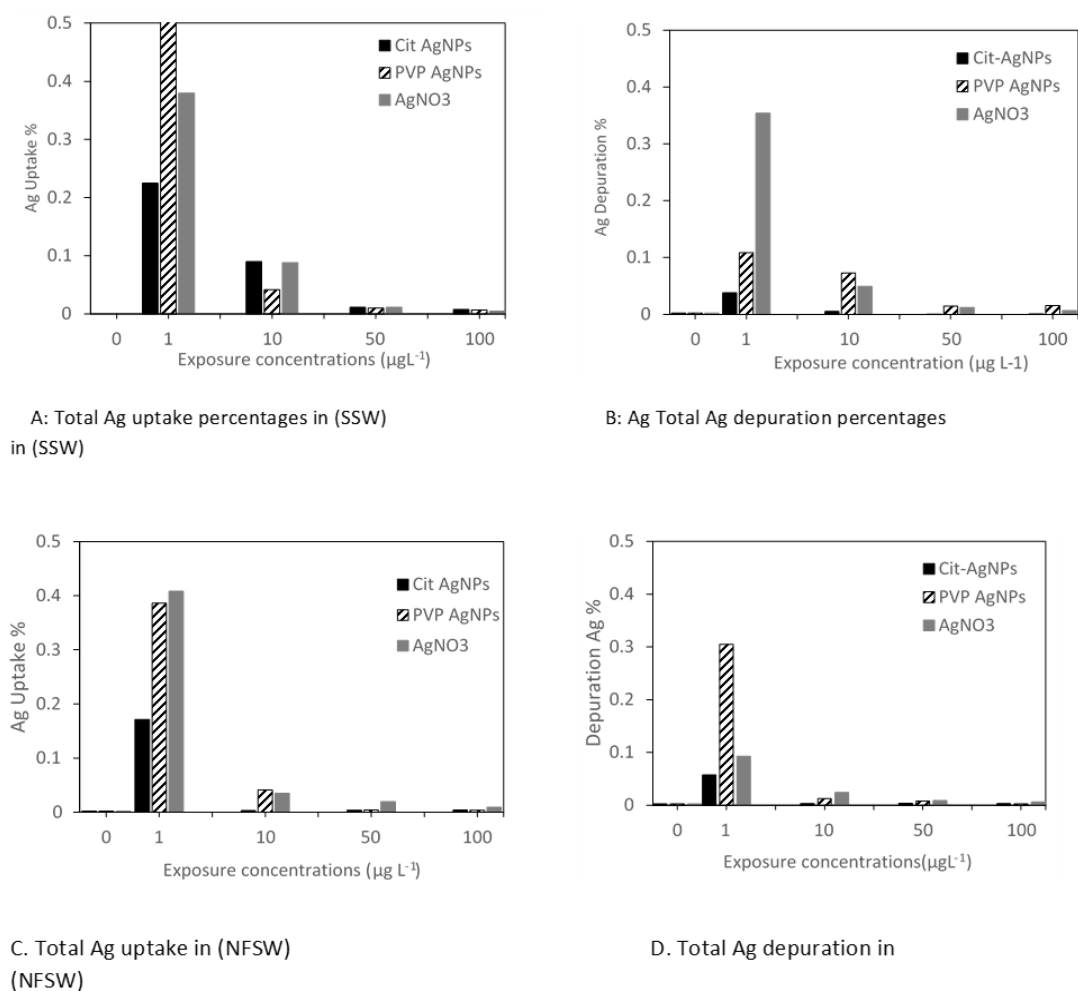
**Figure. 4.2** Typical transmission electron microscopy (TEM) of synthesized of Cit- Ag NPs in (NFSW) at time 0.0 h. and 24 h., (A). Cit. AgNPs, 50 µg L<sup>-1</sup> at time 0.0 h, (B). <sup>1</sup>Cit. AgNPs 100 µg L<sup>-1</sup> at time 0.0 h, (C).50 µg L<sup>-1</sup>Cit. AgNPs at time 24 h, (D).100 µg L<sup>-1</sup>Cit. AgNPs at time 24 h.



**Figure. 4.3** Typical transmission electron microscopy (TEM) of synthesized of PVP Ag NPs in (SSW) at time 0.0 h. and 24 h., (A).  $10^{-1}$  PVP AgNPs  $50 \mu\text{g L}^{-1}$  at time 0.0 h, (B). PVP AgNPs  $100 \mu\text{g L}^{-1}$  at time 0.0 h, (C).  $50 \mu\text{g L}^{-1}$  PVP. AgNPs at time 24 h, (D).  $100 \mu\text{g L}^{-1}$  PVP. AgNPs at time 24 h.

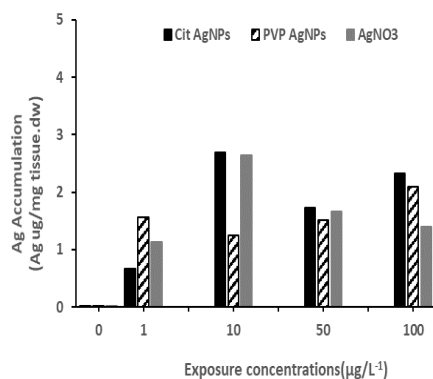


**Figure. 4.4** Typical transmission electron microscopy (TEM) of synthesized of PVP Ag NPs in (NFSW) at time 0.0 h. and 24 h., (A).50  $\mu\text{g L}^{-1}$  PVP AgNPs at time 0.0 h, (B). PVP AgNPs 100  $\mu\text{g L}^{-1}$  at time 0.0 h, (C).50  $\mu\text{g L}^{-1}$  PVP. AgNPs at time 24 h, (D).100  $\mu\text{g L}^{-1}$  PVP. AgNPs at time 24 h.

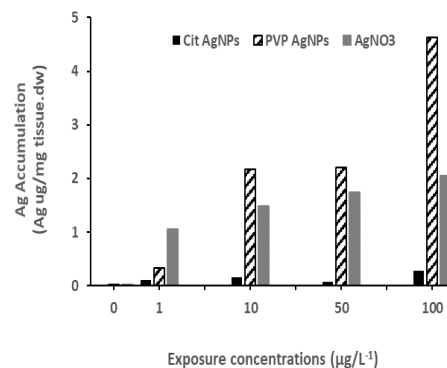


**Figure 4.5** Total uptake percentages of Ag in clams. Percentages of the total silver uptake in individual clams based on initial addition of Cit. AgNP, PVP-AgNPs, and AgNO<sub>3</sub>. The (A, B) represent the uptake and depuration the acute exposure in (SSW) and (C, D) are uptake and depuration in the acute exposure in (NFSW).

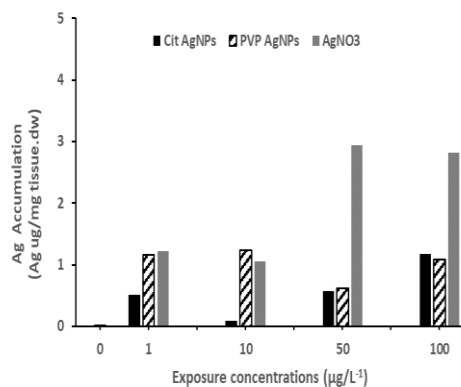




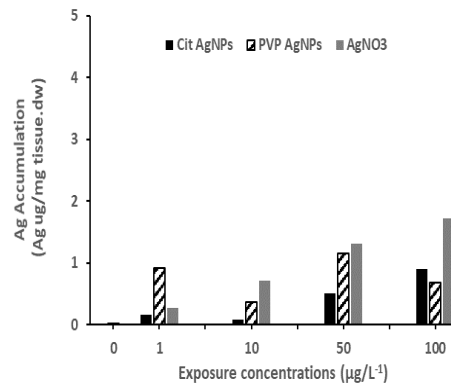
(A): Uptake phase in (SSW).



(B): Uptake phase in (NFSW).

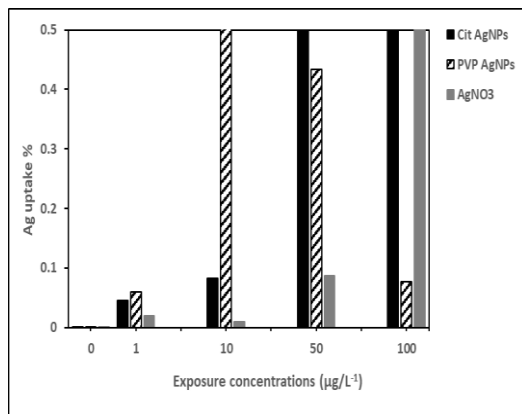


(C): Depuration phase in (SSW).

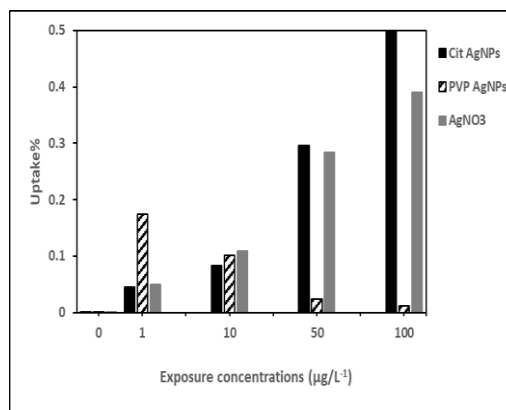


(D) Depuration phase in (NFSW).

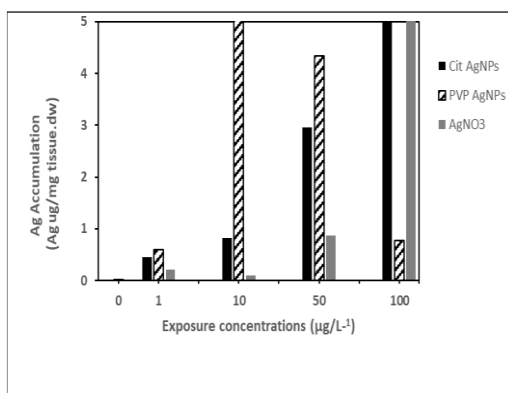
**Figure 4.6** Distribution of Ag concentrations to the experimental treatments AgNO<sub>3</sub>, Cit. AgNPs, and PVP-AgNPs of *M. mercenaria* after 24 h acute exposure. (A). uptake phase in (SSW). (B). uptake phase in (NFSW). (C). depuration phase in (SSW). (D). depuration phase in (NFSW).



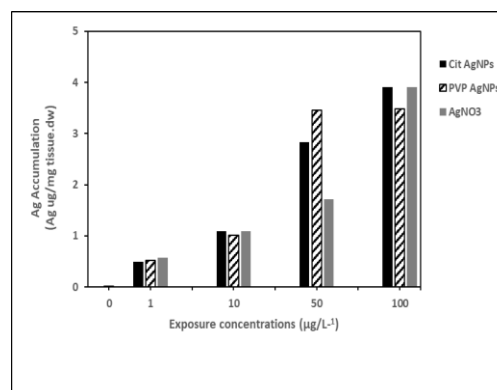
(A): Uptake phase in (SSW).



(B): Uptake phase in (NFSW).



(C): Ag concentrations in (SSW)

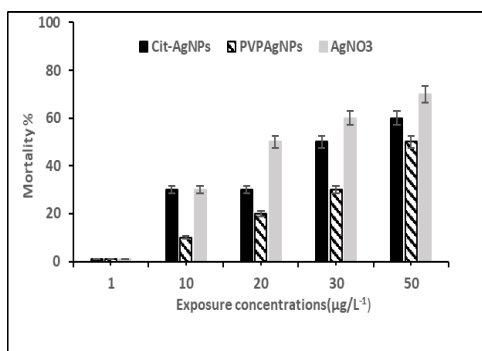


(D): Ag concentrations in (NFSW)

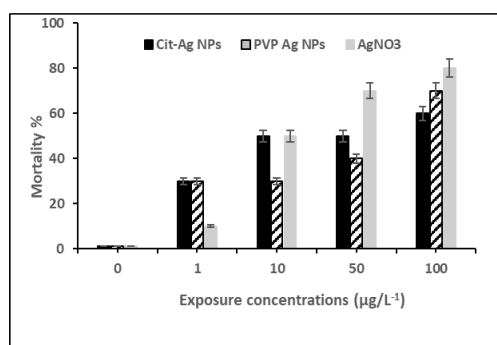
**Figure 4.7** Chronic exposure of AgNO<sub>3</sub>, Cit. AgNPs, and PVP-AgNPs to juvenile bivalve's hard clam *M.mercenaria*. (A). uptake phase in (SSW). (B). uptake phase in (NFSW). (C). distribution of Ag concentrations in (SSW). (D). distribution of Ag concentrations in (NFSW).

**Table 4.1** Toxicity test (24h), and chronic toxicity test (7 days) with AgNO<sub>3</sub>, Cit-AgNP, PVP AgNPs to *M. mercenaria*. Effect concentrations and corresponding 95 % confidence intervals are all in (µg L<sup>-1</sup>).

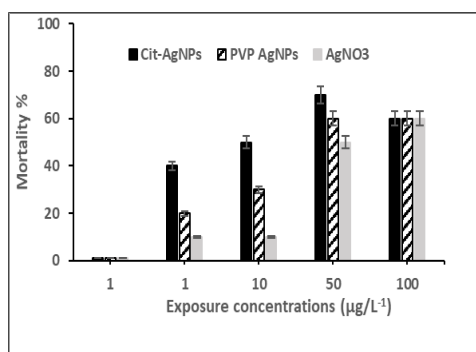
Exposure time	NPs type	Media	LC50	Lower and upper (95% CI) confidence limits (µg/L <sup>-1</sup> )	NOEC	LOEC
Acute exposure (24h)	AgNO <sub>3</sub>	SSW	LC <sub>50</sub> =25.6 ± 1.54	1.04 3.64	0.110	1
	Cit. AgNPs	SSW	LC <sub>50</sub> =27.51 ± 1.70	1.19 4.28	0.219	1
	PVP-AgNPs	SSW	LC <sub>50</sub> = 40.10 ± 1.63	0.62 3.61	0.145	1
	AgNO <sub>3</sub>	NFSW	LC <sub>50</sub> =35.80 ± 1.65	1.07 3.84	0.178	1
	Cit. AgNPs	NFSW	LC <sub>50</sub> =46.56 ± 1.38	1.06 3.68	0.188	1
	PVP-AgNPs	NFSW	42.93 ± 1.89	0.63 3.39	0.123	1
Chronic exposure (7d)	AgNO <sub>3</sub>	SSW	LC <sub>50</sub> =46.56 ± 1.38	0.91 3.22	0.120	1
	Cit. AgNPs	SSW	LC <sub>50</sub> =35.87 ± 1.70	1.04 3.62	0.172	1
	PVP-AgNPs	SSW	LC <sub>50</sub> =66.07 ± 1.56	0.97 3.62	0.226	1
	AgNO <sub>3</sub>	NFSW	LC <sub>50</sub> =46.56 ± 1.38	0.91 3.22	0.120	1
	Cit. AgNPs	NFSW	LC <sub>50</sub> =26.53 ± 1.70	1.14 3.99	0.160	1
	PVP-AgNPs	NFSW	LC <sub>50</sub> =69.62 ± 2.09	0.61 4.12	0.335	1



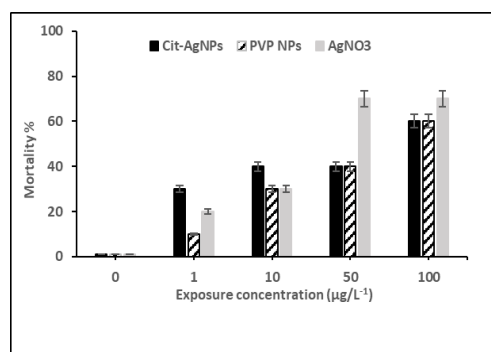
(A): Acute clams mortality percent in (NFSW)



(B): Acute clams mortality percent in (SSW)

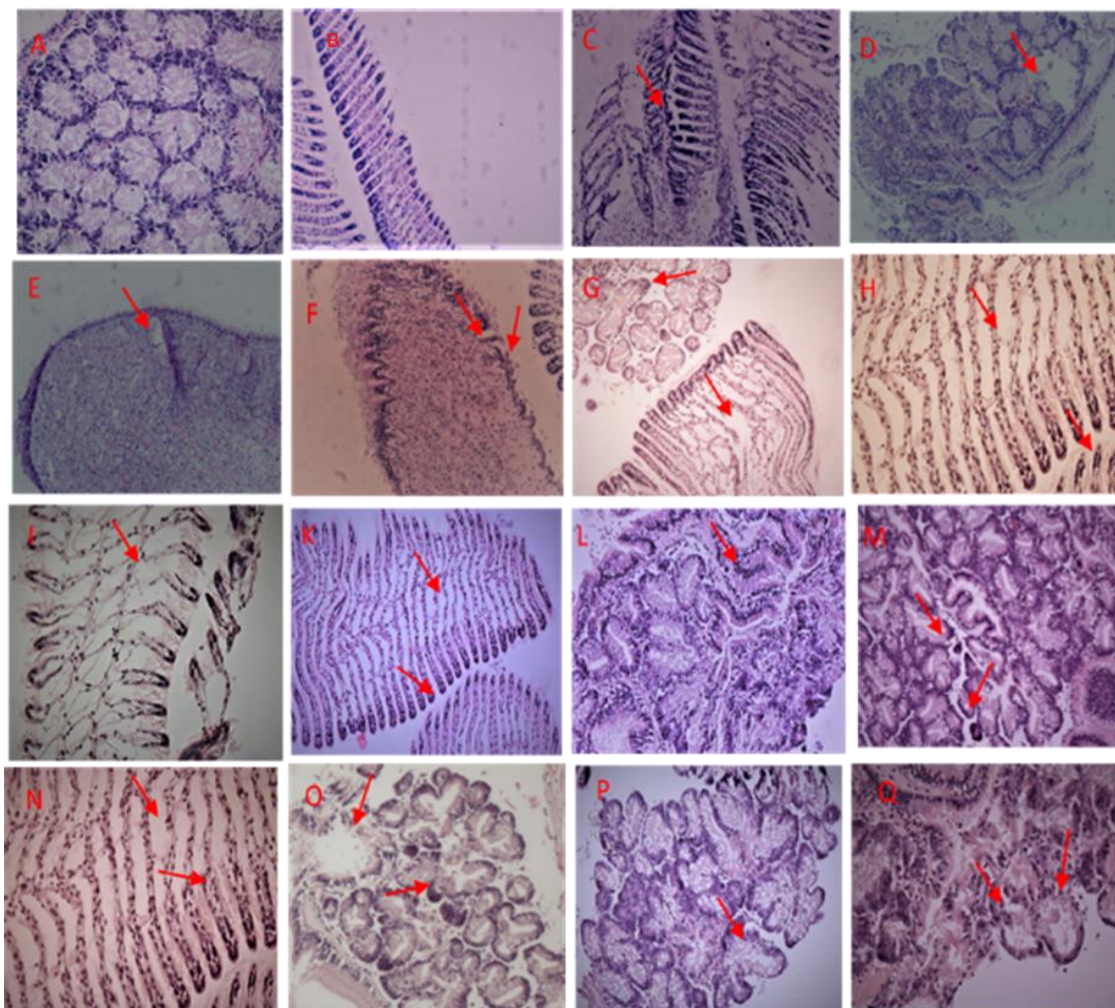


(C): Chronic clams mortality percent in (NFSW).



(D): Chronic clams mortality percent in (SSW).

**Figure 4.8** Mortality percent's of Ag acute toxicity test (24h), and chronic toxicity test (7 days) with AgNO<sub>3</sub>, Cit. Ag NP, PVP-AgNPs to *M. mercenaria*. (A.) Acute clams mortality percent in (NFSW). (B). Acute clams mortality percent in (SSW): (C). Chronic clams mortality percent in (NFSW). (D). Chronic clams mortality percent in (SSW). Effect concentrations and corresponding 95 % confidence intervals are all in ( $\mu\text{g L}^{-1}$ ).



**Figure 4.9** Histological sections of *M.mercenaria* tissues from animals that were acutely and chronically exposed to Cit. AgNPs, PVP AgNPs, and AgNO<sub>3</sub> (0.0, 1, 10, 50, and 100 µgL<sup>-1</sup>) for 24 h and 7 days. (A) Gill structure of control, (B) Digestive gland structure of control after exposure to Ag, (C) Gills in 50 and 100 µgL<sup>-1</sup> Cit. AgNPs in SSW (D) digestive gland structure of 100 µgL<sup>-1</sup> Cit. AgNPs in the acute exposure 24h, (E,F,G) Mental tissues and digestive gland of 50 and 100 µgL<sup>-1</sup> AgNPs in the acute exposure 24h in SSW. Gills in the chronic exposure (7days) in the images (H, I, N) for PVP AgNPs treatment in (NFSW). (K, L) Clams gills in 100 µgL<sup>-1</sup> AgNO<sub>3</sub> in SSW in the chronic exposure. (M, O, P, and Q) Digestive gland structures of 100 µgL<sup>-1</sup> PVP AgNPs at 7 days in NFSW.

## CHAPTER 5

### OVERALL CONCLUSTION

AgNPs is the attractive nanoparticles products and has gain a wide consideration in aquatic ecotoxicology studies. However, there is still not fully known the mechanism underlying behind the AgNPs toxicity. Even though, a great investigation has drawn in this arena. It is highly important to understand how AgNPs are taken up from media, food, and finally depurated out of organism. The debate whether AgNPs is more toxic than AgNO<sub>3</sub> continue to be a hot topic in the scientific research. In the present study we can conclude that AgNPs highly toxicity to juvenile hard clam *M.mercenaria*.

Characterization alterations of AgNPs after spiking in media when AgNPs dispersed in exposure seawater medium, inherent characterization of AgNPs were changed. The size distribution was modified, since AgNPs molded aggregation due to electrostatic force. Depend on original size, surface coatings, and media chemistry, the AgNPs dispersity was shaded. Size distribution of AgNPs in (NFSW) and (SSW) was in dynamic changed. Cit. AgNPs (23±2.5 nm) had the most dramatic alteration on size distribution. After 24 h, hydrodynamic size was in around 168 in the DLS measurements (data not shown). However, the TEM data insure that change in Cit. AgNPs in both (NFSW) and (SSW).

The size was more than (70 nm) in both types of seawater, nearly 3 fold higher than the nominal size. The lowers increase of aggregation AgNPs was in PVP- AgNPs in both exposure seawater medium.

Toxicity of AgNPs is mostly correlated soluble ionic Ag release. *M. mercenaria* were extremely sensitive to Ag and the mortality percent, which are exceeded 50% occurred at (50,100 µg/L) in all treatments. The highest toxic component among the treatments was the AgNO<sub>3</sub> (LC<sub>50</sub>=25.59 ± 1.54 µg/L) in (SSW) at 24h time exposure and the least toxic component was the PVP-AgNP in NFSW at 7days exposure (LC<sub>50</sub>= 69.61 ± 2.09 µg/L). AgNO<sub>3</sub> in SSW was the most toxic compound which was only more toxic than Cit. AgNPs in NFSW and PVP- AgNPs in SSW. All other comparisons in the acute toxicity tests were not significantly different. In the chronic toxicity tests Cit. AgNPs in NFSW was the most toxic compound which was more toxic than AgNO<sub>3</sub> in SSW and both of these compounds were more toxic than AgNO<sub>3</sub> in NFSW, Cit. AgNPs in SSW, PVP-AgNPs in NFSW, and PVP-AgNP in SSW. The AgNPs toxicity is dependent on characteristics of NPs and their behavior in the media. Similar statistically comparisons of LOEC and NOEC values were calculated for all treatments and were not statistically different ( $p > 0.05$ ). This suggests that generally the form of Ag does not appear to affect the onset of toxicity as LOEC values were similar regardless of the form of Ag (AgNO<sub>3</sub>, Cit. AgNPs, or PVP- AgNPs) or the type of seawater exposure (NFSW or SSW). While chronic exposure only showed greater toxicity of Cit. AgNPs in NFSW in *M. mercenaria*, histological results indicated significant sublethal effects in most forms of Ag (AgNO<sub>3</sub> and AgNPs) as histological changes to key tissues were observed. This indicated that AgNPs could induce sublethal stress to the clams that may lead to eventual mortality. Thus, it would appear that the major differences in

toxicity were in the median range ( $LC_{50}$ ) of toxicity that was observed in the chronic toxicity tests and the sublethal stress that may lead to significant tissue damage that may precede higher rates of mortality at higher doses of Ag. The result of Ag acute exposure showed that Cit. AgNPs more toxic than PVP- AgNP in both types of seawater. The chronic exposure showed less effectiveness on *M. mercenaria* survival and highly impact on histological changes indicated that AgNPs could induce sublethal toxicity to the clams. Thus, environmental risk assessments should deliberate the chronic toxicity of AgNP and AgNO<sub>3</sub> at environmentally relevant exposure concentrations from different exposure pathways.

Uptake AgNPs from the exposure seawater by *M. mercenaria* and their tissues accumulated significant quantities of both Cit. AgNPs and PVP-AgNPs in all exposure treatments. At environmentally relevant concentration (ng/L levels), Ag dissolution is the process that driving the bioavailability of Ag at lower concentration. However, at the high AgNPs concentration, the bioaccumulation was dependent on the characteristics of the nanoparticle. Thus, at higher concentrations of 100  $\mu\text{g L}^{-1}$  AgNPs aggregation is foremost and, even though dissolution is still happening, it is less important when amplification in the bioavailability of Ag to marine organisms and the bioaccumulation was thus dependent on the characteristics of the nanoparticles. Clams accumulated Ag at lower concentrations of AgNPs (1  $\mu\text{g L}$ ), as there was enhanced of uptake PVP-AgNPs and Cit. AgNPs in filtered seawater (SSW) more than in unfiltered seawater (NFSW), and a higher percent uptake of total Ag added. While AgNO<sub>3</sub> in unfiltered seawater (NFSW) showed, higher Ag uptake as a percent of total Ag added more than Ag uptake percent in filtered seawater (SSW). This results primarily because of dissolution and aggregation of Cit. AgNPs and PVP-



AgNPs, and for AgNO<sub>3</sub> the Ag ions free which form silver chloride complexes and become unavailable to clams. Clams accumulated Ag at lower concentrations of AgNPs (1 µg L<sup>-1</sup>), there was enhanced uptake of PVP-AgNPs and Cit AgNPs in (SSW) more than (NFSW), and a higher percent uptake of total Ag added. While Ag NO<sub>3</sub> in (NFSW) showed, higher Ag uptake percent of total Ag added more than Ag uptake percent in (SSW). This result because of dissolution and aggregation of Cit. AgNPs and PVP- AgNPs, and for AgNO<sub>3</sub> the Ag ions free which form silver chloride complexes and become unavailable to clams. The depuration data confirmed that AgNPs was mainly through water excretion. In the depuration phase, Ag accumulation has a general trend decreasing towards 24h exposure time. The chronic exposure data showed higher Ag uptake for AgNO<sub>3</sub> in (SSW) of initially Ag added in the lowest concentration. While in (NFSW), the higher Ag percent uptake was for Cit. Ag NPs from Ag initially added in (1 µg L<sup>-1</sup>) concentration and this higher rate of Ag uptake likely explains why the Cit. AgNPs were the most toxic compound tested in the chronic toxicity tests.

Our result hypothesis that Ag once is accumulated within clams there is a potential for consumption by human and may be cause adverse effects in human health. Clams are commercial fishery organism and are frequently consumed by humans; therefore, accumulation of Ag in clams can have momentous effects on human health. Our research proposes a complexity of transformations going on and influencing AgNP behavior, uptake, and accumulation. Complementary testing and mechanistic understanding of AgNP interactions with marine organisms should be continued in tandem with AgNO<sub>3</sub> exposures for comparison. With the intention of, fully understand the impact

nanotechnology has on our environment as well as the associated hazards and risks, the relative role of ion and NP in uptake and accumulation need to be determined.

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