Uptake and Toxicity of Silver Ions, Nanoparticles and Microparticles in *Nereis Virens* In a Sediment Environment

Control

Micro size silver

Nano size silver

Silver on ion form

Project spring 2010

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Pictures presented on the front page shows the control with no deformities and some of the deformities observed in the treatment espoused to respectively micro, ion and nano silver. For more information see section 4.
Abstract

The goal of the project is to investigate how the size and form of Ag affects the internal distribution in the gut cells of *Nereis virens* and to investigate if the toxicity can be related to the differences in distribution within the cell compartments. The worms were exposed to the ion, nanoparticle and microparticle form of silver in sediment with a concentration of 90µg/g dw and left for 10 days. After exposure the worms were dissected, the gut were extracted and used for cellular fractionation. An atomic absorption spectrometer (AAS) test was performed to find the concentration of Ag within each compartment. The observations and AAS tests showed that the ionic form of Ag was the most toxic followed by the nano and micro forms respectively. Mortality was highest in the ion treatment and deformities were present in all treatments. A decrease in activity in the worms in the micro and nano treatment was found but worms in the ionic treatment were lethargic if not dead. Organic matter was measured which displayed an increase in all treatments except for a decrease in the control. Silver concentrations that were measured after cellular defractionation showed that the micro treatment had the highest distribution in the components used for detoxification. On the other hand, the concentrations of the nano and ionic forms showed high distribution in the organelles and the enzymes.
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1 Introduction

The uses of silver date back to some of the most lavish cultures known to man. The Greeks, Romans and Egyptians discovered the purifying properties of silver and used it to keep water and other liquids sanitary (Hill 2009). This custom is still being used in aircrafts, space crafts and water purifying systems to prevent the growth of algae and bacteria (Hill 2009). Silver is also used in swimming pools for the same reason and to avoid the harsh effects of chlorine (Hill 2009). Due to its antiseptic properties, silver has made its way into the medical field as well. Silver nitrate has been used to treat skin ulcers, compound fractures and wounds excreting discharge (Hill 2009). Silver in various forms was used for treatment for almost every infectious disease between the 1800s to a good portion of the 1900s, but the discovery of antibiotics drastically reduced the popularity of silver (Hill 2009). However, due to resistance of antibiotics from some strands of viruses and diseases, there has been a renewed interest in silver (Hill 2009). Pharmaceutical companies are investing in silver compounds for anti-microbial applications, which also include spread prevention of the HIV virus (Hill 2009). Some forms of silver are also used in some serious medical procedures today. For example, silver sulfadiazine is currently the first choice in treating burns in the burn centres in the United States (Hill 2009). Artificial heart valves and catheters are coated with silver to prevent bacteria from growing in spots which they were known to form (Hill 2009). Other than medical procedures and water sanitation purposes, silver can be found in every day items.

In natural waters, silver concentrations range from 0.03 to 500 nanograms per liter. Silver is classified as an environmental hazard due to its ability to be toxic, persistent and bioaccumulative under at least some circumstances. When silver is bioavailable in its ionic form, it is more toxic to aquatic organisms than any other metal apart from mercury. However, it is very complicated to determine the potential for toxicity of silver, because its bioavailability is dependent on the physical, geochemical and biological processes that determine metal uptake by living organisms (Luoma, 2008).

Nanoparticles (NPs) of silver have been found to have antimicrobial activity, the antimicrobial activity of silver is most commonly attributed to the dissolved cation rather than the metallic NP form (Klaine et al. 2008). For this reason, silver NPs are most commonly engineered to release silver ions (Luoma, 2008). Today the use of nano-sized silver has found a wide range of applications and currently it is one
of the most commonly manufactured nanomaterials\(^1\) (Elzey and Grassian 2009). NPs by definition are materials that have a size range of 1-100nm in at least two dimensions (Klaine et al. 2008) and they are used in a wide variety of products, ranging from wound dressings, socks, air filters, toothpaste, vacuum cleaners and washing machines (Klaine et al. 2008). NPs of silver are both occurring naturally and are also being produced in industrial processes (Klaine et al. 2008). Due to the high amounts and wide applications of silver NPs, they have a potential to get into aquatic systems. There they may pose as an environmental risk due to the enhanced antimicrobial activity and bioavailability (Elzey and Grassian 2009).

The increasing use of silver in nano size raises an environmental concern about the effects to the environment. For NPs it is important to investigate whether they are more toxic than silver in the forms which are currently used and whose toxicity is more widely known. If NPs are more toxic, this should be included in the risk management of the nano silver particles. Today a clean compound in nano size is looked at in the same way as the compound in larger sizes (Pedersen and Nielsen, 2007). This issue was the background for carrying out the project.

1.1 Aim of the project

The aim of the project is to investigate how the size and form of Ag affects the internal distribution in the gut cells of *Nerites virens*, and to investigate if the toxicity can be related to the differences in distribution within the cell compartments.

\(^1\) Nanomaterials – materials having at least one dimension in a range from 1 to 100nm (Klaine et al., 2008).
2 Background

2.1 Silver and its occurrence in the environment

Silver is a geologically rare element, a transition metal and has the highest electrical and thermal conductivity of all metals. The various applications of silver are a raising concern of environmental pollution especially in aquatic environments. Silver is usually not naturally occurring but comes from anthropogenic sources such as mining, smelting, and due to a discharge of sewage (Yoo et al. 2004).

According to laboratory testing, silver in its ionic form is proven to be one of the most toxic metals to aquatic organisms due to its tendency to accumulate in sediment, water, and soil (Luoma 2008). This is largely due to humans dumping waste into the environment, but regulations have been put in place in attempts to keeping it to a minimum (Luoma 2008). Although silver is considered to be toxic in most habitats, it is naturally found in deposits of the mineral argentite (Luoma 2008). Argentite is predominately mined in the United States and South Africa, but through a process of smelting silver can also be derived from nickel (Luoma 2008). Between the 1970s to the 1980s, silver mining reached its peak in developing countries, but due to the passing of the Clean Water Act, contaminants including silver had to be captured to prevent them from reaching water bodies and the atmosphere (Luoma 2008). Although developed countries were able to meet the agreements criteria, rapidly developing such as countries found in east and central Asia were unable to hold up to the agreement (Luoma 2008). Amounts of silver in these countries are not available but data on other contaminants found are good indicators of silver emissions (Luoma 2008). The concentration of silver in the environment does determine the impacts but these concentrations tend to be low, making it hard to retrieve reliable data (Luoma 2008). Because silver is so rare, the quantities produced and released to the environment seem small especially when compared with mass discharges of other metals. In 1978, the estimated loss of silver to the environment in the United States was 2,470 metric tons (Luoma 2008). Of that about 500 metric tons were carried into waterways and streams in runoff from soils, and 1,600–1,750 metric tons went to landfills (Luoma 2008). While the silver in landfills is for the most part immobile and the silver in runoff is mostly natural, the most environmentally damaging silver is predicted to be the portion going to the aquatic environment from human wastes, which was estimated to be about 250 tons per year (Luoma 2008). The table below (Table 1) breaks down silver discharges released from industries that use large amounts of the element annually.
Silver is a trace metal and has highly pronounced antimicrobial properties, due to Ag⁺ ability to bind to electron donor groups in biological molecules (e.g., enzymes) containing sulphur, oxygen or nitrogen. Thus resulting in the loss of their function (Muhling et al. 2009).

In aquatic environments silver is mostly abundant as Ag⁺ ion (Luoma and Rainbow, 2008). Silver ions tend to rapidly associate with negatively charged ions to obtain stability, such as fluoride (F⁻), chloride (Cl⁻), sulphate (SO₄²⁻), hydroxide (OH⁻) and carbonate (CO₃²⁻). The silver reaction with chloride ions is of particular importance in saltwater, but the interactions between the two ions are complex. Once formed, silver chloride (Ag⁺(aq) + Cl⁻(aq) → AgCl(s)) quickly precipitates as a solid compound. Chloride concentrations are quite high in seawater, therefore allowing multiple chloride ions to react with a single silver ion, thus forming complexes that are able to hold silver in solution. This complex makes silver more mobile and reactive (Luoma, 2008).

The AgCl⁰ complex is high in bioavailability. Sunda (1993) has suggested that one reason for this could be the low polarity of the complex which will allow for it to penetrate through the cell membrane. However, the behaviour of silver is strongly dependent on the salinity of water (Sunda, 1993, as cited in Luoma et al., 1995). In seawater Ag favours making soluble Ag-chloro-complexes, where AgCl⁰ is the most abundant and very toxic due to its ability to penetrate membranes. In freshwater where chloride concentrations are low, Ag precipitates as an insoluble AgCl complex or forms strong organo- or sulphide-complexes, which have low bioavailability. Therefore the main concern of silver toxicity is in seawater (Luoma and Rainbow, 2009).

In the water column, silver is readily absorbed onto particles due to its high particle affinity (typical partition coefficient kₘ of 10⁴.5 to 10⁶); thus, sediment constitutes the main repository for silver in marine and estuarine environments (Yoo et al., 2004).

<table>
<thead>
<tr>
<th>Silver Released in Aquatic</th>
<th>Kg silver per million people</th>
<th>Total Discharge (Metric tons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waste Treatment Facilities</td>
<td>350</td>
<td>70</td>
</tr>
<tr>
<td>Photo Developing</td>
<td>325</td>
<td>65</td>
</tr>
<tr>
<td>Photo Manufacture</td>
<td>270</td>
<td>54</td>
</tr>
<tr>
<td>Metals Production</td>
<td>20</td>
<td>1 - 10</td>
</tr>
</tbody>
</table>

Table 1 Silver Disposal to Aquatic Environments 1978: USA (Luoma, 2008)
In the sediment, metals such as silver are partitioned in many forms, including soluble free (hydrated) ions, organic and inorganic complexes, precipitated on metal hydroxides, precipitates with insoluble organic complexes, and insoluble sulphide. However, the movement of metals, their availability and toxicity is dependent on other sediment-associated factors, such as organic carbon content, cation exchange capacity, pH, temperature, grain size and possibly acid volatile sulphate content (Hirsch, 1998a; Hirsch, 1998b).

To our knowledge, there is no information available on sources and fate of MP$s in the environment.

### 2.2.1 Bioaccumulation

A part of silvers fate in the environment is its ability to bioaccumulate. Silver shows strong bioaccumulation in estuarine and marine environments. The AgCl complex is available to biota and accumulates in sediments, where it is available for deposit feeders. Experiments with estuarine clam *Macoma balthica*, which is a deposit feeder, showed that one unit of Ag contamination in oxidized sediment results in 56 units of bioaccumulation in the organism. That indicates that even a little contamination with Ag in marine and estuarine environments increases bioaccumulation greatly (Luoma, 1995). Moreover, comparing to other trace metals, silver has the highest uptake rate constant (K_u – expressed in µg/g/d per µg/L or L/g/d). The uptake rate constant is the slope of the linear relationship of uptake rates and metal concentration ranges, under a given set of geochemical conditions. Uptake rate characterises metal uptake through membrane transport, which reflects the interaction between bioavailable metal concentration and characteristics of the biological transport system (Luoma and Rainbow, 2009).

### 2.2.2 Properties of NPs

The properties of NPs are different when compared to particles above nano size of the same compound, due to the NPs large surface to volume ratio. The reason for those differences is mainly that approximately 40-50% of the atoms in NPs are located on the surface. This causes a much greater reactivity compared to that of bigger particles, where there are fewer atoms on the surface compared to the volume (Farre et al. 2008) which increases the rate of which silver ions may be released (Luoma, 2008).

Studies have shown that aggregation is an important issue when considering the fate of NPs, and the tendency of aggregation is affected by such factors as e.g, pH (see Figure 1) (Elzey and Grassian 2009). In seawater, where the ionic strength is higher than in freshwater, the NPs will tend to agglomerate, aggregate and precipitate to the sediment (see Figure 2). Therefore benthic estuarine organisms may be particularly at risk. Even small increases of salinity (as small as ~2,5‰) can make aggregation and precipitation a lot more favorable (Klaine et al. 2008). However, some types of silver NPs are engineered to stay dispersed in water (Luoma, 2008).
Figure 1: Summary of the behavior trends observed for commercially manufactured silver NPs in aqueous acidic environments. The state of the NPs (top) shows agglomeration at neutral pH levels trending to de-agglomerate isolated NPs (o), and silver ions (+) at low pH levels. The color of the suspension trends from black (neutral) to gray to yellow to a colorless solution at low pH. The electric potential of the suspended particles trends from stable (neutral) to unstable, and becomes more stable after the isoelectric point minimum. The percent dissolved silver (bottom) is small but detectable at neutral pH and increases rapidly at pH ≤ 0.5 (copied from Elzey and Grassian, 2009).

The agglomeration, aggregation and precipitation processes of manufactured NPs in seawater would lead to the deposition of NPs on sediment biofilms, probably resulting in accumulation in the sediment and exposure to sediment-dwelling organisms (Klaine et al. 2008). Information on the fate of Ag-NPs in the sediment has to our knowledge not been published.
2.3 General Toxicity effects of silver on organisms

This section will concentrate on the toxicological effects of silver on living organisms. The purpose of this is to gain an understanding on what to expect when a given organisms is subjected to silver contamination in regards to the lethal concentrations (LC), change in growth rate, causing of defects and changes in behaviour. The toxicity of Ag will also be compared to other metals.

Metals and their different forms, such as ions, have been subject to extensive research due to their release into the environment through industry. According to a study done by Khangarot and Ray 1987, where they used bioassays to estimate the toxicity levels of different metals (Ag, Hg, Cu, Cd, Zn, Ni and Cr) on the amphibian tadpoles *Bufo melanostictus* over 12, 24, 48 and 96 hours. They found that Ag is the most toxic substance followed by Hg. According to their results, Ag has a higher toxicity level by a factor of around 8 compared to Cu (Ag LC$_{50}$ = 0.0062 mg/L vs. Cu LC$_{50}$ = 0.0456 mg/L over 48 hours). Another study, Lima et al. 1982, performed a similar toxicity test with Ag exposed to organisms in an aquatic environment. Fathead minnows, Flagfish, Scuds, also known a freshwater shrimps, and Midge larvae were exposed to AgNO$_3$ for 24, 48, 72 and 96 hours. Fathead minnow, Flagfish and Scud saw a LC$_{50}$ at 10.6, 25.9 and 4.7 μg/L, respectively, over 48 hours, where Midge larvae were far more resilient with a LC$_{50}$ at 3160 μg/L over 48 hours.
It is known that the Ag⁺ ion has a strong affinity for binding with sulfhydryl, amino and phosphate groups, as well forming complexes with phosphotides, proteins, RNA and DNA (Petering and McClain 1991). This indicates that Ag has the potential of causing damage to vital parts of an organism, such as the membranes.

The dietary source of metal may offer the major route of metal uptake to many aquatic invertebrates (Rainbow et al., 2006). It is known that benthic organisms are able to accumulate metals from their environment via different routes including ingestion of sediment and subsequent accumulation over gut epithelia as well as from porewater and overlying water via diffusion over the body surface (Selck & Forbes, 2004). The extent and routes of Ag transfer from sedimentary reservoirs into deposit-feeding organisms are especially poorly known (Yoo et al., 2004). However, it is known that silver that is bound to sulphate (Ag₂S) is considerably less toxic to aquatic organisms, when compared to silver ions because of it being chemically inert and having a low solubility product (Hirsch, 1998a). In solution, when silver is strongly associated with sulphides or bound to sulphur ligands, the bioavailability of silver can be reduced (Luoma and Rainbow, 2005).

In general, silver uptake from diet is more important than uptake from solution. But the exact contribution of diet to bioaccumulation depends on silver uptake efficiency by the gut (also called the assimilation efficiency). If an organism takes silver up efficiently from food or prey and retains it for long periods before excreting it, it will very likely accumulate higher concentrations of silver than the concentrations in the food (called biomagnification). There are many invertebrates especially invertebrate predators that have high assimilation efficiencies, slow loss rates and potential to biomagnify silver. For example, polychaete worms assimilate 24-34% of ingested silver from food (Luoma, 2008). High metal concentrations may decrease the feeding rate of the organism, which can sometimes be caused by poisoning of digestive processes, which prevent gut transit of the food. Metals can inhibit the enzymes or gut transit, which causes starvation of the organism and reduced growth (Luoma & Rainbow, 2008).

In all the uptake scenarios metal has to cross the membrane of the cell in order to accumulate in the organism. Hydrophilic metal species are usually transported across the membrane with help of carrier proteins and major ion channels. Metals are able to sustain an inward diffusion gradient by rapid binding of intracellular metal in non-diffusible form. However, the specific routes of silver uptake are dependent on environmental factors and the particular form of silver (e.g. AgCl⁰ can easily diffuse across the cell membrane due to its hydrophobic properties).

There is not much information available specifically about silver micro particles (MPs), but generally they are thought not to be as toxic comparing to NPs, due to the surface-to-volume ratio. The experiment conducted by Cha et al. showed that silver MPs (2-3.5μm) were not as toxic as silver NPs (13nm), when applied to
human hepatoma cell line. The study showed that NPs induced higher cytotoxicity and DNA damage than MPs (Cha et al., 2008). Midlander et al. came to the same conclusion when exposing copper and copper oxide NPs and MPs on cultivated lung cells (Midlander et al., 2009). Another study conducted by Chen et al. on copper NPs and MPs showed that NPs induced head injuries in internal organs of mice after oral administration, whereas MPs exposure did not show such effects (Chen et al., 2006). Study by Meng et al. indicated that nanosized copper particles became quickly dissolved in artificial gastric fluid, while for MPs this process occurred significantly slower (Meng et al., 2007).

There is very little information available on the biological uptake and accumulation of NPs in whole organisms. However, it is clear that organisms will tend to incorporate NPs within their bodies, mainly using the gut, but leaving a possibility of translocation within the body, e.g., to reserve fat droplets (shown in experiment with Daphnia magna and fluorescent carboxylated NPs) (Klaine et al. 2008). On a cellular level, NPs are able to enter cells by diffusing through the cell membrane, through adhesion and endocytosis (Klaine et al. 2008). Endocytosis appears to explain toxicity of silver NPs in higher organisms, such as marine invertebrates, in which the bioaccumulation of silver in general is fast, comparing to other trace metals. In the ionic form silver can be taken up by transporters, because its properties are most similar to sodium and copper ions (Luoma, 2008).

Silver NPs may have multiple mechanisms of causing toxicity on a cellular level. Morones et al. (2005) has reported possible pathways of silver NPs, which include adhesion of NPs to cell membrane surface altering its properties, thus affecting cell permeability and respiration; penetration inside bacterial cell and causing DNA damage; and release of toxic Ag⁺ ions (Morones et al., 2005; Klaine et al. 2008). There has also been reported degradation of lipopolysacharide molecules, formation of pits in the membrane and changes in membrane permeability due to silver NPs (Klaine et al. 2008). It has been proposed also that the release of silver ions could be one of the toxic mechanisms of silver NPs (Luoma, 2008).

NPs can also generate reactive oxygen species (ROS) (Klaine et al. 2008, Luoma 2008), which cause cell membrane damage by lipid peroxidation - oxidizing the double bonds of fatty acid tails of phospholipid molecules in the membrane. That results in increase of membrane permeability and fluidity, making cells more susceptible to osmotic stress or disturbing nutrient uptake. Peroxidized fatty acids can help generating other free radicals, resulting in more cell membrane and DNA damage (Klaine et al. 2008).

Metal ions can bind to carrier proteins, which pass through the membrane and then release them on the other side. For teleost it has been found that epithelial proteins transporting iron, copper and zinc ions can carry silver. Trace metals in their ionic form have high affinity to nitrogen and sulphur, which are the elements of side
chains of various amino acids (e.g. nitrogen in histidine and sulphur in cysteine), therefore trace metals have a possibility to bind to several carrier proteins (Luoma and Rainbow, 2008).

When metals are released from the carrier proteins in the cell, they do not remain in their ionic form for long. As cell interior is protein-rich, the ion has a high chance of binding to various molecules, thereby maintaining the intracellular concentration of free metal ion equal to zero and allowing other metal ion transport down the gradient into the cell. This transport is primarily concentration dependent, meaning that the bigger the metal concentration outside the cell, the higher will be the uptake rate. However, the intracellular molecules that have extra high affinity for the incoming metal will limit its toxicological potential and begin detoxification (Luoma and Rainbow, 2008). One pathway of detoxification is also that metals can be directed to lysosomes and further on transported out of the cell by exocytosis (Selck & Forbes, 2004).

2.3.1 Cellular compartmentalization of metals

Trace metals can be accumulated within an organism in five operational fractions, representing the cellular compartmentalization of the metal – metal-rich granules (MRG), cellular debris (CD), organelles (insoluble fractions), heat sensitive proteins and metallothionein-like proteins (soluble fractions). Organelles and heat sensitive proteins are metabolically available fractions (Luoma and Rainbow, 2008). Metal cations can cause modifications of molecular function and bind to essential enzymes and respiratory protein complexes in the mitochondria, thereby reducing efficiency of energy conversion and causing oxidative damage (Selck & Forbes, 2004).

When metal first enters the body, if it is metabolically available, it will have a potential to bind to various molecules in the cell. Furthermore, it gets accumulated as detoxified store which can be permanent or temporary. The detoxified stores include MRG, inclusions within intracellular organelles (e.g. lysosomes) and binding to metallothioneins. The fate of the metal in the organism can end either with excretion, forming metabolically available component or forming a detoxified store (Luoma & Rainbow, 2008).

2.3.2 Tolerance to metals

It is assumed that juvenile *N. virens* are generally more sensitive to contaminants than adults (Jenner, H.A. & Bowmer T., 1990). In their study of accumulation of cadmium by *N. virens*. Ray *et al.* (1980) demonstrated that the rate of uptake depends on worm size. Smaller worms had a faster uptake than larger ones primarily from the aqueous phase. Under extreme circumstances, where *N. diversicolor* lived in a region with a long history of mining for metals it has been shown that *N. diversicolor* was tolerant to both copper and zinc (Rainbow *et al.*, 2004).
2.4 Methallothioneins

2.4.1 Description and function of the proteins

The metallothioneins (MTs) are non-enzymatic proteins with low molecular weight and high cysteine content (Amiard et al., 2006), which have a function of binding and detoxifying metals. Synthesis of MTs can be induced by the presence of such trace metals as e.g. Zn, Cu, Cd, Ag and Hg. Due to the presence of –SH functional group in the cysteine residues, MTs have high affinity to metals and ability to detoxify them by sequestering them in the cytoplasm and reducing their metabolic activity (Luoma and Rainbow, 2008). MT binding to metals are also preventing them from binding to the intracellular metalloproteins (see Figure 3) (Amiard et al., 2006). MTs are usually not saturated by a single metal but can contain several atoms of Cu, Zn, Cd, Hg or Ag (Amiard et al., 2006).

Besides providing protection from metals, MTs are a part of the antioxidant mechanism used in the protection against ionizing radiation (Cai et al., 1999). (Viarengo et al., 2000; Cavaletto et al., 2002; Correia et al., 2002b; Rodriguez-Ortega et al., 2002). MTs also play a role in the homeostatic control of essential metals such as Cu and Zn. This is due to the MTs ability to act as an essential metal storage ready to fulfil enzymatic and other metabolic demands (Brouwer et al., 1989; Viarengo and Nott, 1993; Roesijadi, 1996). MTs have limited lives within the cell, like many other proteins. MTs will eventually be either expelled from the cytoplasm via lysosomes, which by hydrolyzation can break down macromolecules (Luoma and Rainbow, 2008).

2.4.2 Isoforms of MTs

In a study conducted on snails, evidence of different MTs being specifically dedicated to a specific metal have been found. For example, one has been found to be dedicated to cadmium detoxification, while another is dedicated for copper detoxification (Dallinger et al. 1997). Furthermore, similar results have surfaced for *drosophila melanogaster* (Lauverjat et al. 1989) and *Callinectes sapidus* (Brouwer et al. 1992).

2.4.3 Induction of MTs

Metal contaminants such as Ag, Cd, Cu, and Hg have displayed the stimulation of MT production in many species (annelids, molluscs, crustaceans, fish) (Amiard et al., 2006). Because MT synthesis in the cell is induced by exposure to trace metals, MTs are used as biomarkers in European procedures and are examined in the framework of biological effect quality assurance in monitoring programs (BEQUALM) (Mathiessen, 2000). The regulation of MT genes was discovered by Palmiter (1994) to be a zinc sensitive inhibitor (Figure 3). The affinity for the inhibitor has been found to be in the order illustrated in Figure 4.
Figure 3 Illustration of how introduction of unwanted Cd will outcompete the essential metal Zn on a ligand. This will lead to induction of MT’s, with the binding of Zn to the metal transcription inhibitor. The MT’s will then bind Zn and replace it with the Cd, thereby preventing Cd from harming the cell. MTF (Metal Transcription Factor), MRE (Metal Regulatory Element), MT (Metallothionein), I (metal transcription Inhibitor) after Rosijadi, 1996

Other metals, that have stronger affinity to MT than Zn, are able to replace Zn from the protein. Subsequently Zn can bind with a metal transcription factor, which leads to induction of MT gene expression and MT production to protect the cell (Luoma and Rainbow, 2008; Vasak, 1991).

\[
\text{Hg}^{2+} > \text{Cu}^+ > \text{Ag}^+ > \text{Bi}^{3+} > \text{Cd}^{2+} > \text{Pb}^{2+} > \text{Zn}^{2+} > \text{Co}^{2+}
\]

Figure 4 the protein affinity of some different metals after Vasak (1991)

2.4.4 The Fate of MTs in the Cells

The MTs will eventually be either expelled from the cytoplasm via lysosomes, which by hydrolyzation can break down macromolecules. Amiard et al. (2006) suggests that MTs in some cases are built into the lysosomes, which leads to high concentrations of sulphur and metals in the lysosomes. However this theory has still not yet been proven, since experiments with rat lysosomes showed that the breakdown of MTs depended on which metals were bound to the MT. Mehra and Bremmer (1985) discovered that MTs bound to Cd and Zn could be degraded, and
MTs bound to Cu could not be degraded due to disulphide links in their molecular structure (Mehra and Bremmer, 1991, as cited in Amiard et al., 2006).

2.5 *Nereis virens*

Sediment bioassays are instruments used to test the toxicity and bioavailability of chemical compounds in sediments to benthic organisms. In the present study we selected the marine polychaete *Nereis virens* which is common species in estuarine sediment and is often impacted by anthropogenic persistent compounds. Therefore, infaunal deposit-feeding are used as model organisms in experimental microcosms (Jørgensen et al., 2005; Morales-Casalles et al., 2008).

Currently, the scientific name *Nereis virens* is in discussion. According with World Register of Marine Species, *Alitta virens* would be the name accepted, although the older scientific name *Nereis virens* is the most frequently used. It shows that there is no definite consensus. Even Ushakova and Saranchova (2003), and Ushakova and Saranchova (2004) in their articles used in the first one *Alitta virens* and in the last one used *Nereis virens*. In this report the marine polychaete will be referred as *N. virens*.

2.5.1 Model organism, *Nereis virens*

In the present study the marine polychaete *N. virens* was chosen because it is a common species in estuarine sediment which is known to select and ingest large amounts of fine sediment particles that tend to be enriched in organic material, and, hence, metals as well (Selck et al., 1998). In comparison with others polychaetes, *N. virens* is a large species that is able to reach around 20-40 cm in length. Therefore, *N. virens* could provide a greater amount of gut tissue in order to achieve measurable concentrations.

The common polychaete *N. virens* is commonly found in shallow brackish soft-bottoms of intertidal zone, such as muddy and sandy areas, mussel beds and salt marsh root systems (Nielsen et al., 1995; Oliver et al., 1996). *N. virens* occurs from western Pacific through the Siberian and White Seas. It has also been recorded in the Bering Sea, the North Pacific from Alaska to Central California, and in the western North Atlantic Ocean from the Canadian Arctic south to Virginia (Breton et al., 2004). So, *N. virens* is regarded as one of the most abundant intertidal invertebrates throughout the northern hemisphere, sometimes reaching densities > 700 ind/m² (Breton et al., 2003). The widely distributed polychaete *N. virens* is known as important bioturbator of shallow coastal sediment which live in more or less U-shaped burrows, preferring more sandy and organic-poor sediments than *N. diversicolor* (Christensen et al., 2000). Immature individuals of *N. virens* migrate down shore in the fall of their third year and compete for burrows with mature individuals already established. Encounters between immature and mature
individuals are highly aggressive and are often terminated by the expulsion of the intruder (Lemieux et al., 1997).

The species *N. virens* is described as an omnivorous and opportunistic species, which is able to use many feeding modes such as deposit feeding, predation, filter feeding and scavenging, though adults are almost exclusively carnivorous (Palmqvist *et al*., 2006; Oliver *et al*., 1996).

The term opportunistic has been used in the past several decades to describe certain species that can occupy new habitat readily, respond quickly to environmental changes or disturbances and expand with high densities of population, thus dominate the first stage of succession (Pearson and Rosenberg, 1978; Whitlatch and Zajac, 1985).

Thus, this species shows a high tolerance to variations in environmental factors such as temperature, oxygen content and salinity (Jenner & Bowmer, 1990). It has been reported that *N. virens* is seldom found in environments below 15 ‰ in salinity. The marine polychaete achieves high maximum specific growth rates at salinities of 15 to 20 ‰. It is also known that *N. virens* reacts to decreasing dissolved oxygen tensions by a compensatory response in the ventilation pumping activity (Kristensen, 1989; Nielsen *et al*., 1995). According to Vismann (1990), *N. virens* could during long term exposure to hypoxia, by which after 10 days of exposure, only 17% of them were present on the sediment surface.
3 Materials and methods

This section will explain how the experimental design was planned and executed. The first part of the procedure consists of gathering the worms, sediment and the different forms of silver. The worms were acclimatized in clean sediment in order to avoid the worms from being stressed before the experiment. Clean sediment was spiked and put into beakers where the worms would be exposed to the different silver forms. After 10 days the worms were dissected and their guts were homogenized and centrifuged into different fractions (cellular fractionation). The fractions were thereafter tested by atomic absorption spectrometry (AAS) in order to determine the silver content (in µg pr. gram of gut weight in dry weight (dw)). All details are described throughout this section.

3.1 Collection of the worms, sediment and silver

Worms were bought at Jan & Bo’s Lystfiskerbutik in Roskilde, Denmark. Sediment was collected at Munkholmbroen, in Roskilde fjord, Denmark. The silver MPs and NPs were produced by Sigma Aldrich (see Appendix 8.2).

3.2 Worm Acclimatization

Four 40 x 30 plastic containers were filled with sediment up to 2 cm in height. 17% water was added to each container and 15 worms were placed in each container. Air tubes were supplied to each container and a lid was placed on top to prevent of water evaporation. Worms were fed with pieces of shrimp and fish food. Water salinity and food supply was checked twice a week, and after 10 days the worms were removed from the container and placed into separate beakers in 17% water, where the worms were starved for 24 hours.

3.3 Preparation of the Sediment

3.3.1 Sediment Sieving

All the sediment was sieved in stainless steel sieves and afterwards was left for 24 hours to settle. For acclimatization and exposure sediment 1000 µm and 500 µm sieves were used, respectively. Once the sediment was settled, the water was extracted and the sediment was rinsed with 17% salt water and again left to settle overnight. Afterwards, the water was removed and the sieved sediment was placed into one container and homogenized. Then the sediment was distributed into smaller containers and placed into a freezer for 48 hours (-20C). Afterwards, the sediment was taken from the freezer and thawed for minimum12 hours.
3.3.2 Loss on Ignition (LOI)

The digels were prior to use burned at 550 °C for 4 hours. Afterwards, the digels were weighed and approximately 3 grams of wet sediment was added to each digel, digel and sediment were weighed and all the digels were placed in a 105 °C oven for 24 hours. Afterwards, the digels were taken out and weighed, and then placed in a 550 °C oven for 6 hours.

3.3.3 Spiking the sediment

Four containers containing 4000 ml of sediment were prepared to be used as stock sediment for the three forms of Ag with a concentration of 100µg/g dw and a control.

*The spiking of sediment with Ag*+

3.9995g of AgNO₃ (specification sheet, See Appendix 9.2.3) was weighed and mixed with 2ml MilliQ water until dissolved. Due to its high solubility in water the AgNO₃ will rapidly dissociate into Ag⁺ and NO₃⁻ ions. 400µl of the solution was added to 200g ww (0.82 dw/ww ratio) sediment followed by 100ml 17‰ saltwater. This mixture was left on a shaking table for 24h. Thereafter, the Ag⁺ sediment-mixture was added to 3800g ww clean sediment together with 250ml of 17‰ saltwater, adding up to 4350g of spiked sediment mixture, which was thereafter thoroughly homogenized.

*Spiking of sediment with Ag NPs and MPs*

The Ag NPs (specification sheet, See Appendix 9.2.2) and MPs (specification sheet, See Appendix 9.2.1) were weighed 0.5102g and 0.5104g, respectively, and added to their corresponding beakers containing 200g ww sediment and 100ml 17‰ saltwater. The two mixtures were left on a shaking table for 24h. Thereafter, each mixture was added to 3800g ww clean sediment together with 250ml of 17‰ saltwater, adding up to 4350g of spiked sediment mixtures, which were thereafter thoroughly homogenized.

All three stock sediment silver concentrations were tested with AAS. The concentrations of NPs and MPs for the treated sediment were too high, so 1602g ww and 874g ww (dw/ww ratio 0.82) clean sediment was added to micro and NP contaminated sediment, respectively.

3.4 Experimental Set-up

For each treatment (Control, silver Ion form, silver MPs and silver-NPs) 10 samples were prepared. A glass beaker for each worm was prepared with a volume of 800ml. Preparation of beakers was done by adding 400 g ww (295.44 g dw) of contaminated spiked sediment. Therefore a new dw/ww ratio was calculated in order to have the same dw sediment in all beakers. Furthermore, dw/ww ratio was tested
using LOI and the obtained values were comparable to the calculated dw/ww. Finally, 500 ml of 17% salt water was added to each beaker.

<table>
<thead>
<tr>
<th></th>
<th>Ion</th>
<th>Micro</th>
<th>Nano</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated dw/ww ratio</td>
<td>0.7757</td>
<td>0.7616</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measured dw/ww ratio</td>
<td>0.7386</td>
<td>0.7595</td>
<td>0.7500</td>
<td>0.8008*</td>
</tr>
</tbody>
</table>

Table 2: A comparison between the calculated and measured dw/ww ratio and *the measured dw/ww ratio for the control (clean sediment) right before exposure, while a dw/ww ratio of 0.82 for the clean sediment was used in the calculation (measured two weeks prior).

Figure 5 The overall setup of the experiment. In the top of the figure there is the acclimatization in four large plastic containers and in the bottom the actual treatments and their nominal concentrations.
**Adding worms**

Prior to be added to the beakers, the worms were dried with a napkin and weighed to obtain the weight at $T_0$ (i.e., at experimental start). Then each worm was put in a marked beaker. For each treatment 10 beakers were prepared and worms were distributed according to their weight so that each treatment would have comparable amount of big, medium and small worms. Thereafter beakers were covered with parafilm and air tubes were added to ensure the air supply for the worms.

The worms were left in the beakers for 10 days without food supply. In the middle of the exposure time, the water salinity was checked. After 10 days the worms were taken out of the beakers and put into clean beakers containing only 17% salt water. There, they were starved for 24h to ensure that they emptied their guts. Afterwards, the worms were weighed to obtain the weight at $T_{10}$ (i.e., experimental end).

### 3.4.1 Dissection

Each worm was dissected with a set of dissecting tweezers and scissors on a pre-frozen OASIS block. Once the worm was pinned five segments from peristomium to the block, it was cut from the dorsal wall with scissors along the dorsal blood vessel to open the coelom. Approximately half of the gut was cut free. For each form of silver and the control, 3 pooled samples were made. Each pooled gut sample consisted of 3 guts taken from a large, medium and small sized worm. However, due to the mortality rate of 50% in the worms exposed to the ion form of silver, only 2 pooled samples were made. One sample consisted of 3 guts and the other only consisted of 2. For the nano treatment only one worm died and another was so dissolved that it could not be dissected, therefore 1 of the 3 pooled samples only consisted of 2 guts. The sample containing 2 guts were taken from worms that had inflamed heads. After all samples were taken, they were stored in a freezer at -20 °C.

### 3.5 Cellular fractionation

In a procedure explained in Wallace & Louma 2003, Wallace et al. 2003 and Wallace et al. 1998 the cellular fraction can be categorized into five distinct fractions; organelles and enzymes (metal-sensitive fraction), MTs and MRG (biologically detoxified metal) and cellular debris. The organelle fraction will most likely contain nuclear, mitochondrial and microsomal sub-fractions. A step-by-step procedure can be seen below.

**Step 1**

For each treatment the nine gut tissue samples were evenly pooled together into 3 samples and were placed into glass tubes (each glass tube containing 3 gut
tissue samples). Thereby, there were 3 ion-, nano-, micro- and control samples, which gave a total of 12.

Every glass tube was weighed with and without the gut tissue in order to determine the actual weight of the tissue, which was used for adding the right amount of TRIS buffer solution. The glass tubes were thereafter marked with an appropriate label and kept cool (preferably at 4ºC).

**Step 2**

Each tube was added cold 20mM TRIS buffer solution (pH 7.6) at a 1:10 tissue to buffer ratio. The samples were thereafter homogenized with a Polytron homogenizer for 30 sec. at medium speed.

The homogenized tissue from each treatment was thereby transferred to 12 centrifuge tubes. Also, a sample of homogenized tissue from each treatment was transferred to a separate container for future AAS measurement. It was essential that the centrifugation tubes opposing each other in the ultra centrifuge weighed exactly the same in order to prevent damaging the centrifuge. Thereby, the samples had to have the same weight or the blank tubes with equally weighing medium were inserted before centrifugation. All tubes were marked with the appropriate label.

**Step 3**

**First Centrifugation (homogenate):**
The homogenized tissue was centrifuged at 1450 \(\times\) g, 15 min, 4ºC in a Sorvall RC 28S ultra centrifuge. The supernatant (S1) (containing the cytosol) was removed as well as the pellet (P1) (containing tissue fragments and cellular debris). Both the pellet and the supernatant were each put into a new centrifugation tube. The supernatant was transferred by autopipette and the tubes were thereafter labelled. The pellet was re-suspended and digested in 2ml 1M NaOH.
Second Centrifugation (P1):
The re-suspended pellet 1 was centrifuged at 5000 x g for 10 min. This produced supernatant (S2) which contained metal associated cellular debris. The pellet (P2) consisted of metal rich granules. The two phases were each transferred to a labelled glass tube and frozen down for storage.

Third Centrifugation (S1):
Supernatant S1 was centrifuged at 100000 x g for 1h at 4ºC to produce an organelle pellet (P3) and supernatant (S3) containing the cytosol. The supernatant S3 was transferred by autopipette to a new centrifuge tube, where the pellet (P3) was transferred to a glass tube and frozen down for storage.

Fourth Centrifugation (S3):
The supernatant S3 was heated to 80ºC for 10 min and centrifuged at 80000 x g for 10 min. The pellet (P4) contained heat-sensitive proteins (mostly enzymes) and the supernatant (S4) contained the heat-stable proteins, such as MTs. The two phases were each transferred to a labelled glass tube and frozen down for storage.
3.6 Atomic Absorption Spectrometry (AAS)

The measurements of silver were carried out by two methods of Atomic Absorption Spectrometry (AAS), by flame for sediment and graphite for gut analysis. For the determination of silver content in the sediment three replicates from each sample was taken out and dry-frozen for 24h and approximately 0.3 g of sediment were weighed and used for the procedure. For the gut analysis, the different fractions from the cellular fractionation were used for the procedure. For a detailed overview see appendix in section 8.3.

3.7 Statistical Analysis

The statistical analysis of organic content included 2-way ANOVA with the Tukey’s post hoc test (95% confidence) to test the variability between treatments and exposure time (i.e., at experimental start and end). The variability of silver content in the sediment was done with same procedure to test the significance between treatments and exposure time.

Statistics on the percentage change in weight of the worms from $T_0$ to $T_{10}$ was done by arc sin transforming the data (i.e., % weight decrease from $T_0$ to $T_{10}$). Thereafter, a test for equality of several variances was made and a one way ANOVA was performed with the Tukey’s post hoc test (95% confidence). All the statistical analysis was done by SYSTAT version 11.00.01.
4 Results

The following section will focus on two parts; sediment and gut tissue. The sediment part will describe the changes in the organic and silver content of the sediment. The second, focusing on the tissue, will illustrate the results collected from the AAS (silver content) and the toxic effect the silver had on the worms. The data will be compared through statistical analysis.

4.1 Organic content

The organic matter content at $T_0$ and $T_{10}$ was measured with LOI (see Table 3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Organic Content $(T_0)$ [%]</th>
<th>Organic Content $(T_{10})$ [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.702±0.065</td>
<td>0.647±0.095</td>
</tr>
<tr>
<td>Micro</td>
<td>0.775±0.037</td>
<td>0.933±0.082</td>
</tr>
<tr>
<td>Nano</td>
<td>0.663±0.074</td>
<td>0.971±0.061</td>
</tr>
<tr>
<td>Ion</td>
<td>0.776±0.158</td>
<td>1.102±0.099</td>
</tr>
</tbody>
</table>

Table 3 List of organic matter content for each treatment at $T_0$ and $T_{10}$ (Mean±SD) (n=3)

Figure 6 shows a tendency that the organic content of the contaminated sediments has been increasing from $T_0$ to $T_{10}$, whereas it is the opposite for the control sediment.
**Figure 6:** Difference in percentage of organic content between T₀ and T₁₀ for the four treatments, calculated from the mean values presented in Table 3. * T₁₀ significantly different from control T₁₀ (two way ANOVA, Tukey p > 0.05). + statistically significant difference between T₀ and T₁₀ within treatment. (n=3)

Before analysing the data with the two way ANOVA a Levene’s test was performed showing that the variances were not significantly different (Levene's, p=0.82). The data was then analysed by an analysis of variance (two way ANOVA, p<0.05) for differences in the percent of organic matter content in T₀ and T₁₀ for treatments and between treatments. Further the data was analysed with a Tukey test, the results are shown in Table 4. The p-values will not be mentioned, but can be found in the table. The two way ANOVA showed that the organic content was not statistically significantly different in the T₀ samples (see Table 4).

The organic content increased significantly over time for ion T₀ and T₁₀ and for nano T₀ and T₁₀. For control and micro there was no statistically significant increase or decrease from T₀ and T₁₀. There was a statistically significant (two way ANOVA, Tukey, p<0.05) higher organic content at the end of the experiment at T₁₀ in the three contaminated treatments compared to the control T₁₀, which also is also shown on the figure above (see Figure 6).

Furthermore, it was found that there was no significant difference between the organic content for micro treatment at T₀ and T₁₀. For the nano treatment a significant increase was found between nano T₁₀ versus nano T₀, and nano T₁₀ versus the control T₀ and T₁₀. Relating this to Figure 6 indicates that the organic content has
increased from nano T0 to nano T10. There was also a significant increase in organic content from ion T10 and ion at T0, which indicates that the organic content also increased in the ion T10.

<table>
<thead>
<tr>
<th></th>
<th>Ion T0</th>
<th>Ion T10</th>
<th>Micro T0</th>
<th>Micro T10</th>
<th>Nano T0</th>
<th>Nano T10</th>
<th>Control T0</th>
<th>Control T10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion T0</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ion T10</td>
<td>0.008</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micro T0</td>
<td>1.000</td>
<td></td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micro T10</td>
<td>0.443</td>
<td>0.358</td>
<td>0.432</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nano T0</td>
<td>0.783</td>
<td>0.000</td>
<td>0.793</td>
<td>0.035</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nano T10</td>
<td>0.213</td>
<td>0.646</td>
<td>0.206</td>
<td>0.999</td>
<td>0.013</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control T0</td>
<td>0.967</td>
<td>0.001</td>
<td>0.971</td>
<td>0.093</td>
<td>0.999</td>
<td>0.036</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Control T10</td>
<td>0.656</td>
<td>0.000</td>
<td>0.669</td>
<td>0.023</td>
<td>1.000</td>
<td>0.008</td>
<td>0.994</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Table 4 The p-values from analysis of variance (two way ANOVA, Tukey) comparing the % organic content for T0 and T10 from the different treatments (n=3).

4.2 Silver concentration T0 and T10

The silver content in the sediment was measured at T0 and T10 (see Figure 7). In all cases except for the ion treatment, the tendency is that the silver concentration declines.

![Sediment concentration in T0 and T10](image)

Figure 7 The silver concentration in the sediment at T0 and T10 from all the treatments. The concentrations have been corrected for the content in the blanks. * Statistically different from control T0 and T10 (two way ANOVA, Tukey p > 0.05) + statistical difference between nano T0 and T10 (two way ANOVA, Tukey p < 0.05). (n=3)
Before analyzing the data with the two way ANOVA, a Levene’s test was performed showing that the variances were alike (p=0.33). A two way ANOVA (p<0.05) was then performed, followed by a Tukey test. The analysis showed that there was a statistically significant (two way ANOVA, Tukey p<0.05) decrease from control T0 and T10. For the rest of the treatments only the silver concentration in the nano treatment significantly decreased from T0 to T10 (two way ANOVA, Tukey p<0.05). The silver ion concentration from T0 to T10 showed an increase, which was not significant (two way ANOVA, Tukey p>0.90), the increase does not comply with our expectations. Therefore, another test was conducted to investigate the possible increase. The other test showed that the T0 ion concentration was higher than the one presented above in Figure 7, which meant that there was no sign of increase. The data obtained from the new test showed for ion at T0 (98.4 µg/g vs. 90.5 µg/g) where the T0 values for the NPs and MPs show similar numbers (nano 116.4 µg/g vs. 118.5 µg/g and micro 104.8 µg/g vs and 99.7 µg/g).
4.3 Observations of the worms

Observations of the worms at T₁₀ regarding their growth and differences in behaviour and appearance will be presented in the following section because the observations can be related to the effects of the different treatments.

4.3.1 Growth

One of the measured endpoints was growth, which in this case was a loss of weight. Before analysing the data with the ANOVA a Levene’s test was preformed showing that the variances were not significantly different (Levenes, p=0.13). Weight loss was dependent on treatment (ANOVA, p=0.017) such that worms in the ion treatment lost more weight compared to the control worms (ANOVA, Tukey P<0.016) but not compared to the other treatments (ANOVA Tukey P all > 0.12). There were no significant difference in weight loss among control, micro and nano treatments (ANOVA, Tukey, p>0.6). There seems to be a tendency that the weight loss is higher in all the exposure scenarios compared to the control. This tendency was only significant (ANOVA, Tukey, p=0.016, see Table 7 in the appendix section 8) for the ion treatment.

![Figure 8: Decrease in weight from T₀ to T₁₀. Only the living worms at T₁₀ were included (control and micro n=10, nano n=9 and ion n=5). * statistically significant different from control.](image-url)
4.3.2 Toxic effects

Another endpoint was survival. In the control and the micro treatment all of the worms survived, while 1 died in the nano and half of the worms died in the ion treatment (see Table 5). For the rest of the observations done at T10, the toxicity effects were the highest for the ion treatment. When looking at the activity of the worms, 0 worms for the ion, 4 for nano and 8 for micro were active and all control worms were active. The activity was observed relative to the activity of the worms in the control when they were transferred from the sediment to saltwater only.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0/10</td>
</tr>
<tr>
<td>Micro</td>
<td>0</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>6/10</td>
</tr>
<tr>
<td>Nano</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>5/9</td>
</tr>
<tr>
<td>Ion</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3/5</td>
</tr>
</tbody>
</table>

Table 5 Observations done at T10, the numbers represent the number of worms in the category. *n=10, ** only the worms that were alive at T10 were observed.

Deformities were observed at T10 in one or more worms in all cases where the worms were exposed to silver. No deformities were observed in the controls. The deformities were in the head region and the tail for all deformed with the exception of one, which had a deformity on the side of its body (see Figure 9). It should be noted that the deformities were mostly observed on the worms exposed to NPs. The worms exposed to micro size particles had the second highest occurrence of deformities. Two worms with deformities were seen in the ion treatment (see examples in Figure 10).

![Figure 9 Picture of worm from ion treatment at T10. The picture shows a deformity on the dorsal side of the body.](image)

Besides the deformities observed in the nano treatment, there were three cases where the worms were bleeding from the head region (see example Figure 10 g).
Figure 10 Pictures of the worms after T₁₀ were taken through a microscope. Pictures were taken of the control (a and b) and the worms which showed deviation from the control, examples of those pictures are presented in this figure (ion – c, d; micro – e, f; nano – g, h).
4.4 Silver in cellular fractions in the gut

The AAS results for the different fractions were given in concentration (μg silver/l) in a 25 ml solution. Thereby, the silver concentration for all fragment samples was converted into silver content (amount) by dividing the concentration with a factor of 40, resulting in the amount of silver found in the fractions. Thereafter, the silver content was converted to amount of silver per g ww of gut tissue, as well as silver per g dw gut tissue (dw/ww ratio 0.33).

The AAS data showed that the control samples have a fairly high amount of silver for the MRG, organelles and MTs. This might be due to some amount of silver in the non-spiked sediment, the worms accumulating silver prior to experimental exposure or contamination during dissection or the AAS procedure. The mean values for the control in each treatment were thereby subtracted from each sample within the same treatment in order to exclude the concentrations in the different fractions that are around or below the control concentration, showing whether an uptake in the different fractions has taken place (see Figure 11). As shown, high deviation for the CD and MT fractions for the micro treatment was observed. The same can be said about the nano silver organelles fraction.
Differences among the four treatments and different cell fractions were observed. The AAS results indicate that cells from the micro treatment accumulated the most silver into MRGs, CD and MTs, whereas no silver was observed in the enzyme fraction. However, the pattern was different for NPs and ions. The data shows (figure 3) that the concentration for the ion treatment was highest in the enzymes and organelles, however the enzyme fraction show no variation due to two replicas were below the AAS detection limit. The concentration in the MT and MRG fractions for the ion treatment are the lowest in its treatment. The NP fractions show concentrations in a similar magnitude compared to the ion fragments. However, the highest concentration for the NP treatment was found in the organelles (high variation can be observed). The second highest concentration was found in the enzymes and CD. The NP MT fraction had a higher concentration compared to the ion fraction. The NP MRG fraction, like the ion treatment, had a very low concentration.
4.4.1 Differences between treatments:

It was investigated whether there were significant differences between the treatments (control, micro, nano and ion) in the same cellular fraction. According to the Tukey’s test, MRG from the micro treatment showed significant differences when compared to control, nano and ion treatments (two way ANOVA, Tukey p<0.001, p<0.001 and p<0.002, respectively). However, none of the other fractions showed significant differences among each other.
5 Discussion

5.1 Behaviour of the worms

The behaviour of the worms is an important factor for the understanding of our observations and measurements throughout the discussion. In the following sections we argue that the behaviour of the worms had an effect on the different results we obtained, therefore we will briefly present the most relevant observations.

In the control treatment all the worms were buried in the sediment at the end of the experiment. They were active when taken out from the sediment and had no deformities. For the three treatments - MP, NP and ion - the number of active worms decreased in the following order: MP, NP and ion the same pattern accounted for the number of dead worms. In all three cases where there were a little over half of the worms found on top of the sediment, which most likely indicates that they were not feeding the entire duration of exposure. Moreover, the activity of the worms was in many cases lower in comparison to the control worms, which could also suggest a decrease in feeding. Some of the worms had deformities in the head region, especially the ones within the NP treatment, and to a lesser extent within the micro and ion treatments. Some of the deformities were so severe that the worms were not able to feed, which would definitely lower the dietary uptake of silver from the sediment.

5.2 Sediment: Organic content and silver concentration

In the analysis of the sediment there are 3 factors that are inter-linked. Weight loss, organic matter and silver concentrations in T₀ and T₁₀ can play a contributing factor to the overall picture of the experiment. A correlation can be drawn when observing Figure 6 and Figure 8, which exhibit the percent difference in organic content between T₀ and T₁₀ in all treatments and the percentage of decrease in weight, respectively (ion having the biggest difference, nano having the second largest etc.).

It was expected that there would be a decrease in the organic content due to feeding by *N. virens*. Instead, it was found that there was an increase in organic content at experimental termination. There seems to be a tendency that high percentage differences from T₀ to T₁₀ in organic matter also show a high percentage of weight loss. This could be due to a part of the worm tissue dissolving into the sediment. However, more testing is needed in order to confirm this phenomenon due to the variation for the different treatments.
The highest mortality was observed within the silver ion treatment (50% of worms were dead after exposure), and the worms were surfacing. 3 out of 5 of the living worms were found on the surface of the sediment at the end of the exposure. The ion treatment resulted in the highest amount of organic content in the sediment and highest loss of weight in worm gut weight. For the nano treatment, 5 out of 9 worms were found on top of the sediment, while the head was deformed in 3 out of 9 living worms. This would cause a decrease of the feeding rate and thereby lose weight (lost around 19% of gut weight). In the case of the micro treatment, 6 out of 10 worms were found on the surface of the sediment at the end of exposure. In all silver treatments, where the organic content increased, the worms were less active in and out of the sediment compared to the control. This shows a correlation between the increase in weight loss and the potentially lowered feeding rate of the worms. Feeding rates can be inhibited by toxic compounds as found by Giessing et al. (2003) testing PAH’s on *N. diversicolor*.

The silver concentration in the different sediment treatments displayed in Figure 7, contains unexpected results. In the ion treatment the silver concentration seemed to increase from T₀ to T₁₀ but the increase was not significant (p=0.917). The AAS test was redone as described in the result section and the new data indicated that the silver ion concentration from T₀ to T₁₀ does not change.

The silver concentration in the sediment from T₀ to T₁₀ in the nano treatment dropped by 37%, which is a greater difference when compared to all the other treatments. The difference is also significant from a statistical viewpoint (two way ANOVA, Tukey, p=0.004). One of the explanations for the observed decline could be that silver particles were accumulated in the tissue of the worms. However, the change in the silver concentration in the sediment cannot be explained through the accumulation of particles alone. Another explanation for the high decrease of nano silver concentration could be the tendency of the nano particles to aggregate due to the large surface area in comparison to their volume. This could make it more difficult to have a homogenous distribution of nano particles in the sediment. The decrease from T₀ to T₁₀ could be due to a heterogeneous distribution in the sediment.

In the other treatments there was no statistically relevant decrease of the silver concentration from T₀ to T₁₀. The reason for that could be the large volume of sediment, which makes it impossible for the relatively small worms to take up such great amounts of silver resulting in an observable change in concentration from T₀ to T₁₀.
5.3 Effects

There was no mortality for worms exposed to silver in the MP form and a majority of those worms displayed active behaviour. Two worms had inflammations in the head but did not appear to be as severe as the inflammations found in the NP exposure. The worms in the NP exposure only had one casualty amongst the group. On the other hand, almost half of the worms had inflammations that can be seen in the pictures (see Figure 9 and Figure 10). The deformities in the head were most likely due to the worm’s activity when burrowing and consuming the sediment. This could also explain why some of the worms had mouth and jaw deformations. Previous research has also proven that certain NPs are capable of producing high levels of different ROS levels in water, which could also be a determining factor in the overall effects of the NP treatment on the worms (Griffitt et al., 2008).

Worms exposed to the ion form of silver had 50% mortality. This was due to the high toxicity of Ag⁺ which correlates with the conclusion of the bioassay conducted by Kahngarot and Ray (1987), in which they determined that Ag was the most toxic metal amongst other metals such as: mercury, copper and cadmium. This is due to the high electron affinity that silver ions possess, which resulted in the damaging of vital functions of *N. virens*, ultimately leading to death (Kahngarot and Ray, 1987). *N. virens* also showed a behavioural response: they stopped feeding and lost weight, as indicated in the results section. The worms were also inactive and were lying above the sediment in the water instead of burying themselves in the sediment. This indicates that worms were avoiding the sediment and therefore starving. This behaviour has also been observed for *N. diversicolor* exposed to PAH’s (Giessing et al., 2003). This behaviour was not observed in the other treatments, therefore it can be argued that silver is the most toxic in its ionic form when compared to NPs and MPs. This could be explained by the silver ion tendency to form AgCl⁰ complexes when dissolved in salt water, where silver in this form is highly bioavailable to aquatic organisms due to its ability to penetrate cell membranes (Luoma et al., 1995; Luoma and Rainbow, 2009). The higher toxicity observed for the ion treatment compared to micro and control could maybe also be explained by the distribution in the cell (see Table 6). The ion form, as well as the NPs, was found in a higher percentage in the metal sensitive fractions such as organelles and heat sensitive fractions.

5.4 Silver accumulation

When looking at the microparticle data, there seems to be a higher amount of silver in the MRGs and MTs and a lower amount of silver in the enzymes compared to the other treatments. The high amounts of silver in the MRGs and MTs show that the processes used for detoxification had higher amounts of silver than any other fraction. The low amounts of silver found in the enzymes, which are sensitive to metals, indicate that a large portion of the micro silver is taken into the detoxification
systems as opposed to being absorbed in the metal sensitive fractions (Wallace et al., 2003). From this we suggest that the silver MPs are the easiest to detoxify, thus protecting metabolically available fractions from being damaged. A high concentration was seen in the CD fraction. However, due to CD not being metabolic available (Luoma & Rainbow, 2008) the MPs would not necessarily decrease the worms activity. There was also a considerable concentration in organelles, which could potentially cause damage to essential parts of the cell, such as mitochondria, DNA and RNA (Cha et al., 2008). Toxic effects were observed after exposure, thereby indicating that cell damage must likely have occurred.

Silver NPs are able to produce ROS (Choi and Hu, 2008) which can cross cell membranes into critical organelles (Carlson et al., 2008). Carlson et al. (2008) showed that silver NPs in the size of 55 nm can inhibit mitochondria function in alveolar macrophages. As indicated in the results, the biggest fraction of nanoparticles (around 37%) ended up in the organelles of the cell (the average concentration being very similar to ionic fraction). However, NPs are able to release silver in its ionic form both in a solution (Midander et al., 2009) and in the gut of the worm where it is favourable due to the acidic environment. Due to the NP’s ability to release ions, there were most likely both forms of silver available in the gut of the worm. Therefore, we can not state if the toxicity from the NPs was due to the nano form or the ionic form, since both have shown toxic effects as mentioned above (Carlson et al., 2008; Midander et al., 2009). However, studies have shown that NPs can get more easily taken up by cells in comparison to metal ions. The reason for this is that cell membrane seems to have an improved barrier capacity for metal ions rather than metal particles (Midlander et al., 2009). Furthermore, in the nano exposure only one worm died, which indicates that the nano form of silver was not as toxic as the ionic form. In the accumulation of NPs in the organelles there is a large deviation between the samples. This could be due to some worms consuming more sediment than others (according to the observations mentioned above) or unpredictable behaviour of nanoparticles. The presence of a high amount of NPs in the MTs can be due to the strong affinity of silver NPs to –SH groups, which are abundant in the cysteine residues of the protein (Carlson et al., 2008). A high concentration was also found in the enzyme fraction. Altering the enzymatic activity will most likely affect the metabolism of the worm.

For the silver ions, the highest concentration was found in the enzyme fraction. The second highest concentration was found in the organelle fraction of the cell, which included such components as mitochondria, nucleus and lysosomes (see Figure 13). As known, silver ions have a strong affinity to bind to sulphhydryl, amino and phosphate groups and forming complexes with proteins, RNA and DNA (Petering and McClain, 1991) and it is able to induce ROS production (Klaine et al., 2008; Choi and Hu, 2008). This can lead to damage of essential components of the cell, such as DNA and proteins, as well as membranes. If the membrane is disrupted, the membrane permeability is changing thus making it more permeable to various
ions therefore altering the normal electron transport across the membrane (Klaine et al., 2008; Choi and Hu, 2008). This would explain the death of the worms in the ion treatment as well as inactivity of the worms that survived during the exposure time.

Rainbow et al. (2006) conducted a study for analysing the distribution of radio labelled silver ($^{110}$MAg) in Nereis diversicolor, following the same defractination procedure as in our study. It should be mentioned that they worked with the whole worm excluding the head, whereas only the gut tissue was used in this study, and there was no mention of mortality during exposure. When comparing the distribution of silver (Table 6) in the fractions observed by Rainbow et al. (2006) and this study it can be seen that for MPs the distribution was quite comparable, but the distribution of NPs and ion form of silver were a lot lower for the MRG. In the enzyme fraction the MPs were found at a similar degree as found by Rainbow et al. (2006) while the NPs and ion forms were distributed to a much larger extent in the enzymes, which are most likely due to the alterations in feeding behaviour.

<table>
<thead>
<tr>
<th></th>
<th>Metal rich granules</th>
<th>Metallothioneins</th>
<th>Organelles</th>
<th>Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainbow et al. (2006)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blackwater estuary</td>
<td>35.6 ± 3.6</td>
<td>8.6 ± 5.4</td>
<td>23.8 ± 7.9</td>
<td>3.9 ± 2.5</td>
</tr>
<tr>
<td>Restronguet Creek</td>
<td>45.1 ± 15.5</td>
<td>17.7 ± 7.4</td>
<td>10.6 ± 2.9</td>
<td>5.5 ± 5.6</td>
</tr>
<tr>
<td>(adapted to silver metal pollution)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>This study</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micro</td>
<td>44</td>
<td>20</td>
<td>3.9</td>
<td>0</td>
</tr>
<tr>
<td>Nano</td>
<td>1.5</td>
<td>16</td>
<td>38</td>
<td>22</td>
</tr>
<tr>
<td>Ion</td>
<td>1</td>
<td>10</td>
<td>26</td>
<td>43</td>
</tr>
</tbody>
</table>

Table 6 Percentage distribution of silver in the cell. The data was obtained from a study by Rainbow et al. (2006) on Nereis diversicolor.
6  Summary, Conclusion and Perspectives

The purpose of the project was to investigate how the different silver forms distribute in the cells for *N. virens* and in what way the worms are affected by these forms.

6.1  Behavior

The behaviour of the worms varied between the treatments. No negative effects were observed on the control worms. While the treatments (MPs, NPs and ion form) showed a decrease in activity and deformities in the head region, which we suggest would decrease the feeding rate and thereby the dietary uptake of silver.

6.2  Sediment organic content and silver concentration

We observed that the organic content only decreased in the control. For the three other treatments there was an increase in organic content. This could be related to the weight loss (tissue dissolving into sediment). There was a high decrease in the silver concentration in the nano treatment, which was too high to be explained by the uptake in the worm alone. The high decrease could rather be explained by uncertainties related to the agglomeration, causing heterogenous distribution of the NPs in the spiked sediment. For the MP and ion treatment there was no significant difference in silver concentration between T₀ and T₁₀.

6.3  Effects

After being exposed to ionic silver, 50% of the worms died, whereas in the nano treatment 1 out of 10 worms died. The ion treatment resulted in the greatest weight loss comparing to the other treatments, while the NP and MP treatments also saw a loss of weight compared to the control.

MPs showed the lowest toxicity on the worms, as there was no mortality, and there were fewer deformities when compared to worms after NP and ion form treatment. In the NP treatment there were many deformities observed in the head region, which shows a higher toxicity compared to the MPs.

6.4  Silver accumulation

There were differences in cellular fractions of different forms of silver in the gut of the worms. MPs accumulated most in MRGs, CD and MTs, which are not metabolically available fractions, indicating that most MPs were detoxified within the cells. The highest concentration for the NP treatment was in the organelles,
followed by similar amounts in enzymes, CD and slightly lower in MTs. Ions were also found to a high extent in the metabolically available fractions, having the highest amount in the enzymes. In comparison with MPs, a lot higher fraction of NPs and ions ended up in metabolically available fractions, which can be related to a higher toxicity.

### 6.5 Conclusion and Perspectives

Judging by the observations on behaviour and effects of the worms after exposure the ion form is defiantly a toxic. The NP treatment can also be considered hazardous. Although not having the same kill efficiency as the ion form, it did have a profound effect on the worms, killing one and causing serious deformities to four others. The MPs treatment turned out to be the least potent, resulting in no deaths and few deformities. The data on cellular distribution shows that the ion form had the highest concentration in the enzyme fraction, NPs in the organelles fraction and MPs in the MRG fraction, which could explain the different toxic effects. However, the amount of replicas used in for the cellular distribution varies from 1 to 3. Having more replicas would add a higher degree of confidence in the discussion on how the affects differ from each silver form. Also, judging from the weight loss and mortality in the ion form treatment, 90 µg/g dw sed is too high in order for the worms to most effectively absorb the silver. The same could also be said regarding the NP treatment, since the concentration in the cellular fraction is within the same range for both NPs and ion form. The deformities did most likely also contribute to the decrease in feeding activity.

To understand the role of the NPs further, more investigation on the bioaccumulation and toxicity is needed. Another perspective that should be taken into account when working with NPs is that the toxicity might be size dependent since there is quite a difference in size from NPs to MPs, which thereby yielded quite different results. This study was performed on NPs up to 100nm and it would be interesting to know if smaller NPs behave differently.
7 Reference list


Luoma, S.N., Ho, Y.B. and Bryan, G.W., 1995. Fate, Bioavailability and Toxicity of


8 Appendix

8.1 Appendix statistical analysis from the result chapter

Tables with output from statistical analysis presented in the result chapter.

8.1.1 Organic content (see 4.3.1 Growth)

The comparison of the worms weight loss by a one way ANOVA and Tukey test (see 4.3.1 Growth)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ion</th>
<th>Micro</th>
<th>Nano</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
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</tr>
<tr>
<td>Ion</td>
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<td>Micro</td>
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<tr>
<td>Nano</td>
<td>0.12</td>
<td>0.6</td>
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<td>1.00</td>
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</table>

Table 7 Output from Tukey test on the weight loss from the worms still alive at T_{10} (control and micro n=10, nano n=9 and ion n=5).
8.2 Appendix Specification sheets

8.2.1 Specification sheet for micro size silver

<table>
<thead>
<tr>
<th>Specification Sheet</th>
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<tbody>
<tr>
<td><strong>Product Name</strong></td>
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<tr>
<td><strong>Product Number</strong></td>
</tr>
<tr>
<td><strong>Product Brand</strong></td>
</tr>
<tr>
<td><strong>CAS Number</strong></td>
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<tr>
<td><strong>Molecular Weight</strong></td>
</tr>
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</table>

**TEST**

<table>
<thead>
<tr>
<th>Appearance (Color)</th>
<th>Conforms to Requirements</th>
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</thead>
<tbody>
<tr>
<td>Powder</td>
<td>Grey or Pale Seige</td>
</tr>
<tr>
<td>ICP-Confirms Silver Component</td>
<td>Confirmed</td>
</tr>
</tbody>
</table>

- **Trace Metal Analysis**
  - As2O3 0.0 ppm
  - ppm
  - ppm
  - ppm
  - ppm
  - ppm
  - ppm
  - ppm
  - ppm
  - ppm
  - ppm
  - ppm

- **Purity**
  - Conforms
  - 99.99% Purity Based On Trace Metals Analysis

http://www.sigmaaldrich.com/catalog/DataSheetPage.do?brandKey=ALDRICH&sy... 28-05-2010
8.2.2 Specification sheet for nano size silver

<table>
<thead>
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<tbody>
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<td><strong>Product Name</strong></td>
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<td><strong>Product Number</strong></td>
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<tr>
<td><strong>Product Brand</strong></td>
</tr>
<tr>
<td><strong>CAS Number</strong></td>
</tr>
<tr>
<td><strong>Molecular Weight</strong></td>
</tr>
<tr>
<td><strong>TEST</strong></td>
</tr>
<tr>
<td><strong>Appearance (Celer)</strong></td>
</tr>
<tr>
<td><strong>Appearance (Form)</strong></td>
</tr>
<tr>
<td><strong>ICP: Confirms Silver Component</strong></td>
</tr>
<tr>
<td><strong>Trace Metal Analysis</strong></td>
</tr>
<tr>
<td><strong>Aluminum (Al)</strong></td>
</tr>
<tr>
<td><strong>Arsenic (As)</strong></td>
</tr>
<tr>
<td><strong>Gold (Au)</strong></td>
</tr>
<tr>
<td><strong>Boron (B)</strong></td>
</tr>
<tr>
<td><strong>Beryllium (Be)</strong></td>
</tr>
<tr>
<td><strong>Bismuth (Bi)</strong></td>
</tr>
<tr>
<td><strong>Calcium (Ca)</strong></td>
</tr>
<tr>
<td><strong>Cadmium (Cd)</strong></td>
</tr>
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<td><strong>Cobalt (Co)</strong></td>
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<td><strong>Chromium (Cr)</strong></td>
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<tr>
<td><strong>Copper (Cu)</strong></td>
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<td><strong>Iron (Fe)</strong></td>
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<td><strong>Tin (Sn)</strong></td>
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<td><strong>Vanadium (V)</strong></td>
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<td><strong>Tungsten (W)</strong></td>
</tr>
<tr>
<td><strong>Zinc (Zn)</strong></td>
</tr>
<tr>
<td><strong>Zirconium (Zr)</strong></td>
</tr>
<tr>
<td><strong>Purity</strong></td>
</tr>
<tr>
<td><strong>99.5% based on Trace Metals Analysis</strong></td>
</tr>
<tr>
<td><strong>Size</strong></td>
</tr>
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</table>

http://www.sigmaaldrich.com/catalog/DataSheetPage.do?brandKey=ALDRICH&ey... 28-05-2010
### Specification Sheet for the silver nitrate

**Product Name**: Silver nitrate, BioXtra, >99% (titration)

**Product Number**: 88157

**Product Brand**: SIAL

**CAS Number**: 7774-56-8

**Molecular Weight**: 169.87

#### Test

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<th>Property</th>
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<tbody>
<tr>
<td>Appearance (Form)</td>
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</tr>
<tr>
<td>Solubility (Turbidity)</td>
<td>Clear</td>
</tr>
<tr>
<td>Solubility (Color)</td>
<td>Colorless</td>
</tr>
<tr>
<td>Purity (by Titration)</td>
<td>&gt;99.0 %</td>
</tr>
<tr>
<td>Insoluble matter</td>
<td>≤0.1 %</td>
</tr>
<tr>
<td>Chloride Content</td>
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</tr>
<tr>
<td>Sulfate (SO4)</td>
<td>≤0.05 %</td>
</tr>
<tr>
<td>Aluminum (Al)</td>
<td>≤0.0005 %</td>
</tr>
<tr>
<td>Calcium (Ca)</td>
<td>≤0.0005 %</td>
</tr>
<tr>
<td>Copper (Cu)</td>
<td>≤0.0005 %</td>
</tr>
<tr>
<td>Iron (Fe)</td>
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<tr>
<td>Potassium (K)</td>
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<tr>
<td>Magnesium (Mg)</td>
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<tr>
<td>Sodium (Na)</td>
<td>≤0.01 %</td>
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<tr>
<td>Ammonia (NH3)</td>
<td>≤0.05 %</td>
</tr>
<tr>
<td>Phosphorus (P)</td>
<td>≤0.005 %</td>
</tr>
<tr>
<td>Lead (Pb)</td>
<td>≤0.001 %</td>
</tr>
<tr>
<td>Zinc (Zn)</td>
<td>≤0.0005 %</td>
</tr>
</tbody>
</table>

#### Recommended Reagent Period

3 Years

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28-05-2010

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53
8.3 Procedure for AAS

8.3.1 Procedure for AAS for sediment

**DS/EN ISO 15587-2: Water quality – Digestion for the determination of selected elements in water (and modified for tissue/sediment) using volumetric flask.**

**Part 2: Nitric acid digestion**

*Summary of the method:*

This method-summary specifies the digestion of Ag.

*Reagents:*

5.2 Nitric acid, concentrated, 65 %

5.4 Ammonia solution, approximately 25% by mass.

*Apparatus:*

Milestone furnace

*Equipment:*

Carefully acid-wash digestion equipment in contact with the digestion solution and volumetric ware. Glass equipment is to be preferred due to the adhesion of silver to plastic.

*Sampling:*

The tissue and the sediment has to be freeze-dried before preparation for AAS.

The water can be used without further treatment. If necessary the water can be frozen until it allows for the procedures to be completed the same day.

*NB!! And now is the time for protective screen, rubber apron, gloves and over sleeves to wear during the next steps.*

*Preparation of water:*

1. Add 25.0 ml ±0.1 ml of test water into the digestion vessel (Weflon tube with lid)

2. Add 6.25 ± ml of nitric acid (5.2)

3. Swirl and allow the mixture to stand until any visible reaction has stopped. Then cap the vessel

4. Place on the Weflon tube the protection shield, the adapter plate and the special spring
5. Introduce the vessels vertically into one of the numbered niches of the six-position rotor body
6. Then tighten the HTC screw in the upper part of the rotor body using the Tension Wrench, till you hear a clicking sound informing that the vessel is blocked inside its niche. Place the ring around the rotor to secure the vessels.
7. The rotor unit is now ready for being placed in the microwave cavity of the Milestone unit

Preparation of tissue/sediment:
1. Weigh out approximately 0.3000 g of freeze-dried tissue/sediment or as much as possible to the digestion vessel (Weflon tube with lid). The exact weight is noted.
2. Add 6.25 ± ml of milliQwater and 6.25 ± ml of nitric acid (5.2)
3. Swirl and allow the mixture to stand until any visible reaction has stopped. Then cap the vessel
4. Place on the Weflon tube the protection shield, the adapter plate and the special spring
5. Introduce the vessels vertically into one of the numbered niches of the six-position rotor body
6. Then tighten the HTC screw in the upper part of the rotor body using the Tension Wrench, till you hear a clicking sound informing that the vessel is blocked inside its niche. Place the ring around the rotor to secure the vessels.
7. The rotor unit is now ready for being placed in the microwave cavity of the Milestone unit

Operating procedure:
8. The program suitable for digestion is started, normally program no. 8. Press: Start – Start. Program 8 will appear. Press start. The intervals are:
   Step 1:  6 min  250W
   Step 2:  6 min  400W
   Step 3:  6 min  650W
Step 4: 6 min 250W
Ventilation 5 min

Cooling down:
9. Once the digestion program is completed, very high temperature and pressure are reached inside the vessels. It is therefore necessary to cool down the rotor before opening the vessels.
Place the rotor in the cooling system in the fume-hood and let the water flow for approximately 20 minutes.

Uncapping the vessels:
10. The rotor is dripped off before placing it on the workstation. Carefully loosen the screws in the upper part of the rotor body using the Tension wrench and wait till the pressure is completely released
11. Remove the external protection ring, take the vessels out of the rotor body one by one.
12. Uncap the vessel – there might some fume evaporation. Immediately after uncappping carefully drop by drop add 30 ml 25% ammonia solution (5.4) into the vessel. There might be a violent reaction, so the protection window of the fume-hood has to be as low as possible.

Preparing the samples:
13. While the operating procedure is running, wash the filters once with diluted nitric acid (1:1) and 3-4 times with milliQwater.
14. The sample in the Weflon tubes is transferred through the filter into an acid washed 100 ml volumetric flask. The tube and the filter is washed with milliQwater into the flask and filled to the mark. Now the sample is ready for AAS-measuring not waiting too many hours!!
8.3.2 Procedure for tissue

DS/EN ISO 15587-2: Water quality – Digestion for the determination of selected elements in water (and modified for tissue/sediment) using volumetric flask.

Part 2: Nitric acid digestion

Summary of the method:

This method-summary specifies the digestion of Ag.

Reagents:

5.3 Nitric acid, concentrated, 65 %
5.4 Ammonia solution, approximately 25% by mass.

Apparatus: Milestone furnace

Equipment: Carefully acid-wash digestion equipment in contact with the digestion solution and volumetric ware. Glass equipment is to be preferred due to the adhesion of silver to plastic.

Sampling: The tissue and the sediment has to be freeze-dried before preparation for AAS.

The water can be used without further treatment. If necessary the water can be frozen until it allows for the procedures to be completed the same day.

NB!! And now is the time for protective screen, rubber apron, gloves and over sleeves to wear during the next steps.

Preparation of small amounts of tissue:

1. Weigh out as much as possible of freeze-dried tissue in the Weflon tube with lid). The exact weight is noted.
2. Add 1.5 ± ml of milliQwater and 1.5 ± ml of nitric acid (5.2)
3. Swirl and allow the mixture to stand until any visible reaction has stopped. Then cap the vessel
4. Place on the Weflon tube the protection shield, the adapter plate and the special spring
5. Introduce the vessels vertically into one of the numbered niches of the six-position rotor body

6. Then tighten the HTC screw in the upper part of the rotor body using the Tension Wrench, till you hear a clicking sound informing that the vessel is blocked inside its niche. Place the ring around the rotor to secure the vessels.

7. The rotor unit is now ready for being placed in the microwave cavity of the Milestone unit

**Operating procedure:**

8. The program suitable for digestion is started, normally program no. 8. Press: Start – Start. Program 8 will appear. Press start. The intervals are:
   
   Step 1:  6 min  250W  
   Step 2:  6 min  400W  
   Step 3:  6 min  650W  
   Step 4:  6 min  250W  
   Ventilation 5 min

**Cooling down:**

9. Once the digestion program is completed, very high temperature and pressure are reached inside the vessels. It is therefore necessary to cool down the rotor before opening the vessels.
   
   Place the rotor in the cooling system in the fume-hood and let the water flow for approximately 20 minutes.

**Uncapping the vessels:**

10. The rotor is dripped off before placing it on the workstation. Carefully loosen the screws in the upper part of the rotor body using the Tension wrench and wait till the pressure is completely released.

11. Remove the external protection ring, take the vessels out of the rotor body one by one.
12. Uncap the vessel – there might some fume evaporation. Immediately after uncappping carefully drop by drop add 7.2 ml 25% ammonia solution (5.4) into the vessel. There might be a violent reaction, so the protection window of the fume-hood has to be as low as possible.

Preparing the samples:

13. While the operating procedure is running, wash the filters once with diluted nitric acid (1:1) and 3-4 times with milliQwater.

14. The sample in the Weflon tubes is transferred through the filter into an acid washed 25 ml volumetric flask. The tube and the filter is washed with milliQwater into the flask and filled to the mark. Now the sample is ready for AAS-measuring not waiting too many hours!!