MASTER'S THESIS

Development of a methodology to test acute toxicity of nano-silver on activated sludge

carried out by

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ABSTRACT

This thesis was written as a part of the project "Nano-DESTINARA" leading by Umweltbundesamt Wien GmbH in cooperation with Vienna University of Technology. The project involves tests on four nanoparticles - TiO_2, Ag, CeO_2 and fullerenes – to check their toxicity on the biocenosis of activated sludge by means of tests on acute and chronic effects.

There is big number of articles and different projects of nanoparticles' toxic effects, however, none of them is about their potential influence on the environment via wastewaters. In the frame of this project the role of WWTP in emission this contaminants will be investigated. The quantification of the material flow of nanomaterial in municipal wastewater treatment is essential for the calculation of PEC-values (predicted environmental concentration) in risk-assessment for the aquatic environment.

In the frame of this thesis a literature research was done to collect the knowledge about previous studies on nanoparticles, especially about concentrations causing the toxic effect. The practical part of thesis was preliminary tests conducted on silver nitrate in different conditions, to adjust the proper methodology for further nanoparticle tests. The results were discussed upon the calculated parameters as oxygen uptake rate, MLSS, different forms of nitrogen and phosphorus concentrations. Tests were conducted mostly without the addition of nanoparticles, only in last week the addition of silver nanoparticles were investigated to confirm the proper matching of the created methodology.

The results had presenting that the highest inhibition was observed for the samples with silver nitrate in modified activated sludge at concentrations 50 and 100 mg/L. There was no inhibition in samples with Ag NPs, which might be caused by the dispersant used for sample preparations.

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1. INTRODUCTION

1.1. Nanotechnology and nanoparticles.

Nanotechnology is one of the few groups of science that has grown explosively during the last decade. Innovative methods of synthesizing nanoparticles (NPs) and nanotubes are constantly discovered. In common with growing knowledge of their structure and properties, new methods of fabricating patterned nanoparticles. Today goals of nanotechnology are :

- To master the synthesis of nanostructures and their assemblies of desired properties,
- To explore and establish nanodevice concepts,
- To generate new classes of high-performance nanomaterials (incl. biology-inspired systems),
- To improve techniques for the investigation of nanostructures.

There is no uniform definition of the term „nanomaterials" or "nanoparticle". Different countries inside and outside the European Union have their owns working titles to describe nanoparticles. generally, nanoparticles are characterized as materials by at least one dimension in the nanometer range. The OECD definition for nanomaterials is: "material which is either a nano-object or is nanostructured and has a size range typically between 1 nm and 100 nm". They are building bridges between molecules and infinite bulk systems. Their chemical and physical properties differ from the same material in bulk form. Nanoscience deals with their unique structure and its effects on: chemistry, dynamics, response, characteristic or energetics. Nanostructures and their assemblies are given in Table 1.

Table 1 Nanostructures (G Yushin, A Nikitin, and Y Gogotsi 2006).

Nanostructures and Their Assemblies

Nanostructure	Size	Material
Clusters, nanocrystals Quantum dots	Radius, 1–10 nm	Insulators, semiconductors, metals, magnetic materials
Other nanoparticles	Radius, 1–100 nm	Ceramic oxides
Nanobiomaterials, Photosynthetic reaction center	Radius, 5–10 nm	Membrane protein
Nanowires	Diameter, 1–100 nm	Metals, semiconductors, oxides, sulfides, nitrides
Nanotubes	Diameter, 1–100 nm	Carbon, layered Chalcogenides, BN, GaN
Nanobiorods	Diameter, 5 nm	DNA
Two-dimensional arrays of nanoparticles	Area, several nm^2–μm^2	Metals, semiconductors, magnetic materials
Surfaces and thin films	Thickness, 1–100 nm	Insulators, semiconductors, metals, DNA
Three-dimensional superlattices of nanoparticles	Several nm in three dimensions	Metals, semiconductors, magnetic materials

Size effects are the most important aspect of NPs. This parameter determines the structural, spectroscopic, thermodynamic, chemical and electronic properties of NPs.

Hansen et al. (2007) decided to divide NPs into three groups, each one based on a location of nanoscale structure. As follows:

1. Materials which contain nanostructured particles – defined as unbound molecules nanosized in two dimensions. This group might divide into subcategories depending on environmental conditions, i.e. nanoparticles bound to a solid substance, suspended in an air or liquid.
2. Materials which have a nanostructure on a surface – this category might be divided into subgroups in respect to materials that build the structure of nanoparticles on a surface. First subgroup is where the surface is structured but bulk and surface consist of the same material. Another group is un-patterned nanosized film on a distinct substrate and the third subcategory is a patterned film on a substrate where the film is thickened or the pattern is in nanoscale along the surface.
3. Materials which are structured in 3D – this group can be divided to the subgroups because of the materials consisting of one, two or more distinct materials (Mikkelsen et al. 2011).

Nanoparticles can be also divided in three groups: natural, artificial and engineered. Natural NPs consist of particles created during the combustion processes, particles from volcano ashes and some molecules as DNA or RNA. Nanoparticles from secondary sources – artificial – come from by-products from industrial production .

Connected with EPA ordinations (2007) engineered nanomaterials that are most commonly available can be divided into four groups:

- Composites – that combine NPs with other NPs or larger bulk-type materials, i.e. nanosized clays, are adding to products ranging to increase thermal, mechanical and flame-retardant properties of the products.
- Metal-based materials – this group contains metal oxides (titanium dioxide or cerium oxide) and quantum dots (closely packed semiconductors crystal comprised of hundreds atoms), nanosilver or nanogold.
- Carbon-based materials – composed by carbon and shaped as ellipsoids and hollow spheres referred as fullerenes, and tubes called nanotubes. These NPs have the application in electronics, they may also improve films and coating.
- Dendrimers – nanosized polymers built from branched units. Surface of this form has a lot of chain ends, which can be modified to receive specific chemical functions (useful for catalysis).

1.2. Nanoparticles synthesis techniques

There are two main synthesis techniques of nanoparticles: vapour-phase and solid-state processes. Vapour-phase was very popular in the earliest stages of nanoparticles development. Solid-state processes are used exclusively by the Ferro Corporation to produce e.g. lithium cobalt oxide, for the cathode material in lithium-ion batteries.

1.2.1. Vapour-phase synthesis of NPs

This method has been practiced for a long time before the nanoparticles became the object of interest for industry. Primary, with this technique nanoparticles were produced during inert gas condensation process. NPs with size ≤10 nm were formed when the metal atoms were released from a thermal source and started to rapidly lose their energy as a result of collision with gas atoms. A large glass cylinder was evacuated to a pressure ca. 2×10^{-6} torr with the pump. Then the alumina crucible was slightly heated via radiation from a graphite heater. After outgassing the reduced atmosphere, usually 0,5 to 4 torr of high-purity argon was introduced to the cylinder. The rapidly heated crucible caused almost equilibrium conditions. NPs which nucleate and grow in the gas phase, were collected on a copper surface cooled constantly by water. During one run it was possible to receive 1 g of nanoparticles. Metals usually synthesized by this method were: Al, Co, Fe, Ga, Cr, Mg, Cu and Ni. (Granqvist and Buhrman 1976)

The modification of this method is plasma-based synthesis. The piece of metal was placed on a copper plate cooled by water and heated with a plasma flame. The gas used in as an atmosphere was mixture of helium and 15% of hydrogen. The smoke from plasma-fired metal flowed to the cold cone where the particles were collected. The mean diameter of particles was ca. 20nm and the production of metals like Ta, Ti, Fe, Co, Cu and Al was in a rate as high as 50 g/h. The other less popular processes of vapour-phase are spray pyrolysis and flame-based synthesis.

1.2.2. Solid-state synthesis of nanoparticles.

This method involves the heat-treatment step with the use of milling media. It is very difficult to receive particles with the size lower than 100 nm, that is why a lot of companies is using innovations, such as mechanical attrition. This helps to achieve the size of particles in the range of 10-30 nm. However, this way of synthesizing nanoparticles has serious

disadvantages such as impurity pick up, inability to tailor the exact shape and size of particles and lack of control on the particle size distribution and characteristic of surface.

1.3. Application of nanoparticles

In the age of minimization nanotechnology has a great field of activity. The biggest possibilities have fullerenes and nanosized metals such as nanogold, nanosilver or titanium dioxide.

Carbon nanotubes are already used as tips in scanning microscopes and as field emitters in display devices. Also biochemical and chemical sensors are fabricated with nanotubes. Their surface properties enable to explore catalytic applications, especially after deposition of metal NPs on a surface.

Nanogold particles attached to DNA strands can be used in assaying specific complementary DNAs. A lot of metal nanocrystals or quantum dots is being used in biological sensoring. Drug and gene delivery is one of the fields bound to see further improvement, they definitely will be much more effective with application of NPs and nanocapsules (CNR Rao and AK Cheetham 2006).

1.4. Nanoparticles in pharmacy

For the last ten years one of the most important fields in nanotechnology is their application in drug delivery process. NPs have the ability to deliver a wide range of different substances to specified areas of the body in cyclic periods of time. Moreover, their small sizes are integral for a systemic circulation. In the past it was not possible to use natural polymers, such as polysaccharides or proteins, in drug delivery because of their various purity or recruitments of crosslinking in order not to denature the drug. First attempts in 1980s with using nano- and micromaterials were conducted with poly(alkylcyanoacrylate). Unfortunately, they were too big to cross the barriers in lymphatic system. This problem has been overcome by addition of surface modifications to nanoparticles. Nowadays NPs such as micelles, liposomes or vesicles are proposed as one of the most promising ways of drug delivery, because of their small sizes and hydrophilic surface.

There are several methods of preparing solid NPs, in which drugs can be e.g. encapsulated in a nanoparticle core, surrounded by a polymer membrane, entrapped in a polymer matrix, chemically conjugated to the polymer or bound to the particles surface by the adsorption

process. The most popular way is the emulsification-solvent evaporation technique. This technique was very effective with hydrophobic drugs, however has very poor results with bioactive agents with hydrophilic nature. In this method the polymer which is dissolving together with the compound in an organic solvent is carrying out the solvent evaporation. Dichloromethane is usually used as a poly-lactic-glycolic acid (PLGA) copolymers. The emulsion is prepared by adding water and a surfactant to previous polymer solution. Nano-sized droplets of polymer are induced in homogenization or sonication process. Then the organic solvent is evaporating and NPs are collected in centrifugation and lyophilisation processes.

The method used to encapsulate the insulin in oral delivery is PIN, phase inversion nanoencapsulation. The Zn-insulin is dissolved in Tris-HCl and then recrystallized by the addition of 10% zinc sulphate. Next step is the addition of precipitate to a polymer solution of PLGA in methylene chloride. Mixture is emulsified and dispersed in 1L of petroleum ether. This step results in the spontaneous formation of NPs.

The effectiveness of drug encapsulating processes depends on a few parameters. The first one is surfactant/stabilizer. There is a lot of synthetic and natural substances with different properties that were proposed to nanoparticles preparations. Feng and Huang (2001) were investigating phospholipids as a natural emulsifiers and they discovered that particles covered by DPPC (dipalmitoyl-phosphatidylcholine) had much smoother surface and it was easier for them to be transported through the human body. Research conducted by Barber et al. (2000) has shown that PLGA NPs prepared with addition of DMAB (didodecyl dimethyl ammonium bromide) were smaller than the NPs prepared with PVA (polyvinyl alcohol). One of the most promising stabilizers is an amphiphile D-α-tocopheryl polyethylene glycol 1000 succinate vitamin E (TPGS), it may increase the incorporation efficiency when it is used as a component of matrix. Additionally, it has a high emulsification efficiency and can be used as a cellular adhesion enhancer. The lower concentrations of TPGS decrease the polydispersity and the particle size (Yin Win and Feng 2005).

The next important parameter is the type of the polymer. Most of biodegradable polymers retain their properties for some time and then slowly starts degrading to the materials that can be dissolving and excreting from the body. Polymers which are going to be used in *in vivo* conditions have to obtain special properties like: biocompatibility, sterilization capability or process ability. Very important is also to investigate features like surface charge, hydrophobicity, density or protein adsorption. The most common polymers used with NPs are PLGA (Poly(lactide-co-glycolide)), PLA (Poly(lactic acid)) and PCL (Poly-ε-caprolactone).

1.5. Nanoparticles vs. environmental risk.

There are several sources of NPs in the environment. This might be production, sources not related with others e.g. wearing clothes containing nanoparticles or accidental release of them, wastewater treatment plants or landfills. Fig. 1 presents possible pathways for nanoparticles and their occurrence in environment.

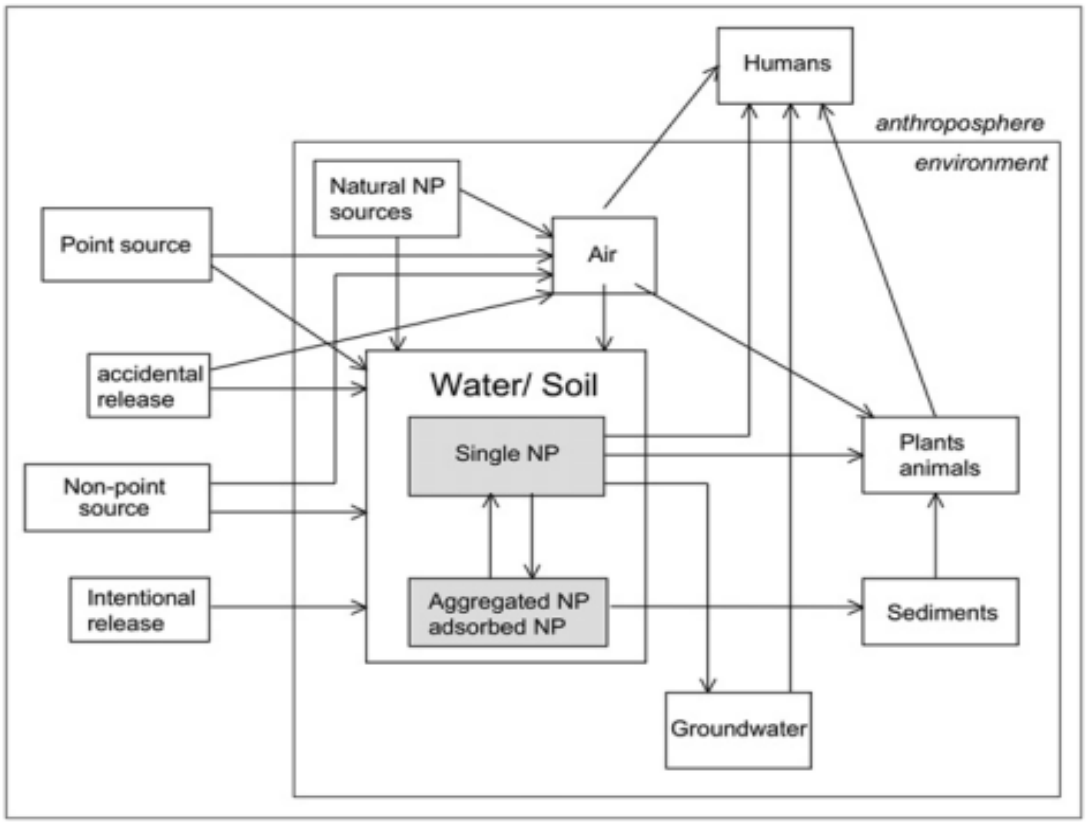

Figure 1 Possible pathways for NPs and their occurrence in environment (Nowack and Bucheli 2007).

However, no matter what is the source of nanoparticles, if it is air or soil, their final destination are groundwater. Human might be exposed on the NPs activity directly via air, water and soil or indirectly via plants and animals. Therefore, the control of NPs present in the environment is an important issue.

2. PURPOSE OF THIS STUDY

The purpose of this study was the development of a methodology, suitable for the assessment of acute toxicity of nanoparticles - for the biocenosis of activated sludge. In the frame of this thesis the parts that were realized were the literature studies on the previous research conducted on these particular nanoparticles and laboratory studies on activated sludge with silver and silver nitrate as a reference.

3. LITERATURE REVIEW

3.1. SILVER

Silver is one of earliest discovered metals. Archaeological excavations suggest that ancient Egyptians have been using it 4000 years B.C. At first it has been used in manufacturing decorations, but it has been noticed that silver has unique properties. They have started to use it in food, water and milk preservation, in antibacterial foils for wounds and burns, and in many other silver solutions that had antifungal properties (Rai, Yadav, and Gade 2009).

Silver nanoparticles are known for about 120 years. Decisive role in preparing Ag NPs has is the addition of stabilizing compounds, to prevent them from growing and aggregating. Synthesis is usually done by reduction of soluble salts with different reduction agents like e.g. glucose, citrate or sodium borohydride. Process might be carrying in water or in organic solvents. "Nanosilver" can be a component in refrigerators, mobile phones, clothes, toothbrushes or cosmetics. Silver nanoparticles are also used in optics, electronic and chemistry using e.g. as a substrates to synthesis, catalytic materials, sensors or conductors (Chernousova and Epple 2013).

Kim et al. (2007) have done research on the effect of Ag NPs on several microorganisms. They were investigating if silver nanoparticles can be applied to control the microorganisms and to prevent harmful diseases. The antimicrobial activity has been evaluated for yeast, *E. coli* and *S. aureus* by determining the minimal inhibitory concentration (MIC) of Ag nanoparticles. The Ag NPs concentrations range was from 0,2 to 33 ng/L. Comparing to the positive control with itraconazole, Ag NPs of 33 ng/L showed similar effects of growth inhibition as the control, and there was also a significant effect for 13,2 ng/L. These results present that the minimal inhibition concentration (MIC) of silver nanoparticles against yeast can be estimated between 6,6 ng/L and 13,2 ng/L. The MIC for *E. coli* may be estimated in range from 3,3 ng/L to 6,6 ng/L, so the growth inhibition effect was stronger than for yeast.

Surprisingly, the MIC of silver nanoparticles against *S. aureus* was estimated to be more than 33 ng/L, so there was no significant effect of inhibition while comparing it with the gentamicin control.

Choi et al. (2008) have been researching the inhibitory effect of Ag NP and two other substances on microbial growth. The aim of their research was to evaluate the impact of distinct Ag substances like Ag NPs, Ag^+ ions and AgCl colloids on autotrophs and heterotrophs at activated sludge in WWTP. As autotrophic organisms mixed and enriched nitrifying bacteria were used and *Escherichia coli* was used as a representative for heterotrophic bacteria. Ag NPs were synthetized during the process of reducing silver nitrate with sodium borohydrate and addition of polyvinyl alcohol, as the capping agent to control the growth and agglomeration of NPs. The addition of 1 mg/L Ag (in Ag NPs form) to the nitrifying suspension caused the inhibition at the level of 86±3%.

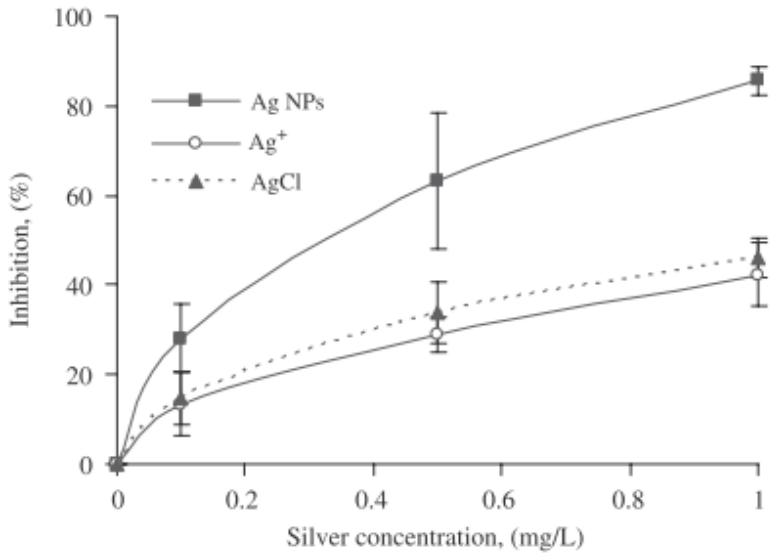

Figure 2 Nitrification inhibition as a function of the concentrations of silver form of Ag NPs, Ag+ ions and AgCl (Choi et al. 2008).

The heterotrophic growth has been inhibited at 55% at concentrations of Ag NPs increasing up to 4,2 μM and the IC_{50} was 4,0 μM Ag NPs. At the concentration 1,0 mg/l Ag there was no sign of growth.

Table 2 Growth races of *E.coli*. and the inhibitions of distinct concentrations (Choi et al. 2008).

Concentration (μM)	Ag NP	
	μ (d^{-1})	Inhibition (%)
1.4	0.40 (±0.03)	17 (±5)
2.8	0.34 (±0.03)	30 (±6)
4.2	0.22 (±0.04)	55 (±8)

Fabrega et al. (2009) investigated the impact of Ag NPs on *Pseudomonas fluorescens.* The suspension has been prepared by dispersing 5 g of silver powder in 1l sterile water and the addition 0,25 mg/L sodium citrate for stabilizing the suspension, after that sonicated (30 min/d, 4 days). The supernatant was left to stand and after 96h the final Ag NPs concentration has been measured by inductively coupled mass spectrometry. The physicochemical properties of Ag NPs were characterized at concentrations between 0 and 2000 ppb (0 and 19 μg/L) Ag NP suspension in minimal Davies Medium at pH values of 6, 7.5, and 9, in the presence and absence of 10 mg/L (4.58 gC/l) SRHA (Suwannee River humic acids). The impact of Ag NPs on *P.fluorescens* were studied under six different growth conditions (pH values of 6, 7.5, and 9; with and without 10 mg/L SRHA in the medium), two different exposure times (3 and 24 h),and four different concentrations, (0.019 – 19 μg/L). Growth of the bacteria was monitored after 3 and 24 h. At 3h reduced growth is observed only at the highest concentration of Ag NPs in the absence of SRHA and only significantly at pH 9 (50% reduced growth). However, this reduced growth at 3h disappears in the presence of SRHA i.e. growth as in the control, without Ag NPs, in the presence of SRHA. After 24h Ag NPs were toxic only at the highest Ag NP concentration (2000 ppb) at pH 9 without SRHA, causing a 90% decrease in the bacterial cell density at 24 h after exposure.

Kaegi et al. (2011) were investigating the behaviour of silver nanoparticles in a pilot WWTP (Figure 3) fed with municipal wastewater. Hydraulic retention time in this WWTP, consisting of a non-aerated and an aerated tank and the secondary clarifier, was 1 day and sludge retention time was 14 days. Nanoparticles were added to the non-aerated part, and samples were collected from the aerated basin and from the effluent.

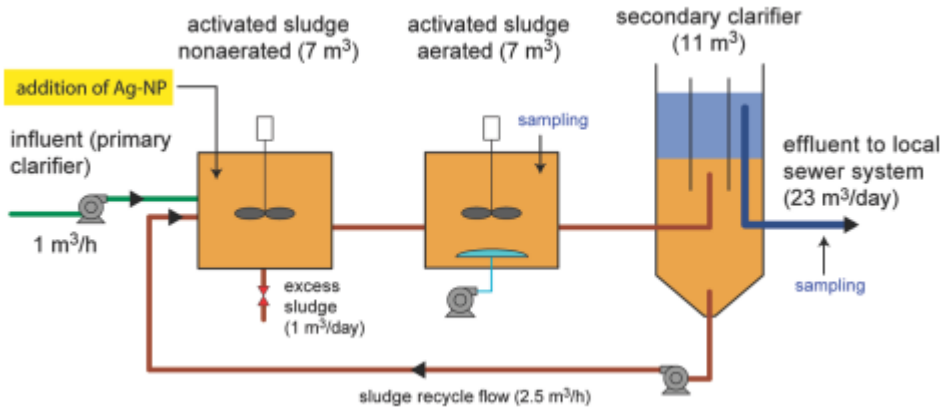

Figure 3Schematic layout of the pilot WWTP (Kaegi et al. 2011).

Analyses have shown that the initial concentration of Ag spiked to the non-aerated tank was 2400 µg/l. The mass balance suggests that ~5% (7.2 g) of the added Ag left the WWTP via the effluent, ~85% (110 g) ended up in the excess sludge and ~5% (7.8 g) still remained in the WWTP when the experiment was stopped after 43 days.

Hou et al. (2012) investigated if the Ag NPs might cause inhibition and loss of bacteria, that could have an impact on COD reduction and nitrogen removal. As mentioned in Choi et al. (2008) 1 mg/L Ag inhibited autotrophic nitrifying organisms from a continuously operated bioreactor by >85%. Here, the research has been conducted for half a month in sequencing batch reactor (SBR). In addition to measuring COD reduction and nitrogen removal, oxygen production was measured to confirm the variations of NH_4 removal in the presence of silver nanoparticles. The wastewater and biosolids were collected from WWTP in Beijing. The Ag NPs (1 mg/L) were added to the influent (1L, 269 mg/L, pH 7,3) and stirring. After that the upper layer aliquots (10 mL) have been collected to measure the silver concentration. The experiment included three treatments which involved adding 0, 0.55 and 2.75 mL of Ag-NPs stock suspension, respectively into the reactors. The COD of the samples collected at the beginning and end of each cycle were determined using the potassium dichromate method. The NH_4 concentrations in the samples were determined using the Nessler reagent colorimetric at 420 nm using a spectrophotometer. The effluent COD values of a particular cycle typically fell in a range of 20–40 mg/L. The average effluent COD in the dosed reactors were not statistically different from the COD in the control reactors in most cycles. In some cases, the COD in the reactors exposed to NPs were even lower than in the controls. These results strongly indicate that dosing of Ag-NPs did not cause any systematic changes in COD removal efficiencies. In the first days of the experiment the removal of nitrogen was statistically lower than in control reactors. This indicates that Ag-NPs slightly inhibited the

respiration of nitrifying organisms at the beginning of the experiment. From day 2 to the end of the experiment, however, the average removal efficiencies in the reactors dosed with Ag-NPs were not statistically different from the average removal efficiencies of the control reactors. These results indicate that Ag-NPs in wastewater at a concentration up to 0.5 mg/L would not dramatically impact the NH_4 removal efficiency of the activated sludge process.

Sun, Sheng, and Liu (2013)were trying to determine the effects of 1 mg/L of silver nanoparticles on microbial communities in activated sludge. The activated sludge was collected from WWTP in Edmonton (Alberta, Canada) right before each of experiments and stored at 15˚C to provide the same conditions as normally in regular wastewater treatment process. Activated sludge samples were allowed to settle by gravity, and after 3h they were removed and divided into two groups: the intact activated sludge and unsettled activated sludge. 50 ml samples with the distinct kind of sludge and with the presence or absence of Ag NPs (1 mg/L) were incubated in 250 mL flasks, at 15˚C and stirred with 200 rpm for 24h in lack of light. Microbial communities were investigated in PCR-DGGE, with the fragment of gene that is coding 16s rRNA from both types of samples. The results have shown that during the 24h treatment with the concentration of 1 mg/L silver nanoparticles, the number of heterotrophic bacteria in intact sludge stabilized at around $5{,}0*10^7$ CFU/ml, and it has not changed significantly in comparison with the control samples. This reveals that silver nanoparticles at the tested concentrations had no impact on the heterotrophic cell culturability of intact sludge samples. For the control samples of unsettled sludge the presence of 1 mg/L Ag NPs caused about 1,5 log units loss in the fast-growing bacteria and one log unit loss in total heterotrophic bacteria number. The decrease started in the 8^{th} hour which might reflect the time taken for silver nanoparticles to diffuse into the activated sludge flocks. DGGE results of intact sludge samples presented the reduction of *Bacteroidetes* and *Proteobacteria,* which may suggesting that these are sensitive to Ag NPs treatment. The microbial community shift in unsettled sludge samples after treatment with Ag NPs was not significant.

Kaegi et al. (2013) were performing batch experiments with different silver and gold nanoparticles connected with activated sludge (AS) to characterize the effect of particle type, coating and size on the removal efficiencies. The sludge was collected from a WWTP in Eawag, usually in the mornings, from the denitrification or nitrification stages. Two sets of experiments were conducted. In the first one 400 g of AS were added to 500 ml polypropylene, and the mixture was exposed to three types of conditions: oxic, anoxic and anaerobic. After 30 min of sedimentation, the supernatant was withdrawn with a pump. The supernatant has been weighted and digested with a mixture of hydrochloric and nitric acids.

This was the blank set. In the second batch, silver nanoparticles were dosed to the AS in anaerobic conditions, and samples were collected after 0.5, 0.8, 1.3, 2.3, 4.3 and 24 h. Results presented that particles attached to the flocks and that they were efficiently removed from the wastewater and that the treatment, size, coating and type of particles has no significant influence on the effectiveness of removal. The removal efficiency was around 99-99.9% after 2 h reaction with the dosage of 150 mg Ag NPs/kg TSS

3.2. CERIUM DIOXIDE

Cerium dioxide NPs (CeO_2) are used in semiconducting as an abrasive, and as components in catalytic converters used in automobile exhaust systems. They are applied as fuel additive, to increase combustion, used in UV radiation absorbance and as electrolytes for fuel cells. They also have an antioxidant activity at physiological pH values what makes them useful for biomedical applications in cells protection (Pelletier et al. 2010).

Limbach et al. (2008) presented their research about removing cerium nanoparticles from a model wastewater treated in a model wastewater treatment plant. Two cerium nanoparticles have been used in this experiment – pure and surfactant stabilized. The activated sludge in the model WWTP was taken from WWTP in Zurich and the respiration rate was checked during the experiments. Nanoparticles were investigated in different media, ionic strengths and pH values. The model wastewater treatment plant consisted of an aeration chamber, followed by a settling vessel (Figure 4).

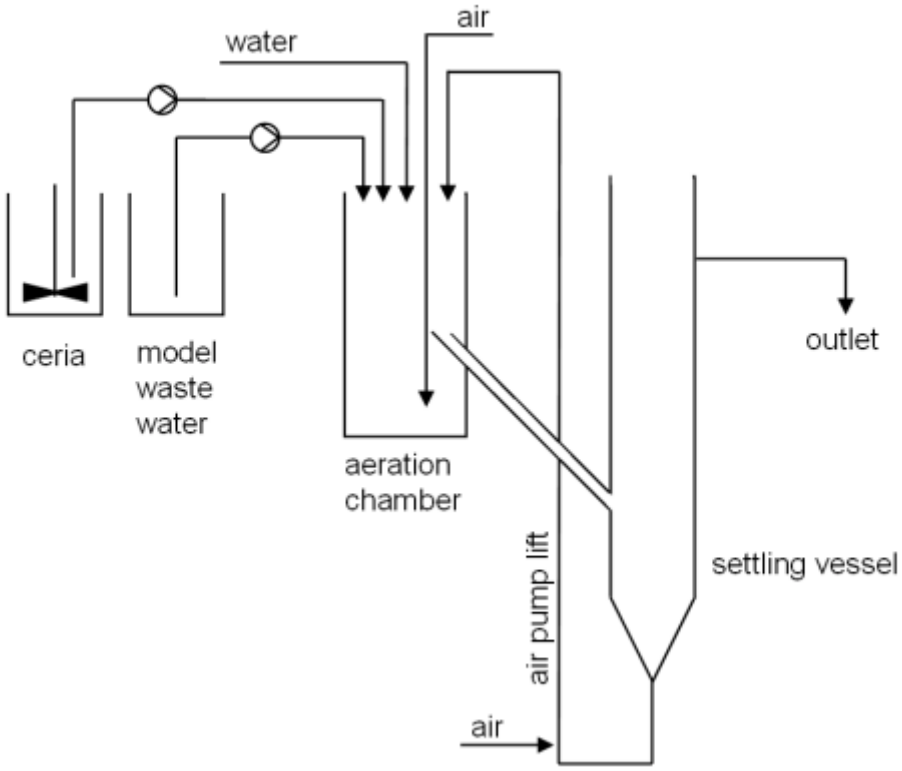

Figure 4Experimental setup of a continuous model wastewater treatment plant according to OECD 303A (Limbach et al. 2008).

The total amount of cerium NPs in effluent was measured with an inductively coupled plasma-optical emission spectrometer. Treated wastewater was analysed by two methods: CeO_2 present as particles of less than 200nm hydrodynamic diameter and total cerium oxide content in outflow. The division has been done to enable larger parts or nanoparticles bound to bacteria. The results present that the presence of cerium dioxide in sludge depends on the dispersant used for CeO_2. After 50h exposure time the sludge had adapted to the presence of benzyl sulfonic acid used to disperse CeO_2 and started to digest the acid, which caused destabilization of cerium nanoparticle. As a result less cerium dioxide have left the sludge. However, NPs concentration (100-1000ppm) in this research had no acute toxicity for the bacteria.

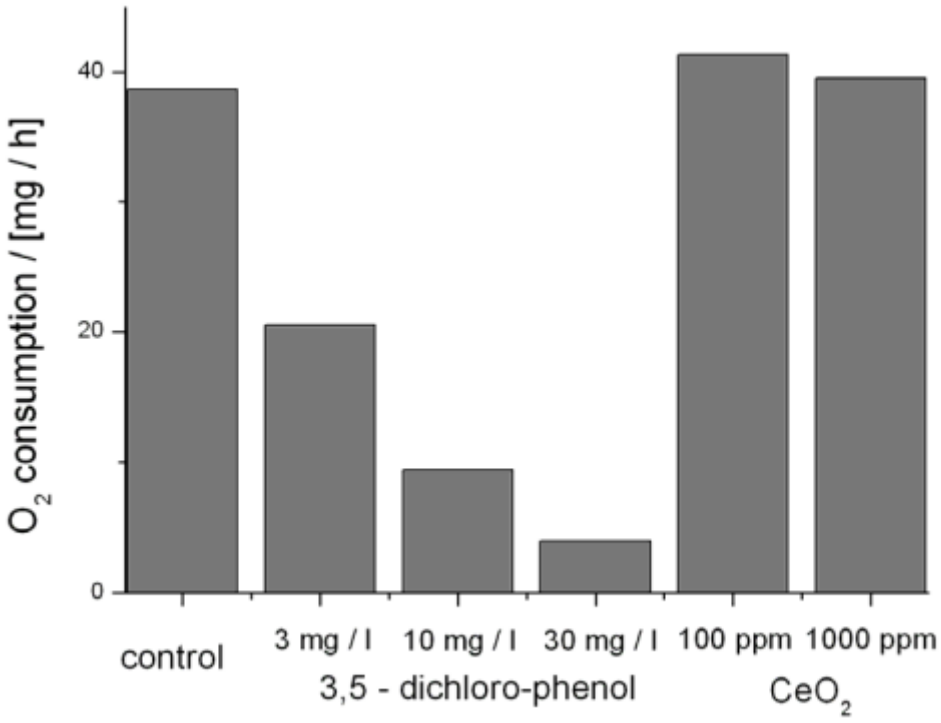

Figure 5 Oxygen consumption of the activated sludge after 3 hours (Limbach et al., 2008).

Pelletier et al. (2010) were conducting research on the effects of nanoparticle concentration, particle size, exposure time, growth medium, and pH on the growth and viability of *E. coli, Bacillus subtilis,* and *Shewanella oneidensis*. The bacterial sensitivity was tested by disk diffusion test. The MIC test were conducted with 50, 100 and 150 mg/L CeO_2 NPs. *Shewanella oneidensis* was more resistant to cerium dioxide due to metal reduction in its metabolism. *E. coli* and *B subtilis* showed distinct responses to applied concentrations of CeO2 NPs. In *E. coli* the growth inhibition effect decreased with increasing particle size. The biggest inhibition was for particles in the size range of 15-19.3 nm – viability of cells (Figure 6) dropped from 100% for the control to ca. 55%, 50% and 35% for concentrations of 50, 100 and 150 mg/L CeO2, respectively. For *B. subtilis* the same size of NPs had no significant effect on growth inhibition.

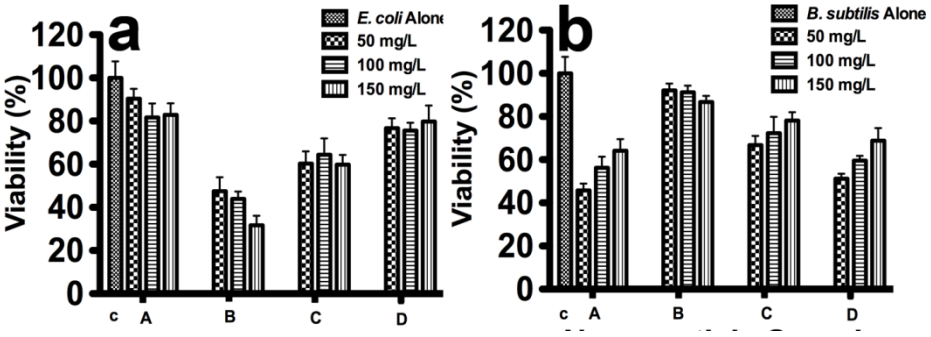

Figure 6 Viability of *E. coli* and *B. Subtilis* at different NP concentrations A – (ammonia solvent, particles size 6-9,5 nm), B – (ammonia solvent, particles size 15-19,3 nm), C – (NaOH solvent, particles size 22-27,7 nm), D – (NaOH solvent, particle size 40-50 nm) (Pelletier et al. 2010).

As a result, nanoparticle size , dispersant applied and the morphology of the particles are the main properties that diversify the bacteria cell response.

García et al. (2011) conducted experiments with acute toxicity tests of three metal nanoparticles, comprising cerium dioxide NPs. The Ce^{3+} ions from $Ce(NO_3)_3$ salt is oxidized at basic pH conditions to Ce^{4+} using hexamethylenetetramine (HMT). Then, CeO_2 nanocrystals precipitate and are further stabilized in aqueous medium with HMT, which forms a double electrical layer to prevent agglomeration. The investigations were done on seeds (*Lactuca sativa*, *Cucumis sativus*, *Solanum lycopersicum*, *Spinacia oleracea*, *Allium porrum* and *Capsicum annuum*), *Daphnia magna* and bioluminescence test on the marine bacterium *Vibrio fischeri.* At the maximum concentration of 0.64 mg/L Cerium dioxide was highly toxic for all tested seeds. A reduction to 10% (0,064 mg/l) did not result in a decreased level of inhibition (~ 80%). In *Daphnia magna* the LC_{50} amounted to 0.012 mg/L. The authors noticed that stabilizers used with the NPs do not provoke any toxic effect on tested organism. In the bioluminescence test cerium dioxide showed a toxicity above 80% at 10% of the maximum concentration (0.64 mg/L).

3.3. FULLERENES

Fullerenes have been discovered in 1985 as a third allotrope of carbon. Their chemical, biological and physical properties have attracted attention in distinct fields of study. Their size, unique cagelike structure, hydrophobicity, electronic configurations and three-dimensionality make them an attractive subject not only in industry, but also in medicine and chemistry. Their specific cagelike structure enables to encapsulate other molecules. This makes fullerenes a perfect medical tool such as medical therapeutic agents, tissue-specific fullerene-based drugs etc. (Wilson 1999). Experiments with human cells in nonaquatic environment revealed that C_{60} might be a very good antioxidant in biomedical applications (Levi et al. 2006). These properties enable them to be used as organic photovoltaic cells, catalysts, polymer additives, organic field effect transistors, and also as antioxidants or biopharmaceuticals. They are already used in personal care products (Murayama et al. 2005).

Carbon-$_{60}$ is probably the most, well recognized nanoparticle so far. It is a prototypical carbon-based nanomaterial. It has low solubility in polar solvents – less than 10^{-9} mg/L. In

contact with water, C_{60} can form - through different ways and in different conditions - a water-stable, colloidal aggregate. Diameters of those "nano-forms" are between 5 and 500 nm. The presence of this aggregated forms enable to reach a concentration of up to 100 mg/L which is about 11 times more than the estimated molecular solubility (Fortner et al. 2005).

One of the earliest researches, conducted by Babynin et al. (Babynin et al. 2002) have presented a toxic influence of fullerenes and their derivatives on organisms. During the experiment, *Salmonella typhimurium* species were exposed to three derivatives of fullerene C_{60} in two distinct media: alcohol and polyvinyl pyrrolidone (PVP). Analyses were conducted on the BA13 strain of *Salmonella typhimurium*. As a result, in each of the investigated media every compound revealed genotoxic activity (counted in His+ revertants number).

The applied researches on microbial response showed that fullerenes are affecting bacteria. In his experiment Fortner (2005) used two well-examined bacteria: gram-positive *Bacillus subtilis* and gram-negative *Escherichia coli*. Both of them are very common, soil species, and have obvious differences in structure of cell wall composition. The indicator of fullerenes influence on bacteria was their growth. The studies were conducted with two different media – one complex and one simple, under both - anaerobic and aerobic conditions. As a result, the presence of nano-C_{60} was inhibiting the growth of organisms. However, depending on which media has been used, different concentrations of fullerenes caused the inhibition. Simple media was minimal David media with 10% of recommended potassium phosphate. The concentration of nano-C_{60} above 0.4 mg/L was inhibited the growth of bacteria, while using the rich media (Luria broth) enabled organisms to take a dose of 2.5 mg/L fullerenes. Distinctions in results could occur because of a probable salting process of fullerenes in cooperation with rich media. It has been shown that the ionic strength has an influence on fullerenes, and might slow down the activity of nano-C_{60}.

Lyon et al. (2006) presented the ways of making fullerene water suspensions (FWS) and the antibacterial activity those resulting suspensions cause. Lyon et al. present earlier works about fullerenes' toxicity, mentioning possible poisonous effects of applied solvents, while researches conducted on powder C_{60} and C_{60} dissolved in solvent have not revealed any toxic properties. Their research aims were to determine whether nC_{60} has antibacterial properties and the solvents used in process of preparing FWS increase or decrease the antibacterial activity. Four distinct methods were: THF/nC_{60} (THF used as a solvent), son/nC_{60} (sonicating C_{60} dissolved in toluene and water), aq/nC_{60} (C_{60} powder stirring in water) and PVP/nC_{60} (applying a solubilizing agent). Tested organism was *Bacillus subtilis*. The minimal inhibitory concentration (MIC) for THF/nC_{60}, son/nC_{60}, aq/nC_{60} and PVP/nC_{60} were 0.09-0.1 mg/L, 0.7-

1.0 mg/L, 0.5-0.63 mg/L and 0.95 – 1.4 mg/L, respectively. These results showed no big differences between the MIC of son/nC_{60}, aq/nC_{60} and PVP/nC_{60}, while THF/nC_{60} might be different because of the way of extraction. It could be demonstrated that solvents used in this research are somehow responsible for the antibacterial activity of the fullerenes, however were not more toxic than in their single form. More important evidence was that the size of nanoparticles (NPs) has an influence on antibacterial activity. The MIC increased when the mean diameter increased. There was no linear relationship between toxicity of nanoparticles and their size which insinuates that other factors might be responsible for higher antibacterial activity. Differently prepared suspensions, despite the same sizes of NPs, had distinct MIC, which was probably the result of differences in the production methods (e.g. temperature, amount of water, reagents, rate of stirring etc.).

Nyberg, Turco, and Nies (2008) performed research on the influence of fullerenes on anaerobic microbial communities. In earlier researches it has been proved that fullerenes neither as nano nor in bulk had any toxic effect on soil microbial respiration, or an impact on phospholipid profiles or Bacterial 16s rRNA genes (Tong et al. 2007). They had tested C_{60} fullerene dissolved in toluene and *o*-xylene and applied it to dried biosolids. Also the aqueous C_{60} has been tested, by using the suspension of C_{60} in methanol and ethanol .The microorganisms were provided with glucose as substrates to gas production in methanogenesis process. Toxicity tests for anaerobic microorganisms were conducted by measuring the production of carbon dioxide and methane for several weeks. After that the microbial community structure has been analysed in PCR and DGGE. Due to the fact of different ways of preparing fullerenes in this project, they had distinct concentrations. Fullerene plated on dried sludge with *o*-xylene and toluene has concentrations of 50000 mg/(kg of biomass)(d/w) and 30000 mg/(kg of biomass)(d/w), respectively, in aqueous conditions 8,6 mg/(kg of biomass)(d/w) and dissolved in MeOH/EtOH 0,32 mg/(kg of biomass)(d/w). Their concentrations have been estimated to $3{,}26\times10^{-6}$ mg/L for samples dissolved in alcohol, and $7{,}96\times10^{-6}$ mg/L for others. Gas production has been measured in 89^{th} day of experiment for *o*-xylene and in 154^{th} for other samples treated with C_{60}. In samples with *o*-xylene there was no significant increase in gas production, but as expected an increase in gas production occurred due to the decay of dead biomass from dried sludge and not because of NPs presence. However, for the sludge treated with *o*-xylene lower gas production was noticed, which might be due to toxic properties of *o*-xylene. The same occurred in toluene samples, no indication of toxic influence of NPs rather the toxicity of toluene, and what is more concerning, no signs of biodegradation. It was also impossible to define the

exact volume of gas formation, because of presence of toluene and *o*-xylene, however it is believed that after long-term exposure these solvents might be degraded anaerobically. genetic analysis of three domains (Archaea, Bacteria and Eukarya) support the conclusion that there is no toxic effect of fullerenes. However, the low concentration of C_{60} that has been applied, limited this work.

Baun et al. (2008) have been researching if the presence of fullerene aggregates may enhance the toxicity of other substances present in water. They used four model chemicals (atrazine, methyl parathion, PCP and phenanthrene), as substances presenting different properties and mixed them with aggregated nC_{60}. Tests were conducted on *Pseudokirchneriella subcapitata* and *Daphnia magna*. The toxicity of nC_{60} had been tested on these organisms before it was mixed with model chemicals. It has shown that at concentrations $\leq$ 50 mg/L there was no toxic signs on daphnids, however, nC_{60}-aggregtes were visible at organisms surface and in the digestive tract. The algal growth was inhibited by 30% at 90 mg nC_{60}/l. Adsorption of nC_{60}-aggregates on algal cells was observed. It could be presented that the presence of nC_{60}-aggregates increased the toxicity of the four chemical compound. Phenanthrene's poisonous activity increased from 720 µg/l without nC_{60} to 430 µg/l with the addition (presented on EC_{50}-value in the algal test). Surprisingly, the addition of nC_{60} water suspension to phenanthrene in the daphnid test decreased the toxicity from 500 µg/l (without nC_{60}) to 680 µg/l (with addition). These concentrations refer to the sum of dissolved and sorbed phenanthrene, and the water phase concentration can be estimated to 48 µg/l with addition of nC_{60}. Also in tests of atrazine, the toxicity increased with the addition of nC_{60} (from 150 µg/l to 99 µg/l). The toxic activity of PCP decreased from 36 µg/l to 70 µg/l without and with the presence of nC_{60} suspension, respectively. Changes in methyl parathion test were not significant.

Choi et al. (2008) presented research with ozonated nC_{60} and its influence on growth of *Escherichia coli*. Many of the recent works presented the toxicity of nC_{60}, however, there is still were only few papers about fullerenes' derivatives. It was suggested that nC_{60} could be less toxic with increased hydroxyl derivatization. Tests were conducted in tubes containing different concentrations of ozonated nC_{60} (0.03, 0.1, 0.25, 0.5, 1.0, 2.0 mg/L) with Davis media and were added to *E. coli.* It could be shown that ozonated nC_{60} with concentration as high as 10 mg/L did not inhibit bacteria growth. *E. coli* was not inactivated during the exposure to 5 mg/L and 20 mg/l ozonated nC_{60} (3h, lack of light, absence of oxygen). Also with the presence of UVA irradiation the inactivation was very small (0.14 log for 150 min by 10 mg/L of ozonated nC_{60}). In the environment without the oxygen, the ozonated nC_{60} did not

have any antibacterial activity. However, in the presence of dissolved oxygen and UVA irradiation, there was a significant level of bacteria inactivation (the inactivation after 150 min of exposure increased from 0 to 1.3 log). The observed effect might suggest that inactivation occurred by the photocatalytic activity of ozonated nC_{60}.

3.4. TITANIUM DIOXIDE

Titanium dioxide nanoparticles (TiO_2) are worldwide used in distinct fields of industry. It is commercially available as a powder for preparing an aqueous suspension. Titanium dioxide is applied to confectioneries, white-coloured sauces and dressings, non-dairy creamers, cottage cheeses and mozzarellas (Kiser et al. 2009). In water treatment nano-titanium dioxide is used as photo-catalyst in production of reactive oxygen. Also is applied as ointments, toothpaste, catalysts, catalyst supports, adsorbents, delustrants, semiconductors, white paint, sunscreen etc. (Mikkelsen et al. 2011).

Kiser et al. (2009) conducted studies on titanium dioxide nanoparticles, focused on the quantification of the Ti-concentration in full-scale municipal WWTP, characterization of the composition and morphology of Ti-based solids in consumer products plus wastewater effluents and biosolids and quantification of the titanium concentration in lab-scale treatment reactors. Samples were collected from a wastewater reclamation facility in Arizona (USA), that is operated according to the activated sludge process and with tertiary filtration, four times each day at different hours (effluent samples from each process unit, activated sludge from the aeration basins, primary and excess sludge, and biosolids). To represents the full-scale WWTP operations, SBRs were used. For analysis, liquid and solid samples were digested with HNO_3 and H_2SO_4 acids and then analysed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). Approximately 91% of titanium dioxide that entered the treatment plant was removed from wastewater, and accumulated in primary sludge, activated sludge biomass and secondary solids. Similar results were observed in other samples collected at other times, except the sample collected at 6 p.m. (probably because of an industrial source discharged into the sewers during the day). In this case the removal of TiO_2 was 56-99%. The concentration of Ti in filtered samples was lower than in non-filtered samples because of higher concentrations of total suspended solids like plant influent and primary effluent. Tertiary effluent had solids concentrations of 4 mg/L TSS, and the samples filtered and non-filtered had very similar results. With the comparison to other samples, the highest concentration of Ti was found in the activated sludge system (i.e. aeration basin),

except secondary solids. This might be caused because of the recirculation of the sludge from the secondary settling tank to the head of the aeration basin, which exposed the activated sludge continuously with inorganic Ti.

Table 3 Average concentrations for four sampling periods (Kiser et al. 2009).

sample description	filtrate Ti (μg/L)	total Ti (μg/L)	total suspended solids (mg/L)	total Ti normalized to solids (mg Ti/g TSS)
headworks	34 ± 3.2	843	336	1.6 ± 0.4
primary effluent	66 ± 51	99	97	1.0 ± 0.1
aeration basin	14 ± 9	2572	2220	0.9 ± 0.3
secondary effluent	40 ± 48	35	7	–[a]
tertiary effluent	20 ± 9	36	6	–[a]
primary solids	–[b]	803	1220	0.7 ± 0.2
secondary solids	–[b]	8464	7542	1.1 ± 0.1

[a] Indicates samples where TSS was too low to accurately quantify normalized Ti concentrations. [b] Solid samples were not further filtered.

In lab-scale reactors, only 12% of Ti was found in the effluent. 88% was associated with the biosolids fraction. It was highlighted that TiO_2 has a very strong affinity for biomass.

Kiser et al. (2010) carried out research about describing biosorption of different types of nanoparticles to wastewater biomass. The main aims were: imaging biosorption of fluorescent nanoparticles to wastewater biomass, quantifying and comparing degrees of biosorption of distinct types of NPs. The TiO_2 suspension was prepared by adding nanoscale 99% TiO_2 (Hombikat, Sigma-Aldrich; St. Louis, MO) to ultrapure water and sonicating for 1h at 200 W/l. Following sonication, the suspension was centrifuged at F = 1000 g for 30 min. The supernatant, which contained suspended TiO_2,was collected and used as stock solution. Activated sludge was collected from a full-scale WWTP in Arizona (USA). All nanoparticle samples were added to the biomass and agitated for 3h. After that biomass was gravitationally settled to simulate settling in real WWTP. Supernatant was collected and analysed. Titanium dioxide NPs was removed from liquid phase to some degree, after exposition to 50 and 400 mg/L TSS. However, TiO_2 NPs removal was very low – 5-6% and 22-24% for 50 and 400 mg/L TSS, respectively. Usually the MLSS in an activated sludge plant is a lot higher (3000-5000mg/l).

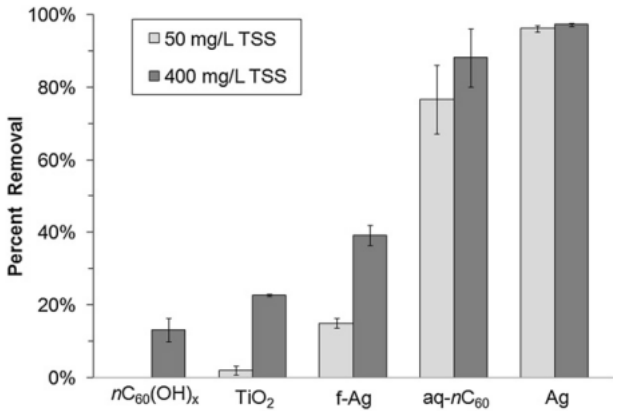

Figure 7 Percent removal of five types of NPs from liquid phase after exposure to 50 and 400 mg/L TSS of whole wastewater biomass (Kiser et al. 2010).

Zheng, Chen and Wu (2011) investigated the effects of titanium dioxide nanoparticles on nitrogen and phosphorus removal from wastewater and on the bacterial community in activated sludge with PCR-DGGE. One of the aims of this work was to check the influence of TiO_2 NPs on the transformation of intracellular polyhydroxyalkanoates (PHA) and glycogen, and the activities of some enzymes associated with biological nutrient removal. The environmentally relevant concentration of titanium dioxide nanoparticles in this study was 1 mg/L, but also the potential effect of 50 mg/L was investigated. Three SBRs were prepared to contain 1 L of synthetic wastewater and 1 L of inoculating sludge obtained from a parent SBR which was operated over 100 days and achieved stable biological nutrient removal (approximately 80% and 99% of nitrogen and phosphorus removal). Afterward, SBR 1 and SBR 2 were fed with 40 and 2000 mL of TiO_2 NPs stock suspension (100 mg/L), respectively, and SBR 3 was operated as the control (i.e., without TiO_2 NPs addition). The experiments were conducted in day 1 and day 70 (as short-term exposure and long-term exposure, respectively). The results indicated that concentration of 1 mg/L TiO2 NPs had no adverse effects on nitrogen and phosphorus removal, which was 79,4%-80,3% and >99%, respectively. The exposure to 50mg TiO_2/l increased the NH_4 in the effluent from non-detectable to 17,5 mg/L. After 70 days of exposure NO_2 and NO_3 in SBR 2 were around 0,68 and 0,73 mg/L, respectively. Total nitrogen removal efficiency of SBR 2 was much lower than in control, 24,4% after 1 day and 80,3% after 70 days. Almost all phosphorus in all SBRs was removed. In further research it has also been presented that Ti NP-concentrations of 1 and 50 mg/L in anoxic conditions had no acute effect on nitrogen and phosphorus removal. However, it was found that 50 mg/L TiO_2 NPs caused the serious inhibition to ammonia oxidation after long-term exposure. The average removal efficiency of NH_4 was observed to

be 30.8%, which was significantly lower than that in the control SBR (>99%). The corresponding effluent NO_3 highly decreased from 5.5 to 0.8 mg/L due to the deterioration of ammonia oxidation.

Wang, Westerhoff and Hristovski (2011) studied the removal efficiency of silver, titanium dioxide and carbonaceous NPs from simulated wastewater and into biosolids using lab-scale SBRs and evaluated the effects of NPs on the activity of bacteria in WWTPs. Typical wastewater treatment systems operate at TSS levels of 1500–2500 mg/L, and this was the target level for most experiments. Lower TSS levels were targeted during the 150 day SBR experiment to demonstrate in continuous flow operation the effect of biomass levels on nanomaterial removal. Reactors were operated for several weeks to reach steady state, which was determined on the basis of consistent total suspended solids (TSS) concentration and effluent chemical oxygen demand (COD), before addition of NPs began. Metal concentrations in liquid samples were determined by acid digestion followed by analysis using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES). Concentration of titanium dioxide NPs added to SBR was in range 0,5-2,5 mg/L. The average COD level of the settled effluent from SBRs without NPs was 64 ± 28 mg/L, and with NPs 45 ± 16 mg/L. Biomass concentrations were also constant with n-TiO2 (average 1.3 ± 0.1 gTSS/L). In control tests approximately 70% of the nano-scale titanium dioxide (n-TiO2) was removed during each SBR loading cycle. The removal of titanium increased from 65% in the absence of biomass (precipitation) to 97 ± 1% with biomass present (1.3 ± 0.2 mgTSS/L). Experiments were not conducted with ionic titanium because of its extremely low solubility.

4. METHODOLOGY

4.1. SLUDGE SAMPLING

The sludge used in this experiment was collected from the Main Treatment Plant in Vienna (MTPV). The MTPV is a two-stage WWTP with biological nutrient removal (BNR) and a design capacity of 4 x 10^6 PE.

20 L (2x 10L containers) of activated sludge were collected every second day from the second, low loaded stage of the MTPV. It was stored in the lab at room temperature, without additional aeration. During the measurement campaign the MLSS (mixed liquor suspended solids) concentration averaged 3,348 g/L and the VSS (volatile suspended solids) 2,302 g/L.

The measurements were conducted on the day of sludge delivery and the next day, respectively; so the sludge was never older than two days.

4.2. Oxygen uptake rate MEASUREMENTS

4.2.1. Experimental set-up and measurement principle

The experimental set-up for measuring the oxygen uptake rate (OUR) consists of a cylindrical reaction chamber (V= 300 ml), a bowl for the chamber and optionally a water batch and a magnetic stirrer. The lid of the reaction chamber has two openings, one for an injection needle and another one for a dissolved oxygen probe connected to a recorder, see Figure 8.

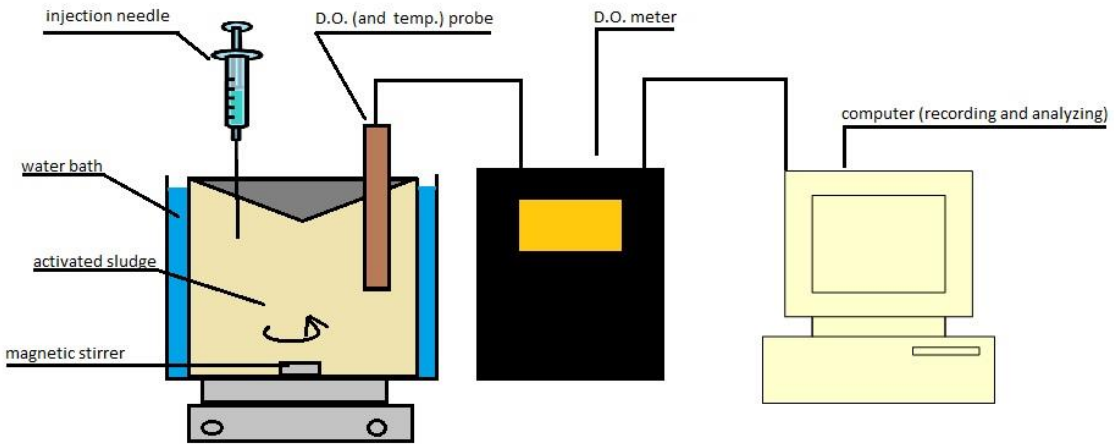

Figure 8 Measuring system.

In order to measure the oxygen uptake rate of activated sludge, which was calculated from the decline of the oxygen concentration the sludge had to be aerobic. There was the aeration of sludge in the big container (10 L of AS) for 1 hour – it ensured enough air for keeping sludge active and constant stirring protect it from decomposition. The pipes were put in to the container and provided the air with previously set pressure.

The activated sludge was mixed and divided into two bottles: A and B. Additionally, specific substances were poured to each of the bottles. In bottle A the total OUR comprised the maximum oxygen uptake rate of the autotrophic bacteria (OUR_{Amax}), i. e. the OUR of nitrifying bacteria and the OUR of heterotrophic bacteria (OUR_H) was determined. This required the addition of ammonia in order to achieve the maximum activity of the autotrophic

bacteria. The ammonia was first added as ammonium chloride and later as ammoniac. Both substances have the stock solution in concentration of 30 mgNH_4-N/L of AS and were given in amount of 1,5 ml. The added nitrogen is oxidised to nitrite by *Nitrosomonas* and in a next step to nitrate by *Nitrobacter*. ATU from a stock solution of 10 g ATU/L of AS was given to bottle B (heterotrophic bacteria) in amount of 0,5 ml to inhibit *Nitrosomonas* and measure the heterotrophic oxygen uptake rate (OUR_h). More details on the calculation of the respective OUR is given in chapter 4.2.2. (Nowak and Svardal, 1993)

After chemical dosage the bottles were aerated for different contact times. The next step was the OUR measurement.

Measurements were conducted in the similar way – activated sludge was poured into the reaction chamber, then the pH and temperature were measured. After that the chamber was closing by the lid and the measurement of OUR was proceeding until it had a constant value for a few minutes. At the end pH and temperature were measured again. The differences between the bottle A and B were:

- For bottle A the analyse was performing as long as has been received the constant line.
- For bottle B after receiving first constant value the 0,3 ml of nitrite (5 gNO_2-N/L) was added and after second constant value 1 ml of Saft has been injected into the reaction chamber.

The composition of Saft was:

- Peptone 16g
- Meat extract 11g
- Glucose 4,6g
- Sodium acetate 9,3g
- Ethanol 3,3g
- Sodium chloride 14g
- Dissolved in 1L of DW

Nitrite was injected to achieve the maximum activity/ OUR of the nitrite oxidising bacteria (the second step of nitrification). It enables in later calculation to estimate the activity of ammonia oxidising bacteria, by subtracting this from OUR_{Amax} and precise on which step of nitrification is the inhibition. Saft is an extra, highly concentrated source of carbon.

After each measurement of bottle A, the sample for MLSS, VSS were taken. Also the filtered samples were prepared to measure dissolve nutrients NH_4-N, NO_x-N, NO_2-N and PO_4-P .

The results of the measurements were analysed and saved in the "OV Messung" computer programme, created by Karl Svardal.

4.2.2. OUR calculations

The results are based on duplicate or sometimes even triplicate measurements. To compensate the temperature differences the equations presented below were used for corrections and all the results were referred to 20 °C:

- Heterotrophic respiration: $f_{T\,h} = 1{,}072^{(20-T)}$
- Autotrophic respiration: $f_{T\,a} = 1{,}103^{(20-T)}$

The results of the measurements are presented as a:

- $OUR_{Total} = OUR_H + OUR_{Amax}$ } Bottle A
- OUR_{H}
- $OUR_{H+NO2\,max}$ } Bottle B
- $OUR_{H+\,NO2max\,+Cmax}$

The parameters that are important for the research are calculated from the ones presented above in the way presented below:

- $OUR_{Amax(20^oC)} = (OUR_{total} - OUR_{H(20^oC)}) \cdot f_{T\,a}$
- $OUR_{NO2max(20^oC)} = (OUR_{H+NO2\,max} - OUR_{H(20^oC)}) \cdot f_{T\,a}$
- $OUR_{Cmax} = (OUR_{H+\,NO2max\,+Cmax} - OUR_{NO2max(20^oC)} - OUR_{H(20^oC)}) \cdot f_{T\,h}$

To compare the results, the values presented above were divided by the value of VSS.

4.2.3. Preparations of the samples

4.2.3.1. Samples with activated sludge

The sludge was aerated in the sludge container right after delivery from WWTP. It was storage in laboratory room. To avoid an increase of pH that could disturb the microorganism activity, the 2,5 g of $NaHCO_3$ was added to the container. This amount helped to maintain the pH on stable level. On the day of measurements the samples for the OUR measurements were collected from the 10L sludge container and poured into a 1L metric cylinder. The sludge was settling for a few minutes. Then the respective amount of silver nitrate was dosed from the stock solution (100 mg Ag/L) and added to the cylinder after removing the same volume of supernatant. The sludge was mixed and divided into two bottles: A and B.

4.2.3.2. Samples with washed activated sludge

In the chronic toxicity tests synthetic wastewater will be used in order to supply a controlled substrate. In order to have similar conditions during the acute toxicity tests and in order to wash out the chloride present in the activated sludge since this would result in the immobilisation of the silver the activated sludge was washed with the synthetic wastewater, i.e. modified.

The basis for the synthetic wastewater was the ISO 9887:1992(British Standards Institute Staff 1995), which was modified by e.g. replacing the chloride with sulphate or HCO_3^- and without the addition of urea since this was dosed separately to bottle A.

10L of feed contained of the following chemicals:

- Peptone 3,2 g
- Meat extract 2,2 g
- $NaHCO_3$ 2,5 g
- $CaSO_4 \cdot 2H_2O$ 90 mg
- $MgSO_4 \cdot 7H_2O$ 40 mg
- K_2HPO_4 280 mg

20L of feed were prepared on the day of the measurement and if they were prepared the day before the feed was stored in the fridge to keep it from decay. The feed was analysed for COD, total nitrogen, total phosphorus. Chloride was measured in the sludge before and after the washing.

After delivery from MTPV the container with the sludge (10L) was settling down. The supernatant was decanted and the sludge was poured into the metric cylinder. The sludge was poured into 4 centrifuge bottles and the weight of each of them filled with a sludge was 400 g. Then bottles where rotating in centrifuge at 2000 rpm for 1 min and the supernatant was decanted. The bottles were filled with synthetic feed up to 400 g and mixed. This procedure (rotating, decanting and filling) was repeating three times. The last repeat was finishing by filling up the 10 L container with the washed sludge and the synthetic feed.

The measurements were carrying out the same way as for the regular sludge.

4.2.2.3 Preparations of the silver nanoparticles.

The silver NPs used in the experiments were supplied by the JRC. The sample ID was 0886 and the name was NM – 300K. The preparation was done according to the JRC scientific and technical report on the Characterisation, Stability, Homogeneity of NM-300 silver (Comero et al. 2011). The NM-300 sample dispersion is yellow-brown; it is an aqueous dispersion of nano-silver with stabilizing agents, consisting of 4% w/w% each of Polyoxyethylene glycerol Trioleate. The dispersion was stored in the refrigerator (4˚C).
The calculations of proper concentration of NPs were:
10w/w% of silver
240 mg NM-300K – 50 ml
24 mg Ag NPs – 50 ml
10 mg Ag NPs– 20 ml
1 mg Ag NPs – 2 ml
0,5 mg Ag NPs – 1 ml

4.2.3. Nitrogen and phosphorus measurements

The activated sludge and washed activated sludge with the silver nitrate has not been testing only by the OUR measurements, but also for different forms of nitrogen and phosphorus. Determination were proceeding with following methods:

- PO_4-P - DIN 38405-D11-3
- NH_4-N - DIN 38406-E5-1
- NO_x-N - DIN EN ISO 13395
- NO_2-N - DIN 38405 EN 26777

5. RESULTS OUR MEASUREMENTS

5.1 ACTIVATED SLUDGE WITH AgNO3

The results of MLSS and VSS presented in Figure 9 demonstrate that there were no significant variations of the values over the project time. The average of the results was 3,2 g/L for MLSS and 2,2 g/L for VSS. Temperature and pH of bottle A are presented in the Tab.2.

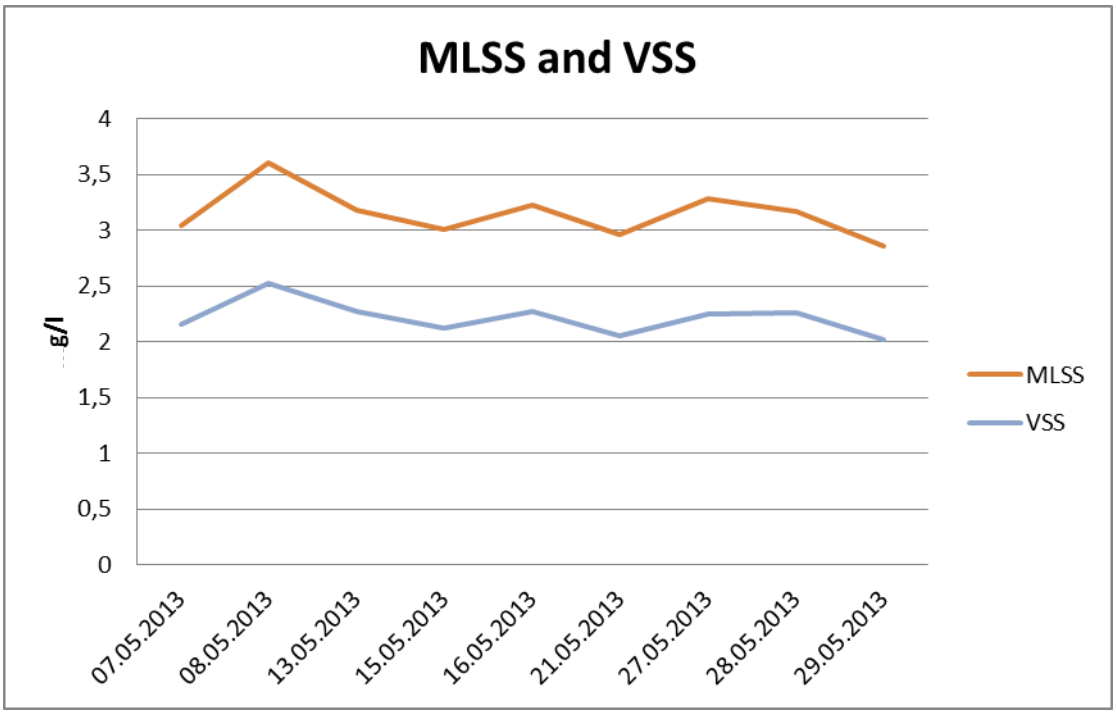

Figure 9 MLSS and VSS of activated sludge with $AgNO_3$.

Table 4 pH and temperature in bottle A

	before measurement			after measurement		
	min	max	mean	min	max	mean
pH	7,1	8,1	7,6	6,6	7,9	7,2
temp	15,3	22,1	18,3	15,8	21,9	18,5

From the second week of the project samples for dissolved nitrogen analysis were taken, always after the measurement. The concentrations are depicted in the following figures (Figures 10-13).

In Figure 10 the results of on bottle A after 30 min aeration are presented. It can be observed that there are slight differences for the silver nitrate concentration 0 and 1 mg/L. There is an increase for concentration of NH_4 and the rest of parameters is decreasing.

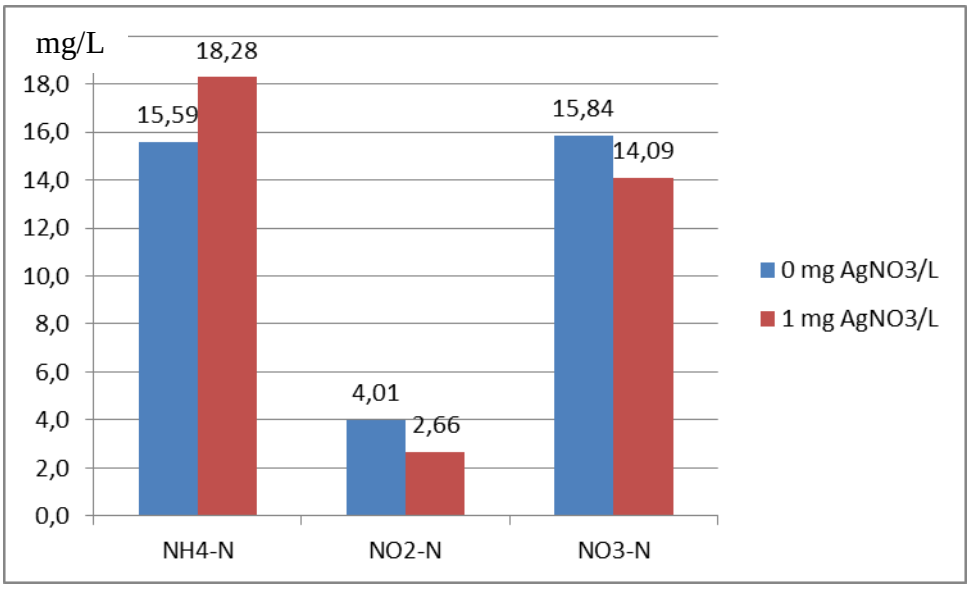

Figure 10 Dissolved nitrogen in bottle A after 30 min aeration of activated sludge with and without $AgNO_3$.

For 1 h aeration (Figure 11) there is again increase of concentration for NH_4-N, and decrease of the rest of parameters, as it was for 30 min aeration.

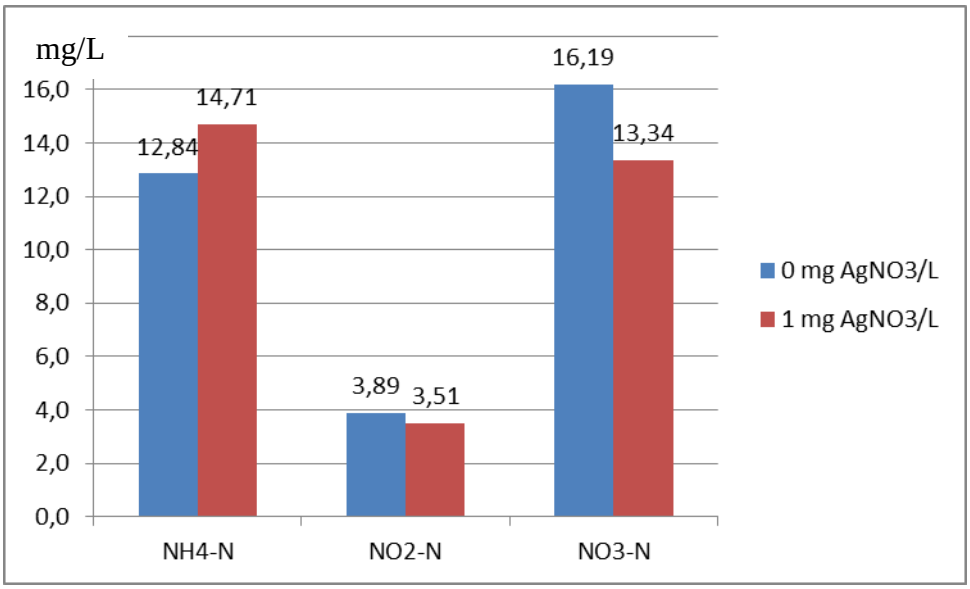

Figure 11 Nitrogen and phosphorus in bottle A with activated sludge with $AgNO_3$.

The concentrations for bottle B are depicted in Figures 12-13.

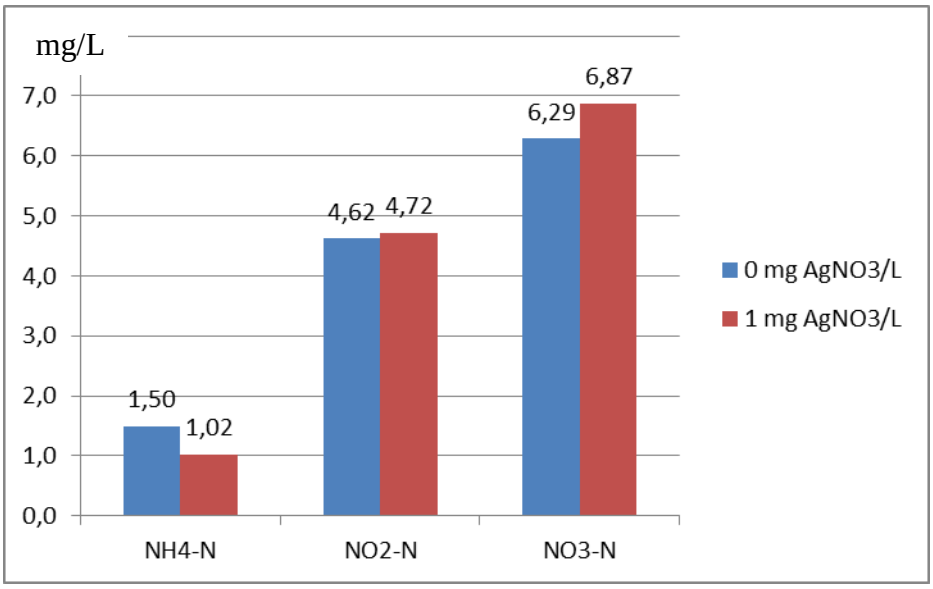

Figure 12 Nitrogen and phosphorus in bottle B with activated sludge with $AgNO_3$.

In the Figure 13 the result of nitrate concentration is lower with silver nitrate addition than without it.

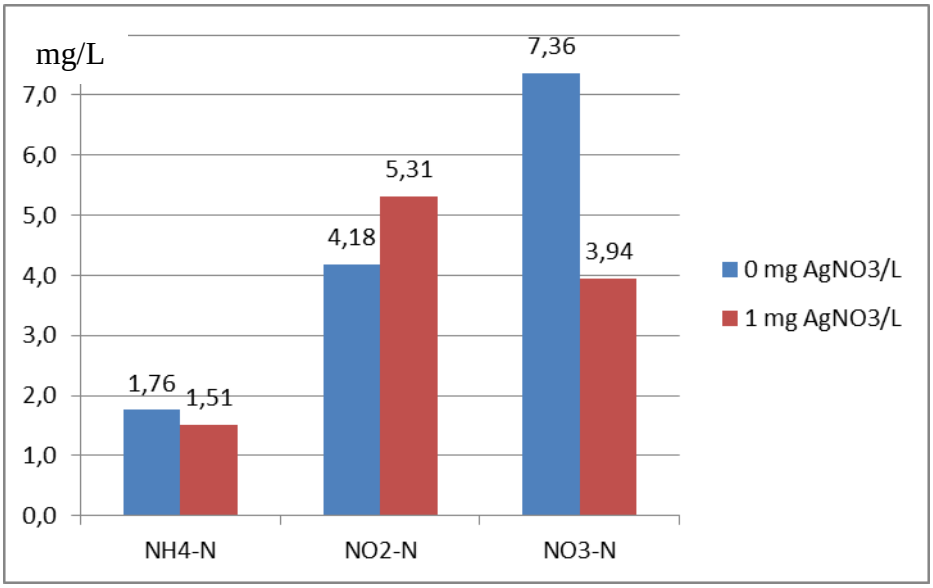

Figure 13Nitrogen and phosphorus in bottle B with activated sludge with $AgNO_3$.

The temperature and pH of bottle B are presented in Tab 3.

Table 5 Temperature and pH of bottle B.

	before measurement			after measurement		
	min	max	mean	min	max	mean
pH	7,4	8,4	8,0	7,0	8,2	7,8
temp	14,7	23,1	18,3	15,6	22,9	18,7

Figure 14-17 present the results of OUR measurements conducted on activated sludge, that has been aerated for 30 min. The respiration activity of the sludge decreased with increasing silver nitrate concentrations, see Fig. 14 and Fig. 16. This effect has not been observed for $OUR_{NO2max.}$

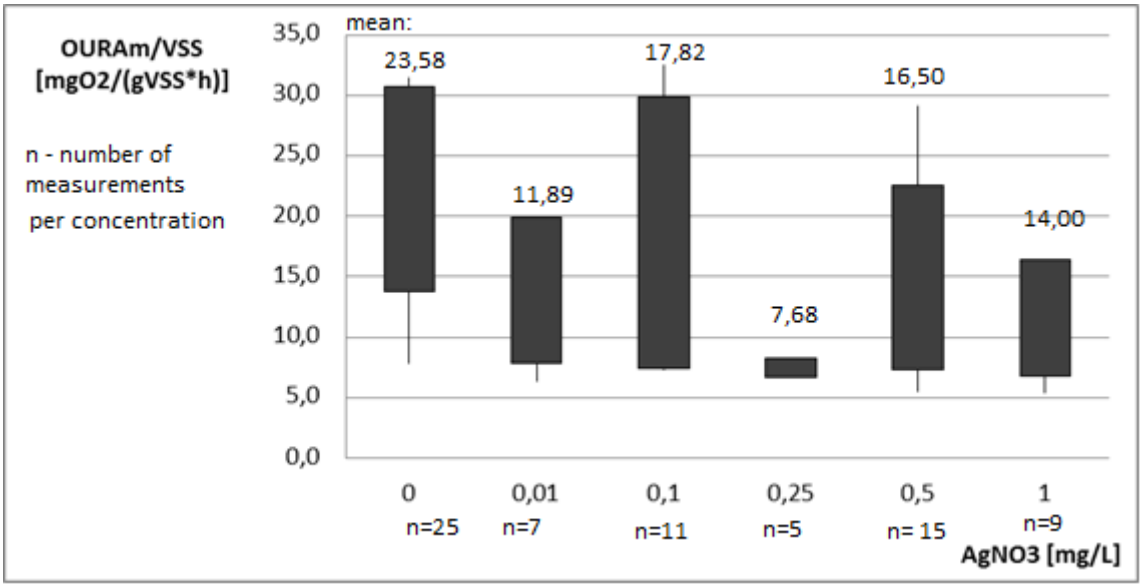

Figure 14 $OUR_{Amax(20^{o}C)}$/VSS of activated sludge after 30 min aeration time.

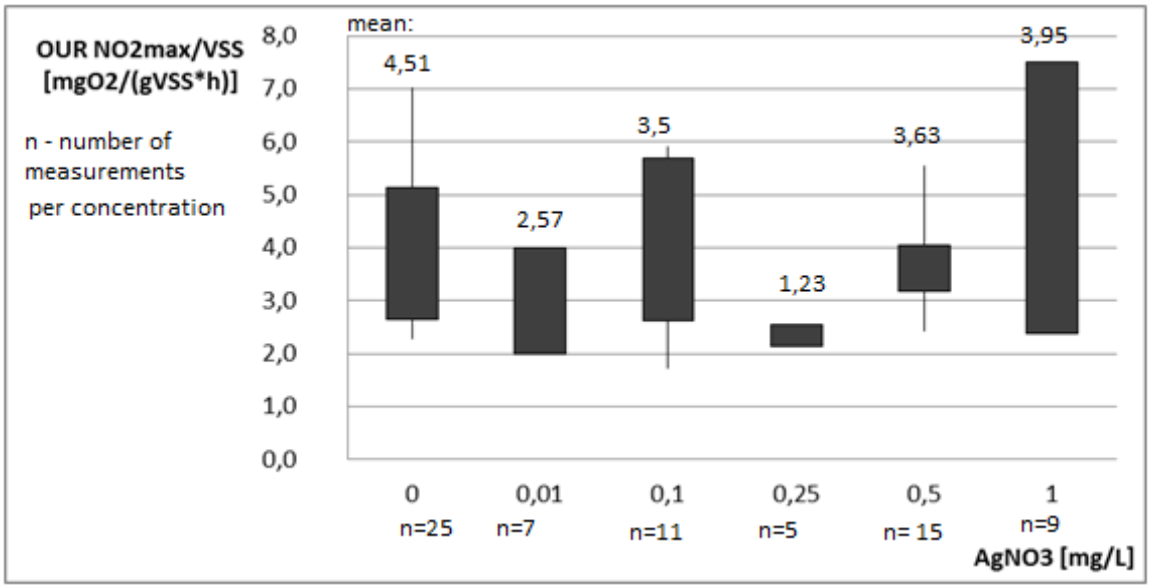

Figure 15 $OUR_{NO2max(20^{o}C)}$/VSS of activated sludge after 30 min aeration time.

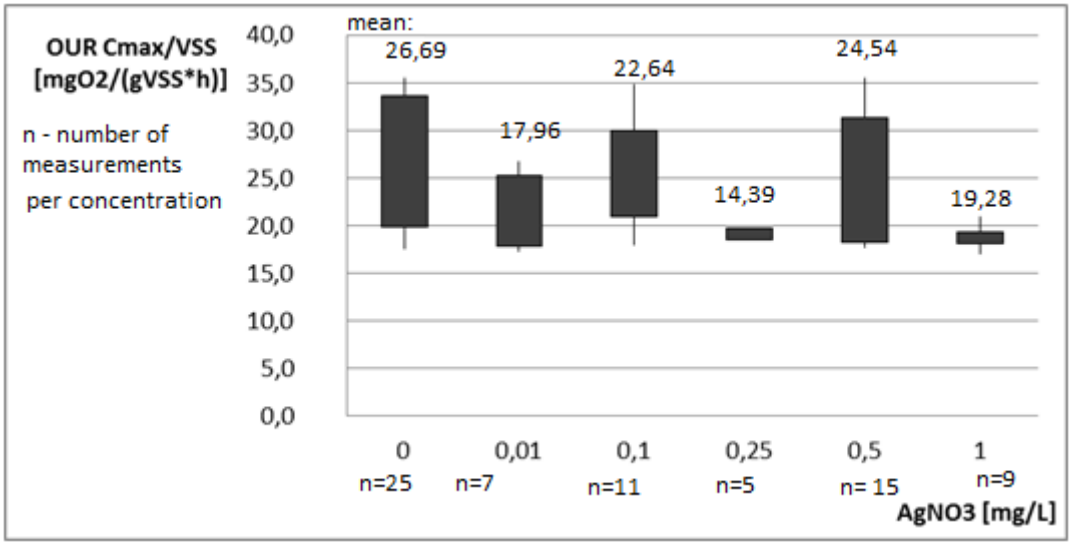

Figure 16 $OUR_{Cmax(20^{o}C)}$/ VSS of activated sludge after 30 min aeration time.

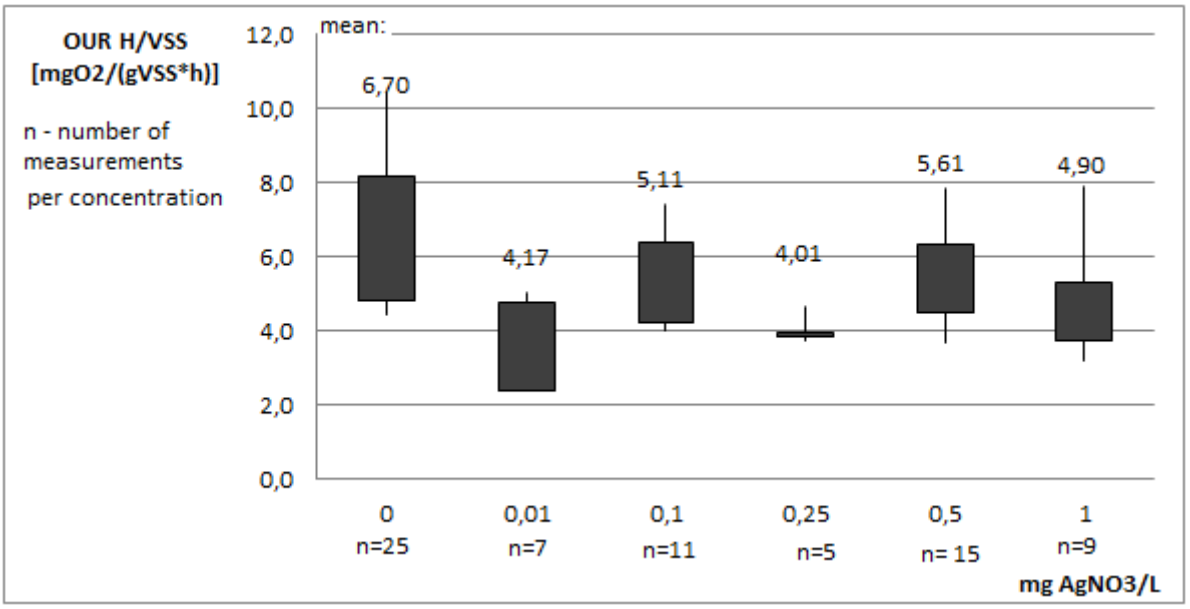

Figure 17 $OUR_{H(20^{o}C)}$/ VSS of activated sludge after 30 min aeration time

At this stage of experiment it was important to compare results of different times of aeration, to decide later which of them is better to be tested with NPs. Results from different contact times will present if with the elongation of time there is an increase in toxicity (higher inhibition) or rather decomposition of contaminant by microorganisms. Figures 18-21 give the results for an aeration time of 1 hour.

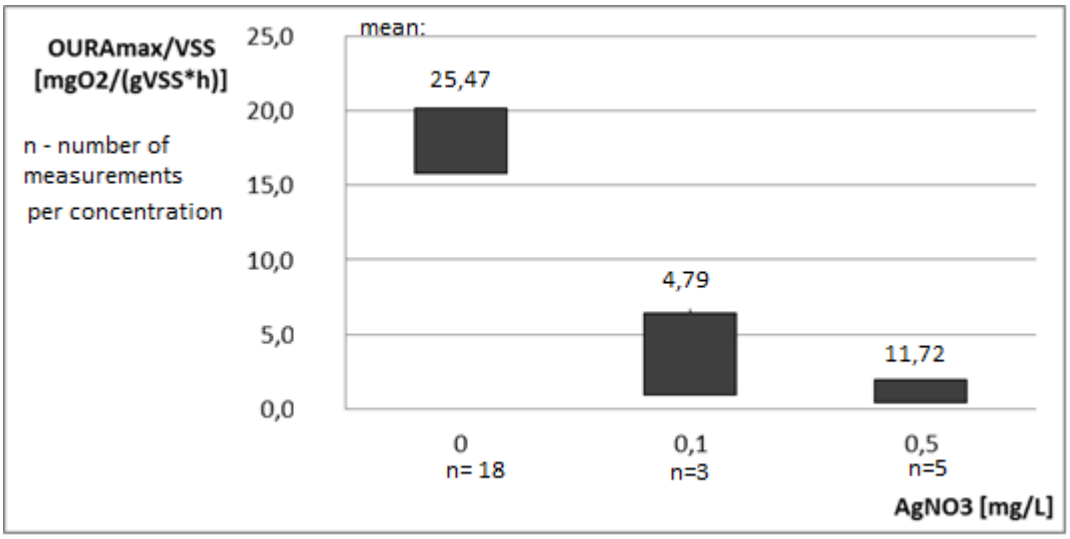

Figure 18 $OUR_{Amax(20^{o}C)}$/VSS of activated sludge after 1 h aeration time.

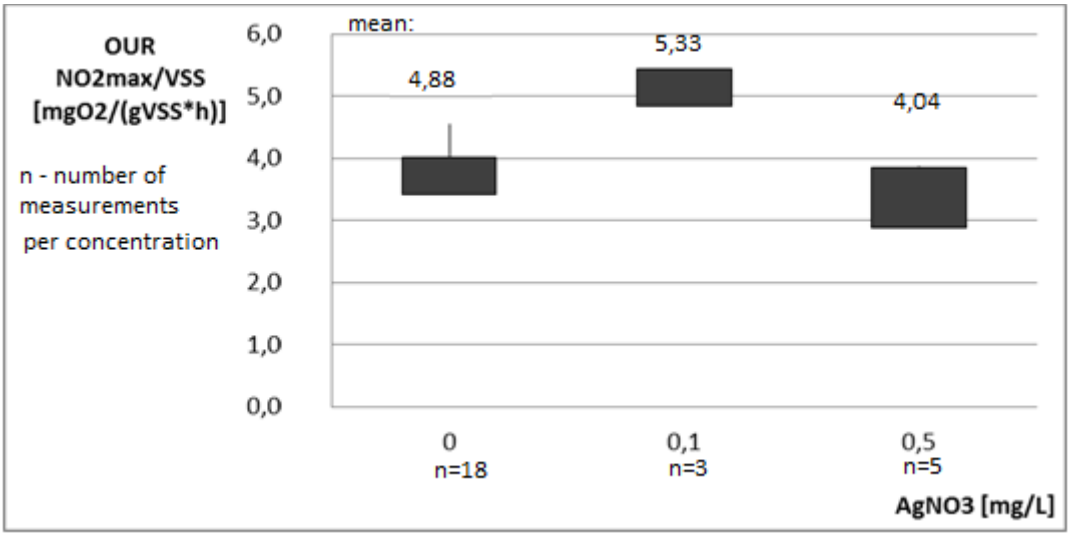

Figure 19 $OUR_{NO2max(20^{o}C)}$/ VSS of activated sludge after 1 h aeration time.

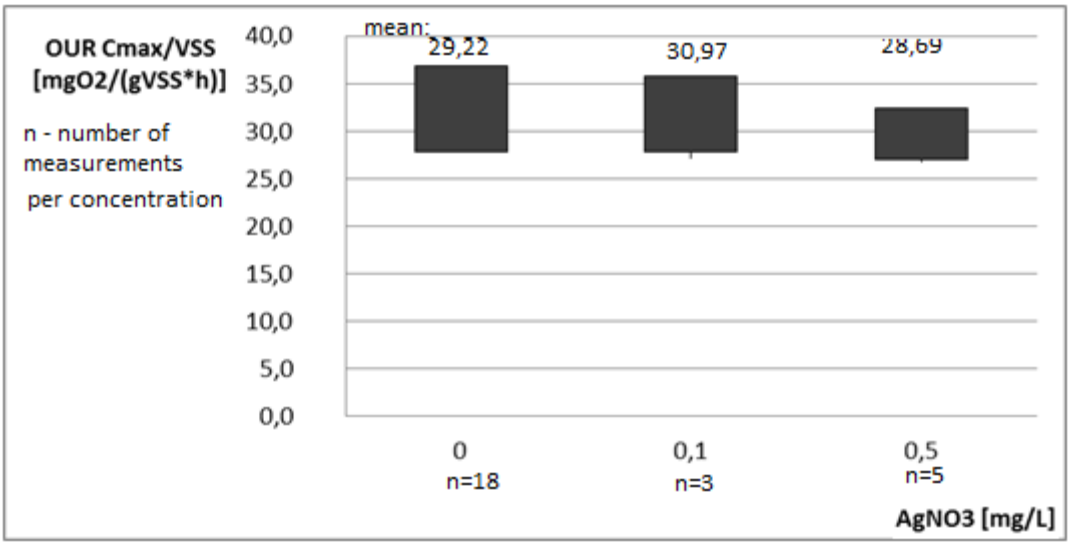

Figure 20 $OUR_{Cmax(20^{o}C)}$/ VSS of activated sludge after 1 h aeration time.

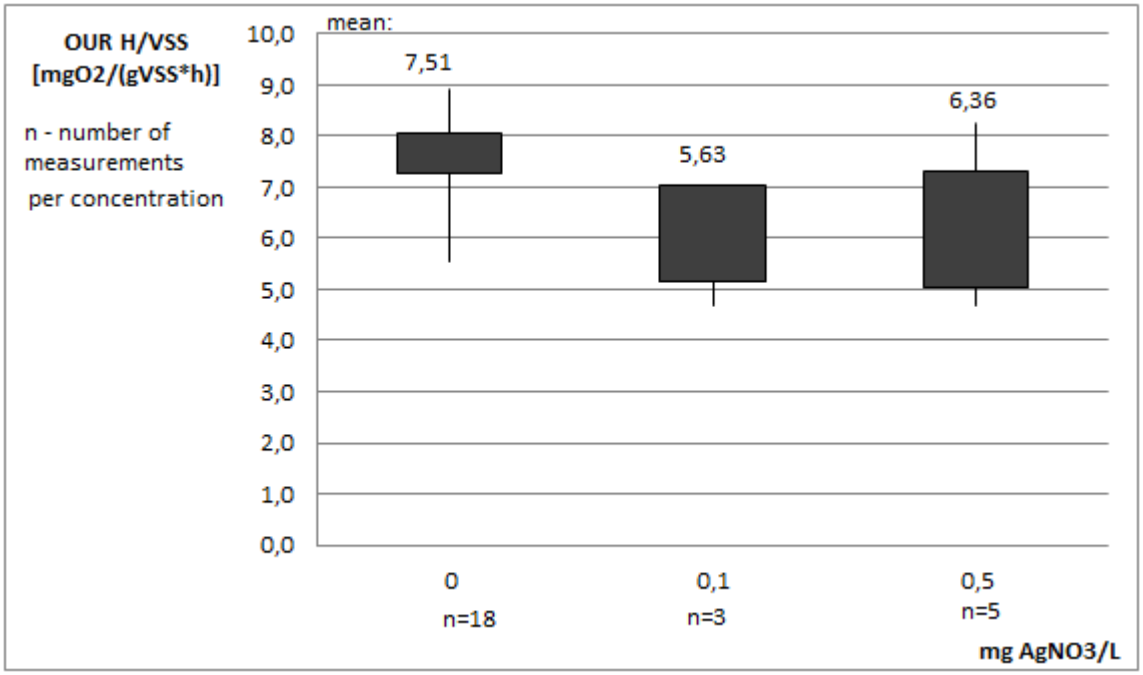

Figure 21 $OUR_{H(20^{o}C)}$/ VSS of activated sludge after 1 h aeration time

It can be observed that the OUR after 1 h aeration is decreasing with the rise of the silver nitrate concentration. OUR_{NO2max} is not changing.

Since 27th of May before the aeration the 1,5 ml of ammonia (stock solution 10 g NH4-N/L) was added to the bottle A instead of 1ml. It turned out that previous amount of ammonia was very fast digest by microorganisms. Additionally before the aeration a buffer was added to the activated sludge container, in order to maintain the stable value of pH. The buffer was $NaHCO_3$ in amount of 2,5 g which gave the final concentration of approximately 250 mg HCO_3^-/L. The results of the tests with silver nitrite after these modifications are presented for 0, 1 and 5 mg $AgNO_3$/L (Figures 22-25).

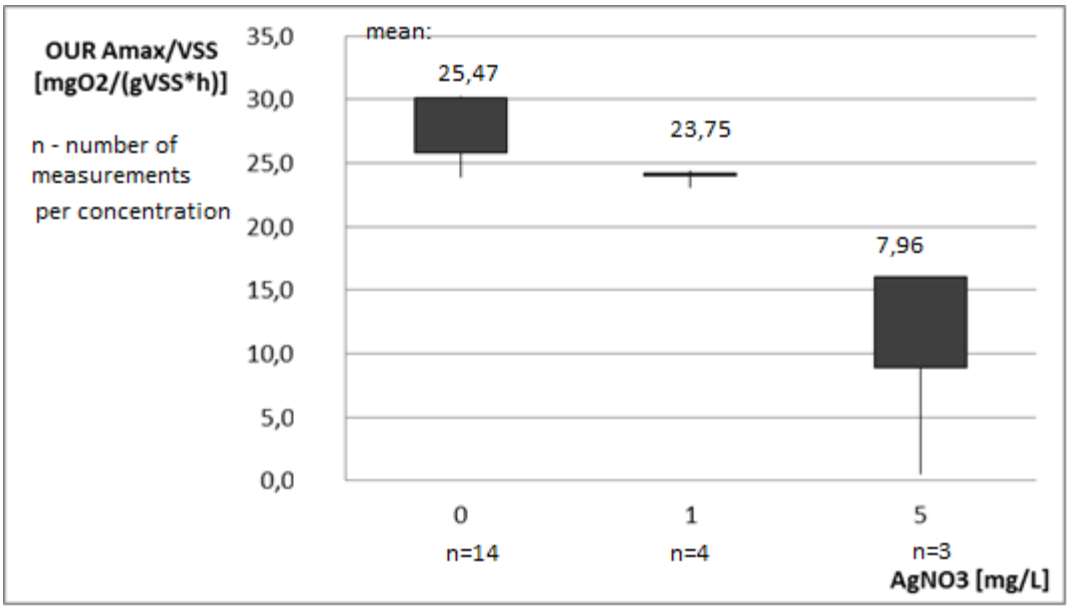

Figure 22 $OUR_{Amax(20^{o}C)}$/VSS of activated sludge after 1 h aeration time (more ammonia + buffer).

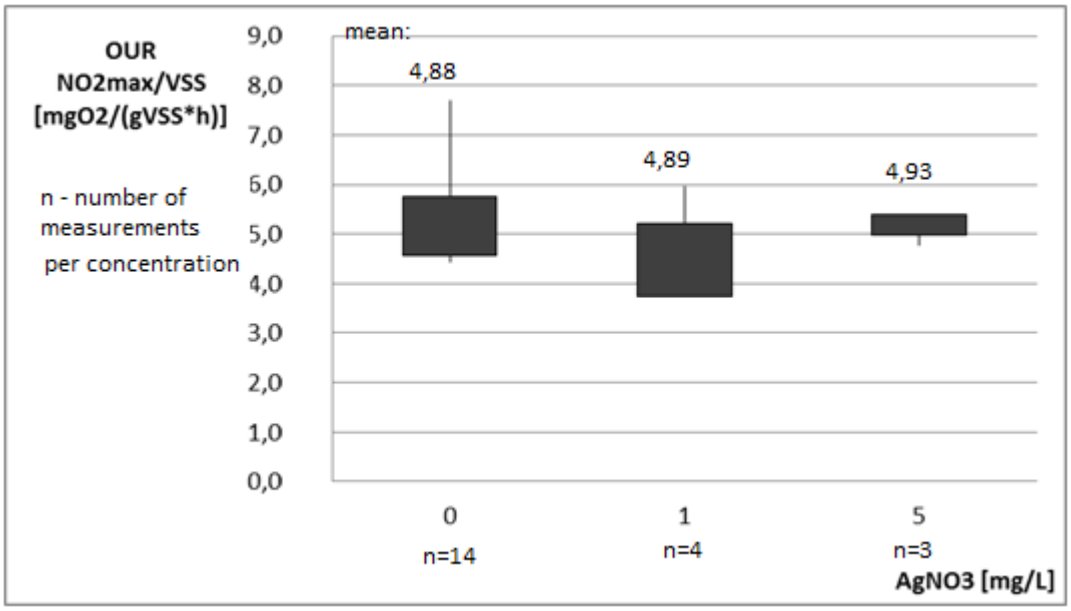

Figure 23 $OUR_{NO2max(20^{o}C)}$/ VSS of activated sludge after 1 h aeration time (more ammonia + buffer).

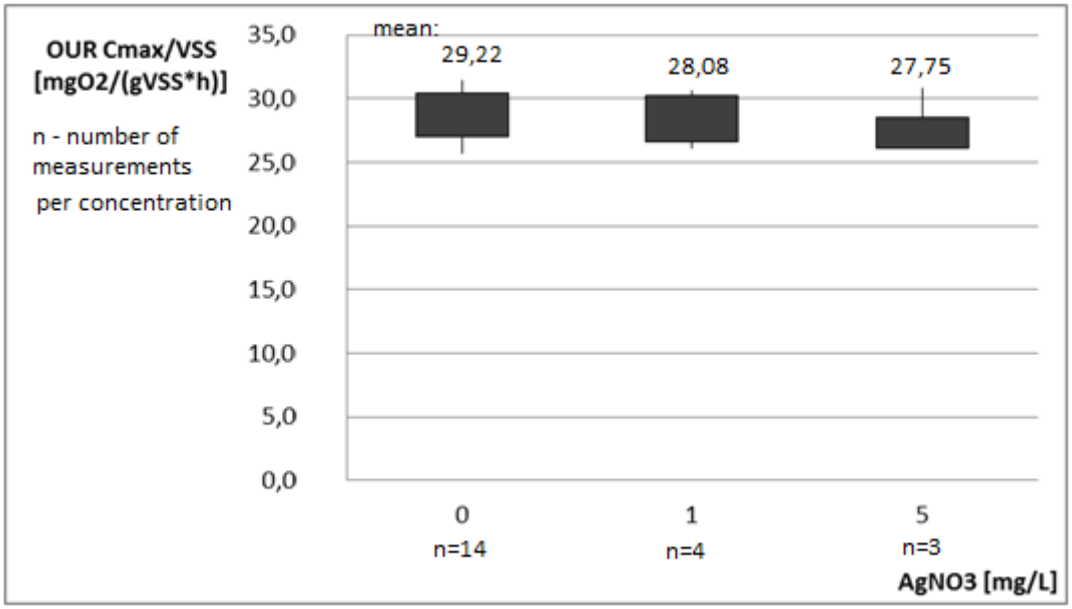

Figure 24 $OUR_{Cmax(20^{o}C)}$/ VSS of activated sludge after 1 h aeration time (more ammonia + buffer).

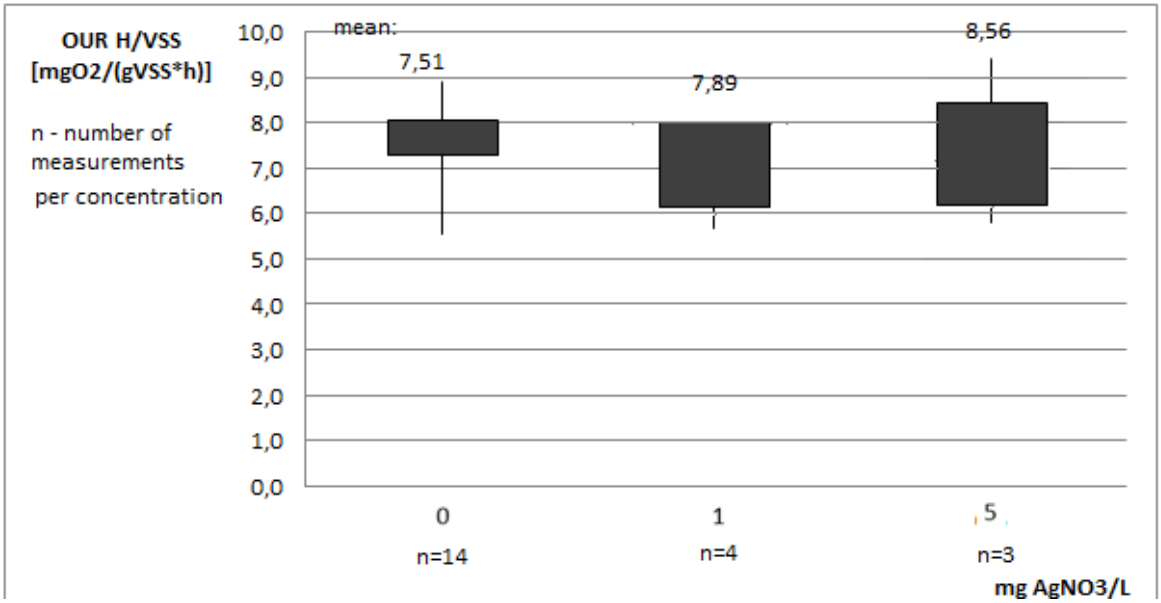

Figure 25 $OUR_{H(20^{o}C)}$/ VSS of activated sludge after 1 h aeration time (more ammonia + buffer).

The results of the measurements presents that the rising concentration of silver in the form of silver nitrate is inhibiting the respiration of the sludge, especially on the first step of nitrification.

5.2. MODIFIED ACTIVATED SLUDGE WITH AgNO3

The measurements with modified sludge were performed with 1 h aeration time. As for the previous stage of the experiment, the samples of sludge were tested also for MLSS (3,4 g/L) and VSS (2,2 g/L). Dissolved nitrogen, see Figures 26 and 27.

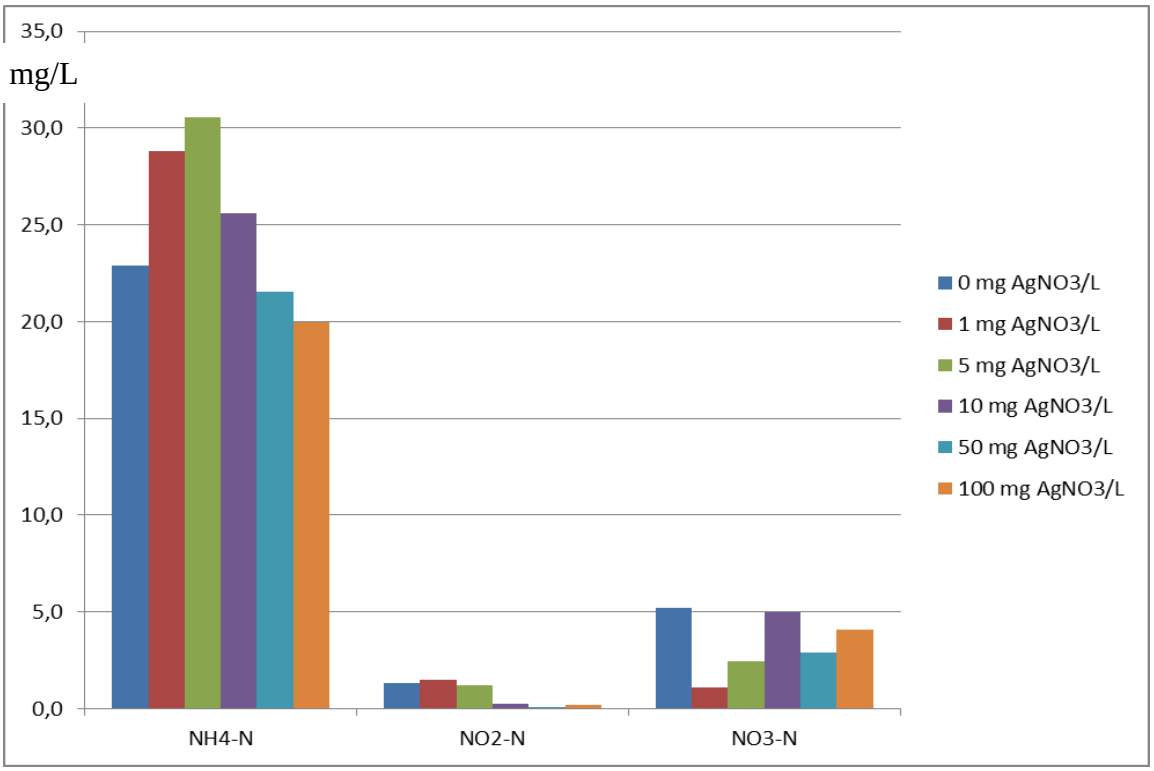

Figure 26 Nitrogen and phosphorus in bottle A with modified activated sludge with $AgNO_3$.

Table 6 Temperature and pH of bottle A

	before measurement			after measurement		
	min	max	mean	min	max	mean
pH	7,6	8,3	7,9	7,1	8,2	7,6
temp	17,7	24,4	20,8	17,8	24,5	20,8

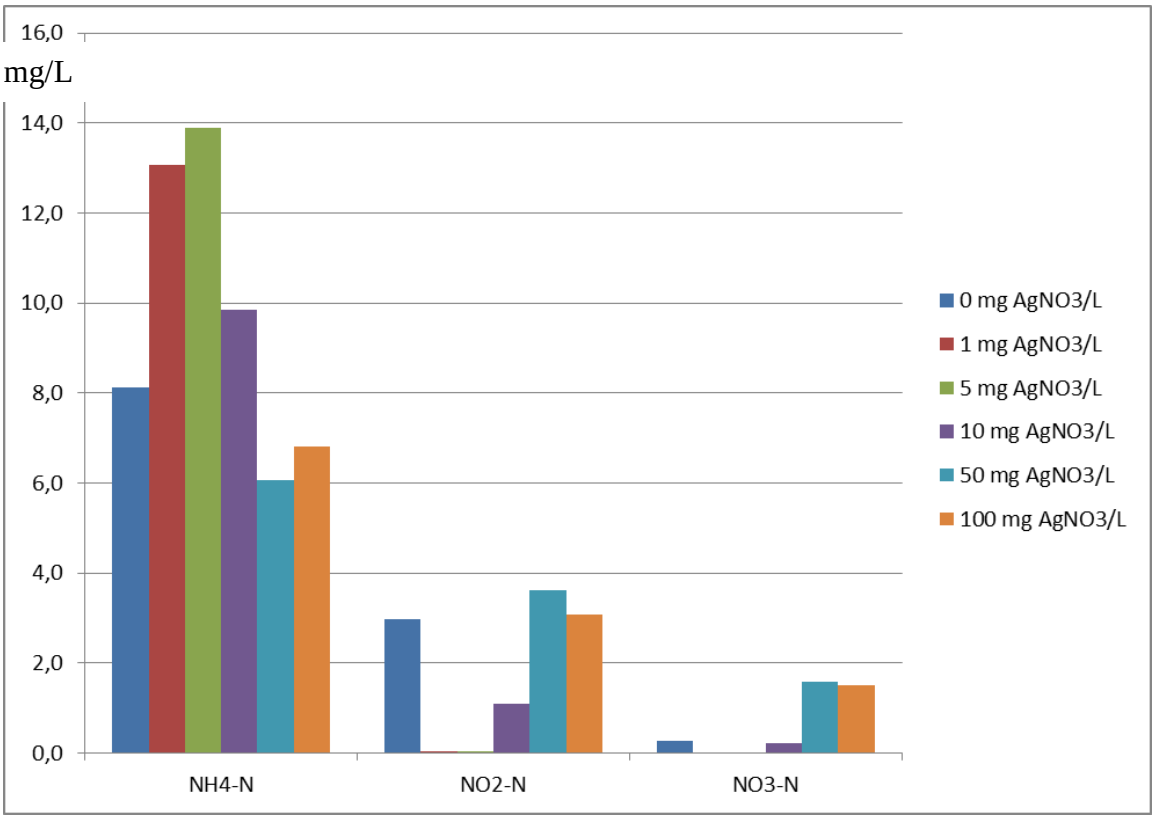

Figure 27 Nitrogen and phosphorus in bottle B with modified activated sludge with $AgNO_3$.

Table 7 Temperature and pH of bottle B.

	before measurement			after measurement		
	min	max	mean	min	max	mean
pH	7,9	8,2	8,1	7,6	8,2	7,8
temp	18,2	24,8	21	18,6	24,9	21,1

The results of OUR measurements conducted on modified activated sludge with 1 h aeration are presented in Figures 28-31.

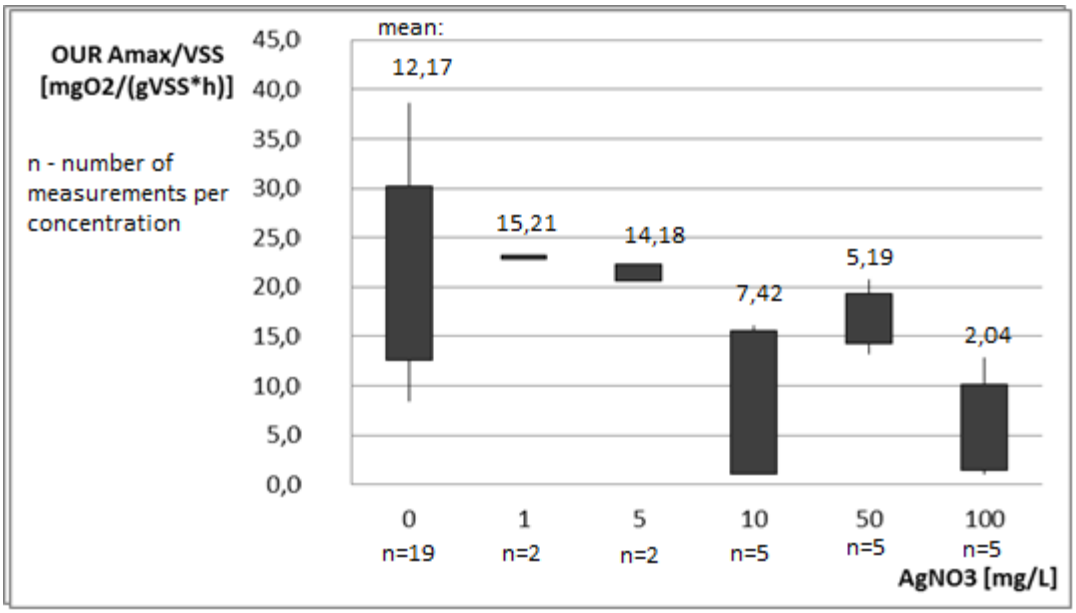

Figure 28 $OUR_{Amax(20^{o}C)}$/ VSS of washed activated sludge after 1 h aeration time

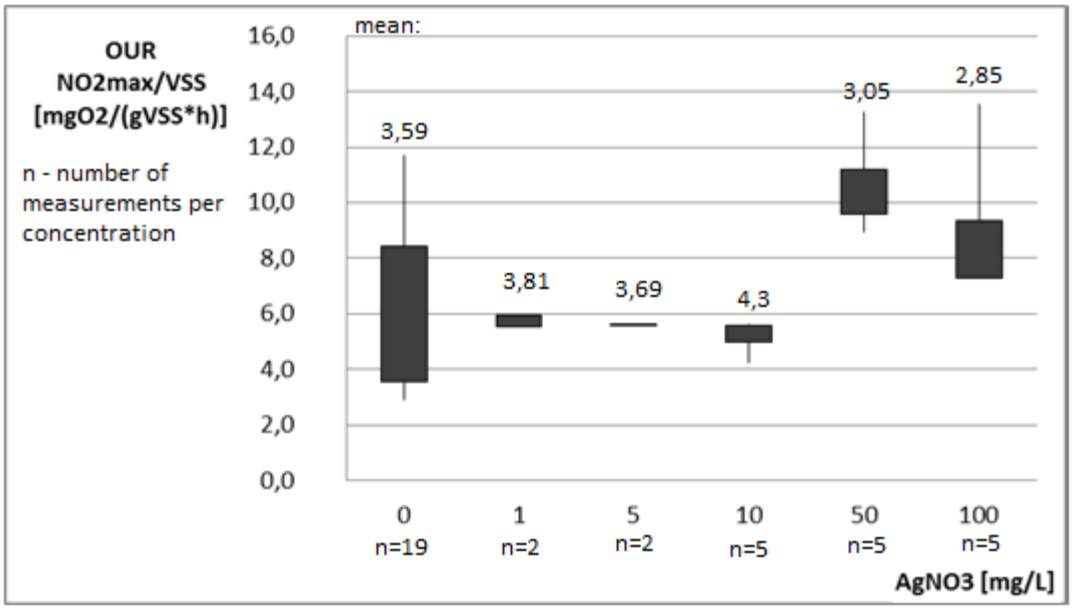

Figure 29 $OUR_{NO2max(20^{o}C)}$/ VSS of washed activated sludge after 1 h aeration time.

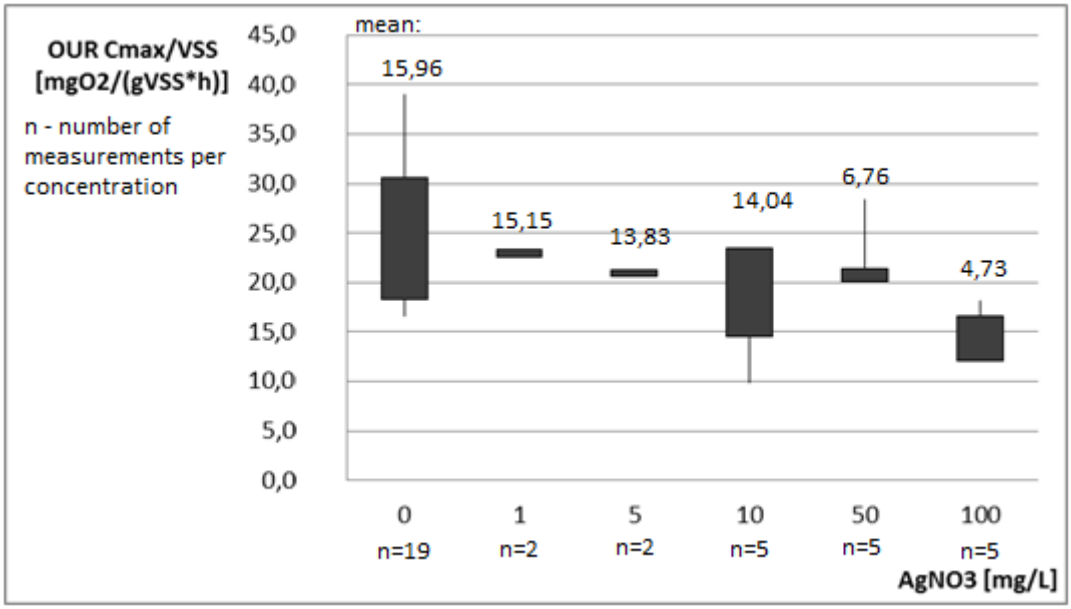

Figure 30 $OUR_{Cmax(20^{o}C)}$/ VSS of washed activated sludge after 1 h aeration time.

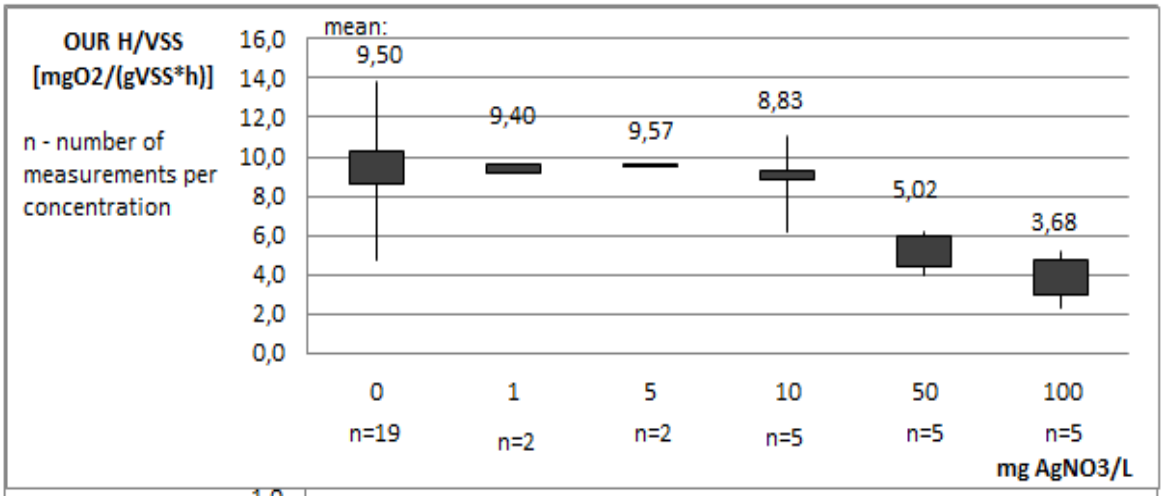

Figure 31 $OUR_{H(20^{o}C)}$/ VSS of washed activated sludge after 1 h aeration time.

Also for modified activated sludge a decrease of respiration for measurements of OUR_{Amax} and OUR_{Cmax} can be observed.

5.3. MODIFIED ACTIVATED SLUDGE WIT Ag NPs

Tests with silver nanoparticles were conducted only once, with concentrations of Ag NPs 0, 0,5, 1 and 10 mg/L. The aeration time was 1 h and the activated sludge was washed with synthetic (modified activated sludge).Results are presented on Figures 32-34.

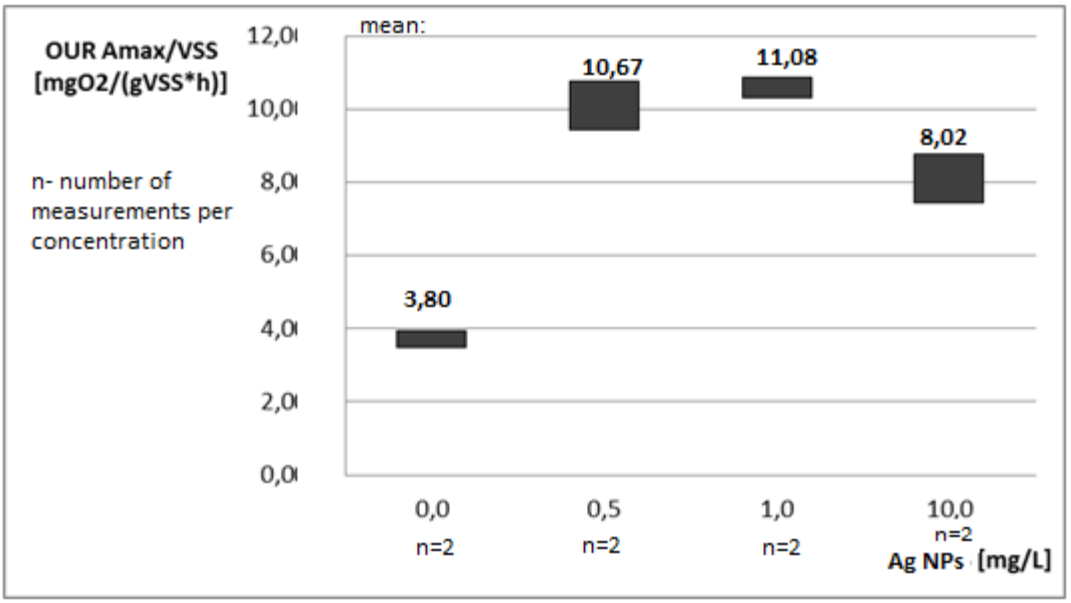

Figure 32 $OUR_{Amax(20^{o}C)}$/ VSS of silver nanoparticles in a washed activated sludge after 1 h aeration time.

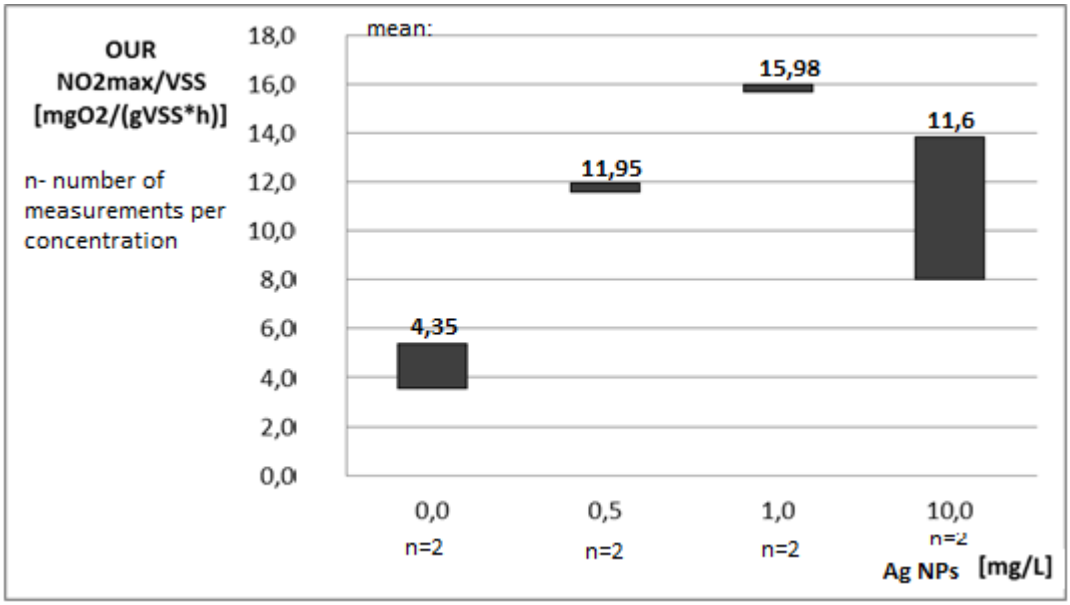

Figure 33 $OUR_{NO2max(20^{o}C)}$/ VSS of silver nanoparticles in a washed activated sludge after 1 h aeration time.

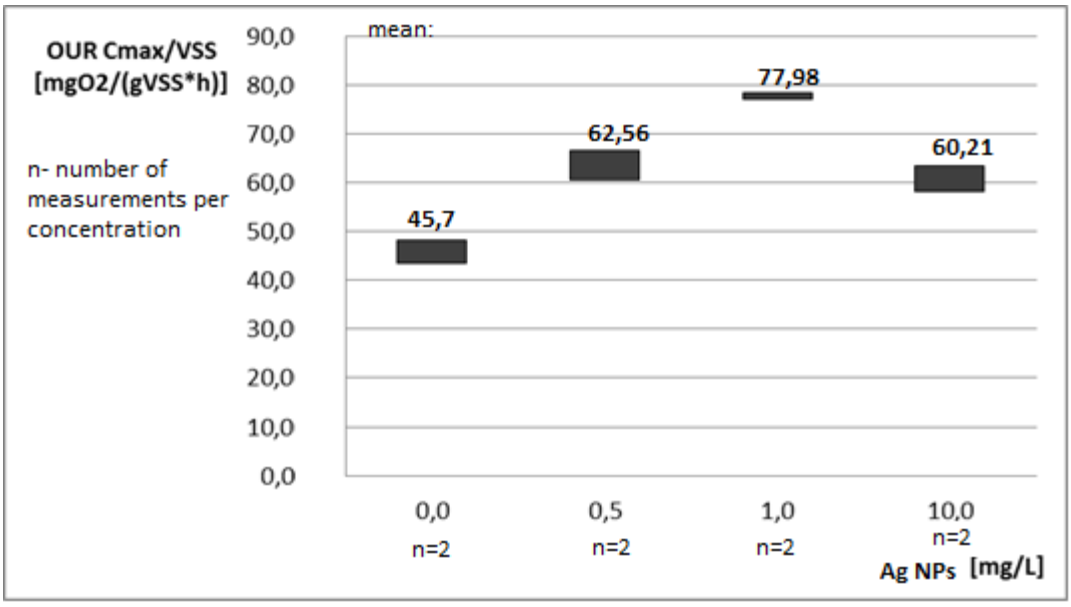

Figure 34 $OUR_{Cmax(20^{o}C)}$/ VSS of silver nanoparticles in a washed activated sludge after 1 h aeration time.

As presented the results are much different than the results for silver nitrate. There is no tendency in decrease of respiration and therefore, there is no inhibition.

6. DISCUSSION ON RESULTS AND DEVELOPMENT OF METHODOLOGY

The primary aim was to measure the inhibition of sludge activity by measurements of oxygen uptake rate for autotrophs and heterotrophs in activated sludge. However, the results received from the first measurements showed that i) the measurement is not trivial, i. e. many details have to be taken into account and ii) the methodology should be changed, to avoid later problems during the tests of nanoparticles.

The first stage of the project revealed big discrepancy of measurements.

Table 8 Results of the first phase of project.

sampling date	Date	substance	aeration time	concentratio	OURAmax in 20degrees	OUR NO2max	OUR Cmax
06.05.2013	07.05.2013	Ag	30 min	0	56,00	5,72	52,86
06.05.2013	07.05.2013	Ag	30min	0	25,14	7,98	53,51
06.05.2013	07.05.2013	Ag	30min	0,01	43,05	5,96	54,46
06.05.2013	07.05.2013	Ag	30min	0,01	33,25	6,59	57,62
06.05.2013	07.05.2013	Ag	30min	0,1	30,83	5,94	51,40
06.05.2013	07.05.2013	Ag	30min	0,1	25,25	12,56	45,20
06.05.2013	07.05.2013	Ag	30min	0,5	32,09	8,37	49,39
06.05.2013	07.05.2013	Ag	30min	0,5	26,31	11,88	42,83
06.05.2013	07.05.2013	Ag	30min	1	35,52	11,34	45,22
06.05.2013	07.05.2013	Ag	30min	1	34,85	16,18	41,66
06.05.2013	08.05.2013	Ag	30min	0	56,30	6,63	46,01
06.05.2013	08.05.2013	Ag	30min	0,01	31,33	8,64	43,69
06.05.2013	08.05.2013	Ag	30min	0,1	28,38	3,71	48,65
06.05.2013	08.05.2013	Ag	30min	0,5	33,00	6,86	47,32
06.05.2013	08.05.2013	Ag	30min	1	32,32	5,77	47,56
13.05.2013	13.05.2013	Ag	30min	0	-5,13	7,77	74,70
13.05.2013	13.05.2013	Ag	30min	0	64,01	6,13	77,09
13.05.2013	13.05.2013	Ag	30min	0	49,34	11,05	66,45
13.05.2013	13.05.2013	Ag	30min	0,5	62,90	7,63	73,29
13.05.2013	15.05.2013	Ag	30min	0	34,82	8,68	44,29
13.05.2013	15.05.2013	Ag	30min	0	19,88	5,73	49,98
13.05.2013	15.05.2013	Ag	30min	0,01	16,05	5,00	43,61
13.05.2013	15.05.2013	Ag	30min	0,01	19,65	8,12	44,96
13.05.2013	15.05.2013	Ag	30min	0,1	18,79	6,62	45,71
13.05.2013	15.05.2013	Ag	30min	0,1	18,49	5,76	45,17
13.05.2013	15.05.2013	Ag	30min	0,25	16,77	5,36	46,42
13.05.2013	15.05.2013	Ag	30min	0,25	20,95	6,42	49,49
13.05.2013	15.05.2013	Ag	30min	0,5	18,40	6,12	45,94
13.05.2013	15.05.2013	Ag	30min	0,5	15,14	6,96	44,66
13.05.2013	15.05.2013	Ag	30min	1	17,21	7,17	45,66
13.05.2013	15.05.2013	Ag	30min	1	13,57	5,99	42,69

The duplicates were not similar that is why it has to be assume that the measurements were not trivial. If comparing the results for the oxygen uptake rate measured after different storage time of the sludge (1, 2 and 3 days which refers to the time from taking sludge from WWTP until proceeding measurements) it can be observed that the most active sludge is the fresh sludge, i. e. the OUR measurements were performed on the day of sludge sampling, cf. 13th May, where the results of OUR for nitrifying bacteria were the highest. Also for that sludge the heterotrophic had the highest rate. The lower results were for two-day old sludge and the weakest was the three-day old sludge, what was presented in Table 8 in the highest inhibition rate. However, in this situation it was difficult to assess whether the toxicity of ionic silver or rather the decrease in activity of the sludge and hence respiration because of the long storage time was the reason for inhibition. In this part of the measurements also the results were very unstable and inconsistent, cf. duplicates and triplicates in Table 8 . The reference sample of

the two-day old sludge on 7th May was 56 $mgO_2/(l*h)$ for the first measurement and only 25 $mgO_2/(l*h)$ for the duplicate.

Table 9 Second stage of project (yellow colour – data not considered reliable).

sampling date	Date	substance	aeration time	concentratic	OURAmax in 20degrees	OUR NO2max	OUR Cmax
13.05.2013	16.05.2013	Ag	30 min	0	71,51	9,89	64,65
13.05.2013	16.05.2013	Ag	30 min	0	69,74	15,14	59,56
13.05.2013	16.05.2013	Ag	30 min	0,1	68,71	10,52	65,37
13.05.2013	16.05.2013	Ag	30 min	0,1	67,81	12,94	60,79
13.05.2013	16.05.2013	Ag	30 min	0,5	12,57	10,17	62,98
13.05.2013	16.05.2013	Ag	30min	0,5	48,16	9,38	63,96
13.05.2013	16.05.2013	Ag	1h	0	45,04	8,11	62,98
13.05.2013	16.05.2013	Ag	1h	0	42,96	7,78	63,02
13.05.2013	16.05.2013	Ag	1h	0,1	2,10	12,35	61,54
13.05.2013	16.05.2013	Ag	1h	0,1	14,62	11,91	62,97
13.05.2013	16.05.2013	Ag	1h	0,5	1,00	6,56	61,34
13.05.2013	16.05.2013	Ag	1h	0,5	0,96	8,80	60,61
13.05.2013	16.05.2013	Ag	3h	0	28,39	8,22	55,95
13.05.2013	16.05.2013	Ag	3h	0,1	-3,80	6,38	61,85
13.05.2013	16.05.2013	Ag	3h	0,5	-2,43	6,06	54,98
21.05.2013	21.05.2013	Ag	30min	0	57,04	14,87	75,14
21.05.2013	21.05.2013	Ag	30min	0	60,92	9,68	71,13
21.05.2013	21.05.2013	Ag	30min	0,1	69,04	12,52	73,92
21.05.2013	21.05.2013	Ag	30min	0,1	64,42	9,48	63,41
21.05.2013	21.05.2013	Ag	30min	0,5	46,07	9,92	75,37
21.05.2013	21.05.2013	Ag	30min	0,5	47,79	8,59	66,48
21.05.2013	21.05.2013	Ag	1h	0	42,75	9,66	78,19
21.05.2013	21.05.2013	Ag	1h	0	33,47	8,52	62,00
21.05.2013	21.05.2013	Ag	1h	0,1	14,28	10,25	75,93
21.05.2013	21.05.2013	Ag	1h	0,5	4,22	8,19	68,69
21.05.2013	21.05.2013	Ag	3h	0	27,09	10,40	72,28
21.05.2013	21.05.2013	Ag	3h	0,1	2,93	10,29	71,77
21.05.2013	21.05.2013	Ag	3h	0,5	2,04	7,11	65,39

The measurements for the different contact times were conducted for 30 min, 1 h and 3 h aeration. However, it appeared that after 3 h of aeration the substances given to each of the measurement bottles have already been digested, which resulted in a limitation of e.g. ammonium and as a consequence, the results were not taken into consideration. The results presented in table above suggest that there is increasing inhibition with increasing aeration time because for each of the concentrations the oxygen uptake rate is lower with the extension of time. Nevertheless, the results were considered as unreliable since this phenomenon also occurred for the reference without any addition of silver. It could suggest that for half an hour the sludge respiration was reduced by 36, 97 and 91% for each concentration, respectively. The problem could be, as mentioned before, the storage time of the sludge, in this case 4 days. Despite the fact, the reduction in OUR for the 1-day old sludge was also high, it has been decided to use the fresh sludge – on the day of delivery from the WWTP and the day after.

This could help to avoid the divergence of the results, and maintain them on a similar level of activity. Moreover, it was decided not to aerate the sludge container if the measurement were planned for the next day. This was considered acceptable due to the nitrate present in the activated sludge which prevented the sludge from becoming anaerobic. On the day of the measurement the aeration of the sludge container was started approximately 2 h before the start of the measurements.

Due to the fact that the results were so diversified, the possibility of probable digestion of ammonium chloride in bottle A (i. e. limitation) had to be resolved. The amount of ammonia given to bottle A was increased from 1 ml to 1,5 ml what gave 30 mg NH_4-N/L. Additionally, also a buffer was added to the sludge container. It was assumed that the buffer (2,5 g of $NaHCO_3$) will help to maintain the pH above 7. If the pH falls below 7 or 6.8 due to nitrification during the measurement in bottle A, it can result in a decrease in activity of autotrophic bacteria. The measurements with the buffer and a larger dose of ammonia were conducted on reference samples. The results (Table 10) show that there are no significant distinctions between 30 min and 1 h of aeration, as expected.

Table 10 Third stage of project.

sampling date	Date	substance	aeration time	concentratic	OURAmax in 20degrees	OUR NO2max	OUR Cmax
27.05.2013	27.05.2013	Ag	30min	0	66,17	13,32	67,04
27.05.2013	27.05.2013	Ag	1h	0	63,04	11,38	63,48
27.05.2013	28.05.2013	Ag	30min	0	63,32	11,03	57,58
27.05.2013	28.05.2013	Ag	30min	0	60,21	12,58	59,09
27.05.2013	28.05.2013	Ag	30min	0	61,54	12,40	57,84
27.05.2013	28.05.2013	Ag	1h	0	59,71	11,12	63,96
27.05.2013	28.05.2013	Ag	1h	0	60,80	10,80	63,71
27.05.2013	28.05.2013	Ag	1h	0	61,44	10,57	62,46
27.05.2013	28.05.2013	Ag	1h	0	61,97	15,81	58,14
29.05.2013	29.05.2013	Ag	30min	0	68,89	13,81	64,14
29.05.2013	29.05.2013	Ag	30min	0	68,95	11,76	59,46
29.05.2013	29.05.2013	Ag	30min	0	68,85	12,33	60,37
29.05.2013	29.05.2013	Ag	1h	0	63,74	12,29	62,54
29.05.2013	29.05.2013	Ag	1h	0	64,32	12,96	61,88
29.05.2013	29.05.2013	Ag	1h	0	67,63	12,25	60,73

After adapting the measurement procedure (buffer addition and ammonium dose) the next step was to observe how the different concentrations of silver nitrate affect the sludge respiration.

Table 11 Fourth stage of project.

sampling date	Date	substance	aeration time	concentratic	OURAmax in 20degrees	OUR NO2max	OUR Cmax
03.06.2013	03.06.2013	Ag	30min	0	54,19	6,78	63,63
03.06.2013	03.06.2013	Ag	30min	0	57,49	9,21	60,06
03.06.2013	03.06.2013	Ag	30min	0,5	57,33	8,23	57,75
03.06.2013	03.06.2013	Ag	30min	0,5	58,00	10,38	60,50
03.06.2013	03.06.2013	Ag	1h	0	57,16	10,11	62,94
03.06.2013	03.06.2013	Ag	1h	0	56,03	8,08	62,53
03.06.2013	03.06.2013	Ag	1h	0,5	56,71	10,22	61,82
03.06.2013	03.06.2013	Ag	1h	0,5	63,56	9,83	56,98
03.06.2013	04.06.2013	Ag	30min	0	55,27	10,72	62,65
03.06.2013	04.06.2013	Ag	30min	0	55,66	14,39	58,45
03.06.2013	04.06.2013	Ag	30min	1	56,17	12,36	58,17
03.06.2013	04.06.2013	Ag	30min	1	54,31	10,37	60,59
03.06.2013	04.06.2013	Ag	1h	0	53,91	11,49	58,17
03.06.2013	04.06.2013	Ag	1h	0	58,38	10,33	61,46
03.06.2013	04.06.2013	Ag	1h	1	54,76	10,65	60,19
03.06.2013	04.06.2013	Ag	1h	1	55,10	13,50	59,10
03.06.2013	04.06.2013	Ag	1h	5	1,24	12,19	64,56

It can be observed that the activity of sludge was rather on the same level, what proved that this concentration of ionic silver was not toxic for the sludge.

The next stage was to test the influence of silver nitrate on the modified sludge. The modification of the sludge was supposed to be investigate during the chronic toxicity test, but in order to wash out the chloride that could precipitate silver it has been decided to introduce modified sludge earlier. In the beginning tests were conducted with low concentrations of silver, increased gradually during the next days (Table 12).

Table 12 Fifth stage of project.

sampling date	Date	substance	aeration time	concentratic	OURAmax in 20degrees	OUR NO2max	OUR Cmax
07.06.2013	07.06.2013	Ag	1h	0	30,642	8,698	40,366
07.06.2013	07.06.2013	Ag	1h	0	36,018	9,323	42,816
07.06.2013	07.06.2013	Ag	1h	0	31,645	8,688	46,728
07.06.2013	07.06.2013	Ag	1h	0	33,417	9,923	44,627
10.06.2013	11.06.2013	Ag	1h	0	27,054	7,263	29,799
10.06.2013	11.06.2013	Ag	1h	0	24,888	7,346	28,150
10.06.2013	11.06.2013	Ag	1h	0	33,588	7,459	31,596
10.06.2013	11.06.2013	Ag	1h	1	32,595	7,915	32,100
10.06.2013	11.06.2013	Ag	1h	1	33,038	8,504	33,256
10.06.2013	11.06.2013	Ag	1h	5	31,733	8,009	30,281
10.06.2013	11.06.2013	Ag	1h	5	29,436	7,929	29,390
12.06.2103	12.06.2103	Ag	1h	0	30,752	8,019	44,645
12.06.2103	12.06.2103	Ag	1h	0	15,403	5,289	30,521
12.06.2103	12.06.2103	Ag	1h	0	42,316	9,687	55,323
12.06.2103	12.06.2103	Ag	1h	10	1,922	7,728	17,944
12.06.2103	12.06.2103	Ag	1h	10	4,671	10,083	26,410
12.06.2103	13.06.2013	Ag	1h	0	27,345	9,147	49,013
12.06.2103	13.06.2013	Ag	1h	0	28,500	9,075	43,493
12.06.2103	13.06.2013	Ag	1h	0	33,829	8,439	49,609
12.06.2103	13.06.2013	Ag	1h	10	29,204	9,059	34,280
12.06.2103	13.06.2013	Ag	1h	10	-10,755	9,879	49,550
12.06.2103	13.06.2013	Ag	1h	10	28,265	10,205	42,544
17.06.2013	18.06.2013	Ag	1h	0	7,832	6,815	18,160
17.06.2013	18.06.2013	Ag	1h	0	17,765	8,594	18,582
17.06.2013	18.06.2013	Ag	1h	0	27,415	9,423	27,459
17.06.2013	18.06.2013	Ag	1h	50	17,475	8,088	18,100
17.06.2013	18.06.2013	Ag	1h	50	16,314	7,548	17,026
17.06.2013	18.06.2013	Ag	1h	100	8,611	7,877	14,021
17.06.2013	18.06.2013	Ag	1h	100	10,901	7,832	15,352

The reference sample was repeated at the end of measuring day. The oxygen rate was higher than in the beginning of measurements, what was expected. The sludge becomes more active over the time if there is a substrate and suitable environmental conditions e.g. air. A comparison of the chloride concentration in the sludge after washing and after the measurement in bottle A revealed a significant increase which was caused by the addition of ammonium in the form of ammonium chloride. In order to avoid the chloride which could lead to a precipitation of the silver the ammonium chloride was replaced by ammoniac, in the same concentration of nitrogen as before.

Table 13 Sixth stage of project.

sampling date	Date	substance	aeration time	concentratic	OURAmax in 20degrees	OUR NO2max	OUR Cmax
24.06.2013	25.06.2013	Ag	1h	0	15,019	3,911	19,383
24.06.2013	25.06.2013	Ag	1h	0	16,422	4,193	17,016
24.06.2013	25.06.2013	Ag	1h	0	19,225	5,821	17,122
24.06.2013	25.06.2013	Ag	1h	50	7,123	5,107	12,545
24.06.2013	25.06.2013	Ag	1h	50	6,596	6,612	14,156
24.06.2013	25.06.2013	Ag	1h	50	8,476	5,568	11,142
24.06.2013	25.06.2013	Ag	1h	100	1,316	4,667	7,584
24.06.2013	25.06.2013	Ag	1h	100	0,512	3,616	8,093
24.06.2013	25.06.2013	Ag	1h	100	0,709	6,749	6,019

Tests with silver nanoparticles were conducted for one day, but the results were not suitable for calculating or assessing an inhibition, since the organics in the dispersant resulted in an OUR and the dispersant without NP which would have been necessary for the reference measurement was not available for the measurements.

As it can be observed in the Table 14 there is no inhibition, probably because of the dispersant of the nanoparticles. The dispersant was organic, what increased the respiration of the sludge as an extra source of carbon for bacteria during the measurement.

Table 14 Seventh stage of project – measurements with Ag NPs.

sampling date	Date	substance	aeration time	concentratic	OURAmax in 20degrees	OUR NO2max	OUR Cmax
27.06.2013	28.06.2013	Ag Nano	1h	0	14,514	2,779	22,516
27.06.2013	28.06.2013	Ag Nano	1h	0	12,863	1,841	25,010
27.06.2013	28.06.2013	Ag Nano	1h	0,5	39,598	5,991	31,380
27.06.2013	28.06.2013	Ag Nano	1h	0,5	34,724	6,177	34,486
27.06.2013	28.06.2013	Ag Nano	1h	1	39,929	8,271	39,864
27.06.2013	28.06.2013	Ag Nano	1h	1	37,908	8,113	40,554
27.06.2013	28.06.2013	Ag Nano	1h	10	27,424	4,137	32,833
27.06.2013	28.06.2013	Ag Nano	1h	10	32,297	7,163	30,101

7. SUMMARY and CONCLUSIONS

The final methodology, suitable for testing the acute toxicity of nanoparticles was created with the following changes compared to the usual OUR measurement procedure:

- Sludge used no longer than one day after delivery from WWTP
- Modified sludge, i. e. activated sludge washed with synthetic feed
- Ammonium addition in the form of ammoniac instead of ammonium chloride
- One hour aeration time of bottle A and B

The next steps in this project should be to test on the rest of nanoparticles.
The dispersant of nanoparticles should also be tested. Each of the substances used to dissolve nanoparticles powder or to dilute nanoparticle liquid should be tested separately for its effect on the respiration and used for the reference measurements.

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