TECHNICAL UNIVERSITY OF LIBEREC
Faculty of Mechatronics, Informatics
and Interdisciplinary Studies

POSSIBILITIES OF USING FLUORESCENCE MICROSCOPY TO DETERMINE THE TOXICITY OF NANOPARTICLES USING PROKARYOTIC MICROORGANISMS

Bachelor thesis

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2. Vybrat vhodný modelový mikroorganizmus - na základě literární rešerše.
3. Vybrat nejméně tři typy nanočástic, jejichž potenciální toxicita bude testovaná pomocí nejméně dvou fluorecenčních metodik, jako je např. stanovení ROS (Reactive Oxygen Species) v buňkách nebo určení životaschopnosti buněk (LIVE/DEAD staining). Pro srovnání výsledků použít klasickou kultivaci buněk bakterií na agarových plotnách.

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[3] HarleyPrescott: Laboratory Exercises in Microbiology, Fifth Edition. The McGraw-Hill Comp. 2002

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Abstract

The bachelor thesis discusses properties and methods for studying toxicity of different nanoparticles using prokaryotic microorganisms. Main aim of thesis was to introduce the principles and comparison of methods for determination of toxicity of nanoparticles: Determination of cell viability, Determination of Reactive Oxygen Species in cells and Cultivation of bacteria on agar media. Nanoparticles (nanodiamond, magnetite and nZVI) were characterized using the CPS disc centrifuge and Zeta Sizer (DLS and DCS methods). The toxicity was investigated on model organism of bacteria *Pseudomonas putida.* Selected methods gave similar results and showed that the nanoparticles are not harmful to prokaryotic *P. putida.* The most accurate method seems to be Determination of cell viability which describes the nanoparticles according to available literature. But each method has its advantages and disadvantages, for example Cultivation of bacteria on agar media is more time consuming. Characterization of nanoparticles provided information about the formation of large aggregates (hundreds of nm) in bacterial growth media which might influence the toxicity tests.

Key words:

Fluorescence, Reactive Oxygen Species, *Pseudomonas putida,* nanodiamond, magnetite, nZVI, nanotoxicity

Abstrakt

Bakalářská práce pojednává o vlastnostech a metodách studia toxicity různých nanočástic s použitím prokaryotických mikroorganismů. Hlavním cílem práce bylo seznámit se s principy a porovnáváním metod stanovení toxicity nanočástic: stanovení životaschopnosti buněk, stanovení reakčních forem kyslíku v buňkách a kultivace bakterií na agarovém médiu. Nanočástice (nanodiamanty, magnetit a nZVI) byly charakterizovány pomocí CPS diskové centrifugy a Zeta Sizeru (DLS a DCS metody). Toxicita byla zkoumána na modelovém organismu, bakterii *Pseudomonas putida.* Vybranými metodami byly získány obdobné výsledky, které ukázaly, že tyto nanočástice nejsou škodlivé pro prokaryotickou *P. putidu.* Nejpřesnější metodou se zdá být stanovení životaschopnosti buněk, která popisuje nanočástice podle dostupné literatury. Ale každá metoda má své výhody i nevýhoda, například kultivace bakterií na agarovém médiu je časově náročnější. Charakterizace nanočástic poskytla informace o tvorbě velkých agregátů (stovky nm) v bakteriálním růstovém médiu, které by mohly ovlivnit testy toxicity.

Klíčová slova:

Fluorescence, Reaktivní formy kyslíku, *Pseudomonas putida,* nanodiamant, magnetit, nZVI, nanotoxicita

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1 Introduction

Nanotechnology, like any new technology, constitutes specific risks which may be large and unpredictable according to some experts (Hunt and Riediker 2011). These are the risks associated with damage to human health (toxicity, teratogenicity, etc.) or a risk to the environment (ecotoxicity, affecting biogeochemical cycles and more). In recent years the theme of safety and potential risks of nanomaterials and nanotechnology is very topical and widely discussed at the national and international levels.

The target of this thesis is to get acquainted with the problems of determining the toxicity of nanoparticles to prokaryotic microorganisms. The work includes preparation and characterization of bacterial cultures and cultivation bacteria growth media. In the thesis are used three types of nanoparticles.

2 Literature overview

2.1 Nanoparticles

Nanomaterial contains particles in one or more external dimensions within the size range 1 nm – 100 nm, for 50 % or more of the particles. In special cases such as concerns for the health, environment or safety may be the number size distribution replaced between 1 and 50 % of the particles. It is a natural, incidental or manufactured material which can exist in an aggregate or as an agglomerate or as an unbound state (ENVIRONMENT DG 2015).

As nanomaterials are considered the graphenes, fullerenes and single carbon nanotubes with one or more dimensions below 1 nm.

Nanomaterials are not intrinsically unsafe per se but we must establish clear and unambiguous criteria to assess their risk (Chair et al., 2012).

2.1.1 Properties of nanoparticles

The behavior of nanoparticles changes with decreasing particle size. These are changes from classical physics to quantum physics. The physical and chemical properties of a nanoparticle can vary due to different composition, size or surface composition.

A sphere-shaped nanoparticles with a radius of 2.5 nm and a density of 5 g/cm^3 has a surface of 240 m^2/g have about 20% atoms at their surface (Borm at al., 2006). But thanks to the adhesive forces, the surface of a nanoparticle is never bare. The forces cause agglomeration or attaching to the next free surface. If we cannot control the surface properties during the technical application, nanoparticles quickly create larger particles called agglomerates. During that particles mostly lose the properties that are dependent on particle size like for example colour and transparency. It is important to control their agglomeration behavior for retain their specific properties which have dispersed nanoparticles (Ngo et al., 2011).

The adverse biological effects increase with small size and active surface of the nanoparticles. Exclusivity of nanoparticles is given by their dispersion in the product. The agglomerates have tendency to sediment and in a polymeric material like an ink, paint, cream etc. they are hardly dispersed (Filipponi, Sutherlan 2013).

For example magnetic nanoparticle exhibit superparamagnetic behavior or quantum quantum phenomena (Jang 2007).

2.1.2 Application of nanoparticles

In different sectors, we can find wide application of nanomaterials like engineering, medicine, electronics, chemical, optical, automotive, cosmetic industry, military industry or environment.

Currently nanotechnology and nanomaterials represent a new, groundbreaking development opportunities in many fields of human activity. It is expected that these new technologies and materials will revolutionize medicine, where due to its size exhibit the ability to enter into individual cells, and for example behave like carriers of drugs or contrast agents in medical diagnostics. Many of the processes and information technology products, as well as the computer industry already cannot be without nanotechnology. Nanotechnology started to be used in many consumer products to improve their surface properties, such as e.g. abrasion resistance, durability, optical properties. These materials promise a high potential in the food industry, especially in packaging materials (Hansen et al., 2008).

In recent years a preparation of nanomaterials moved from laboratory to industrial production and the number of products of nanomaterials increased. Like any new technology, nanotechnology represents a great benefit, but which is also accompanied by the potential environmental and health risks. For ensuring the safe development we cannot do without limitations, legislation that defines risks connected with exposure to nanomaterials and recommendations to safe handling (Maynard et al., 2006).

2.1 Toxicity of nanoparticles

Generally, it is assumed that it is significantly different and specific physico-chemical properties of nanomaterials with their chemical composition which are responsible for their toxic effect and toxicokinetics (their absorption, distribution, biotransformation and elimination in the organism) and, moreover, influence their fate and transformation in environment. It is not yet clear which characteristics are exactly associated with the dangerous potential of various nanomaterials. Even with the same chemical composition, the nanoparticles may exhibit different toxicities depending e.g. on different particle size, surface modification and others (Oberdörster et al., 2007).

Toxicity and toxicological profile changes with decreasing size below 100 nm. The decline of particle size is associated with an increase in total surface area and increasing the free Gibbs energy that reflects the reactivity of the particles. Gibbs energy significantly grows with decreasing particle size, which results in considerably higher chemical reactivity of particles (Rahman 2011).

The properties of the atoms surface layer in the case of nanomaterials in comparison with materials of larger particle size start gradually to dominate over properties of volume, which results also in different behavior of the material in relation to living organisms (Lei 2007).

Other important surface properties that affect the behavior of particles and their resultant toxicity are surface charge, chemical composition of the surface, shape of the surface, defects on the surface, porosity etc. A very important feature of particles and nanomaterials is also their morphology, physical form (whether they are amorphous or crystalline), and if forms aggregates or agglomerates, and which have a shape (particles, tube, etc.).

Principal parameters for expressing the dose in classical toxicology are weight (concentration) as opposed to nanotoxicology it is weight (concentration), size, structure, specific surface, surface modification, and surface charge.

When assessing the dose of nanomaterials we can not rely only on the classical weight concept, i.e. the concentration expression such as $mg*dm^{-3}$. It must be taken into account that the test material due to its increased reactivity may before application in the test pass not only physical (e.g. formation of aggregates, agglomerates), but also chemical changes (e.g. surface oxidation).

In terms of toxicity, the particles are divided into primary (targeted synthesized) and secondary nanoparticles (arising as an unwanted product in a variety of combustion, physical or chemical processes, e.g. products of diesel engines, tobacco smoke, and other).The primary particles (targeted synthesized) may exhibit homogeneous size and shape compared to nanoparticles randomly generated in the environment. Primary and secondary particles can be transformed into other physical or chemical form, as their aggregation or agglomeration. Their structure at nanosize carries new surface properties, which may lead to new characteristics due to toxicity of these materials (Vogel et al., 2014).

The agglomerates and the aggregates after changing conditions in the environment (during the transition to a new environment) may fall apart and again be formed, which affect not only their distribution and toxicity, but also their transport and fate in the environment and organism.

Nanoparticles which form aggregates can concurrently have behavior typical for nanoparticles even microparticles, such as e.g. some nanopowders (To et al., 2009).

Owing to possible leaks of nanomaterials in production or in contact consumer with products containing nanomaterials, it can be expected direct contact of these materials with the human organism, but also with other organisms, animals and plants.

A very important factor influencing the toxicity of nanoparticles is the exposure type, i.e. the place where chemicals or nanoparticles enter the organism. Toxic effect of chemicals (nanoparticles) is then affected by the speed of the onset toxic effect.

A key role for the penetration of nanoparticles into cells plays a cytoplasmic membrane and particle transport there through. Membrane receptors decide what substances and in what quantities will be accepted or excluded from the cell. The lipid nature of membrane blocks

the transfer of ions and large polar molecules. The presence of ionic pumps, transporters and protein channels allows selective ion transport. Macromolecules and nanoparticles can be uptaken through endocytosis, when the substance or nanoparticle is covered and incorporated into the cytoplasmic membrane vesicle (Filipová et al., 2012).

Nanomaterials have diverse effects at the molecular, cellular and tissue level, such as oxidative stress and DNA damage. They can influence the cells in several ways. Nanoparticles in the nucleus can directly interact with DNA molecules or proteins associated with the DNA, which is leading to physical damage of the genetic material. Nanoparticles can affect DNA directly, but also indirectly, when they interact with cellular proteins e.g. proteins in the process of cell division.

Already in the early phase of entry into the cell, increases the production of various carriers (Reactive Oxygen Species, ATP and calcium) and activation of various signaling pathways that lead to cellular responses, such as e.g. the formation of oxidative stress or inflammation, which may result DNA damage also depending on the degree of intensity.

After exposure to the nanoparticles do not necessarily lead to cell death, but can be modified only its genetic information, whether by direct or indirect effects. Although the cell survives, but for the body as a whole, this situation have much more serious consequences. DNA damage can be fixed as a mutation (a permanent change in the genetic information in DNA). Some types of mutations can induce the initiation of carcinogenesis, insofar as they concern gametes, may be affected fertility and the health of future generations**.**

2.1.1 Oxidative stress

The key mechanismus of the nanomaterial toxicity is considered so-called oxidative stress. Many studies show the induction after exposure to atmospheric ultrafine particles, as well as some targeted manufactured nanoparticles. In favorable conditions in the organism exists equilibrium between reactive oxygen species (ROS) and antioxidants. If this balance is broken for some reason, it is created oxidative stress, which constitutes a violation of the balance between formation and removal of reactive oxygen species. This situation is caused by the increased formation of oxygen radicals or reducing the antioxidative system capacity. It is about free radicals ($O_2^{\cdot}$, $HO^{\cdot}$, $RO^{\cdot}$, $HOO^{\cdot}$, $ROO^{\cdot}$, $NO^{\cdot}$), which are molecules or atoms with one or more unpaired electrons in its valence sphere (Linkov, Steevens 2009).

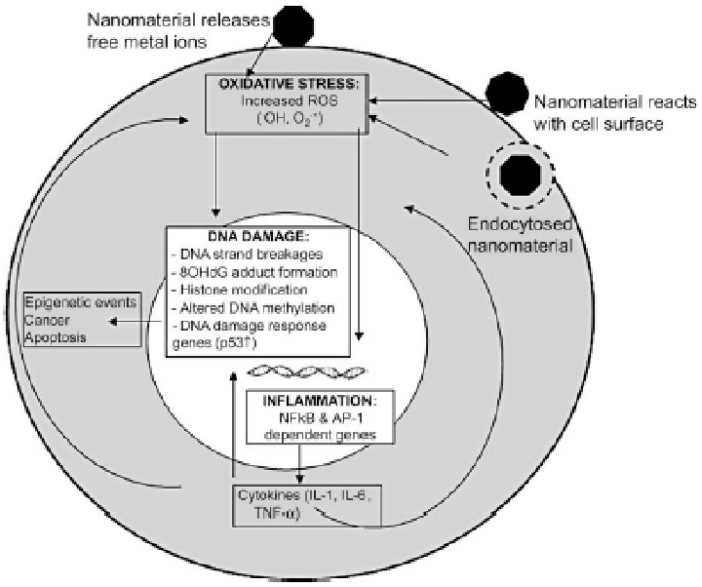


Figure 2.1: Indirect mechanisms leading to damage cells (Filipová et al., 2012)

2.2 Model microorganisms

The model microorganisms are in some way similar and related. When studying the process in model organisms, we are able to predict toxic effect on human and others organism.

As model microorganisms we usually used the bacteria, algae, plants, Crustacea or fish. I used the bacteria of the genus Pseudomonas for my experiment.

2.2.1 Pseudomonas

Pseudomonas is a genus of the family Pseudomonadaceae. Original classification into species was based on the morphological properties of the organism. Currently Pseudomonas genus includes more than 70 species.

Bacteria belonging to the genus Pseudomonas are straight or slightly curved rods with cell wall of gram-negative type. Their size is variable, reaching from 0.5 to 1.0 μm x 1.5 to 5.0 μm. They are moving very well, they are among the fastest-moving bacteria. They use for the movement one or more polar flagella. Bacteria of this genus are rarely unmovable.

Bacteria of genus Pseudomonas are widespread in many different types of environments. They are isolated from soil, water, the surface of plants, animals and fungi, from gastrointestinal tract, may be pathogens and they are used as biocontrol agents (Moore et al. 2006).

They can grow in the temperature range 4-42 ° C in both acidic and alkaline environments. They are able to multiply at pH which is ranging from 4.5 to 8. Also tolerate increased salt concentrations and are often naturally resistant to antibiotic and other toxic substances.

They use simple or complex organic compounds and can survive in distilled water. For the representatives of this genus is very typical formation of pigments. Large group consists of the fluorescent pseudomonads, producing fluorescent dyes, which can be an important taxonomic character.

Pseudomonas putida

The representatives of this species are common saprophytic organisms which are found in most soils and natural habitats where oxygen is present. It is non-pathogenic to humans and it is easily isolable. It has the ability to adapt to the large heterogeneity of their environment, benefit a wide range of available organic compounds and degrade variety of pollutants (Nelson et al. 2002). Thanks to these properties and large plasticity of genome P. putida is especially favorable species for use in various recovery processes for environments. Some of the strains are also used as biocontrol agents and as biopesticides. Much of the research is focused on the development of bioplastics. The temperature optimum for P. putida is 25-30 °C (Patten, Glick 2002).

Characteristics of P. putida are used in defense of the presence of heavy metals. Largely they are present in the genes which encode proteins that are important in the mechanism of resistance to the presence of heavy metals (Cánovas et al. 2003).

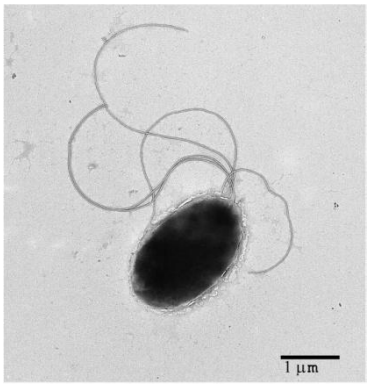


Figure 2.2: Pseudomonas putida (Sagan 2011)

2.3 Fluorescence microscopy

Fluorescence microscopy is based on emission of light of a substance that absorbs light or other electromagnetic radiation. The energy of the emitted photon is reduced transition between energy levels and wavelength of the emitted light is longer than the absorbed photon. Emitted radiation is radiated by an atom which absorbed energy. A phenomenon in which there is absorption of a photon and subsequently emission of a photon due to radiated.

Fluorescence microscope

I worked with a microscope Axiovision IMAGER M2 (Zeiss, Germany). It is a fluorescence microscope for biological, medical and material research. It can photograph in reflected and transmitted light, the possibility of using fluorescence. Zoom of the lens is 2.5 - 100. The microscope has a monochrome and color camera. The camera resolution is 1388 x 1038 px. May occur photobleaching which is damage due to the light.

Figure 2.3: Fluorescence microscope Axiovision IMAGER M2 (Zeiss, Germany)

3 Materials and Methods

Experiments were performed in microbiology laboratories in compliance with laboratory measures at the Institute for Nanomaterials, Advanced Technologies and Innovation.

Exposure time of each measurement was after 24 hours for 3 days. The measurement was repeated for each nanoparticle and each method.

3.1 Nanomaterials

I used three types of nanoparticles: zerovalent iron, magnetite and nanodiamond.

At the beginning of each series of measurements I prepared fresh nanoparticle suspension of a concentration 10 g /L. I weighed 0.05 g of nanoparticles into a test tube and added 5 ml of deionized water (DI). Then I mixed the solution for 10 minutes by a mixer (Miccra D-9 Homogenizer, Germany) to achieve the right homogenized stock suspension.

nZVI

nZVI is zerovalent iron, batch 197 obtained from Nanoiron Ltd. Company, Czech Republic. The zerovalent iron core is passivated by oxidic shell to prevent fast oxidation of the nanoparticles.

Magnetite

Magnetite nanoparticles written into the formula Fe_3O_4, batch ZH09A obtained from Palacky University Olomouc, Czech Republic. Composition is 71,9% Fe (wt), 2,3% O (wt), 25,7% Si (wt).

Nanodiamond

It is cubic diamond, obtained from International Technology Center, USA). The size is approximately 8 nm and a specific surface area 285-295 m^2/g.

3.2 Bacterial growth medium

I used a Tryptone Soya Broth as a medium for cultivation of bacteria. It is generally applicable medium for cultivation of a nutritionally demanding and undemanding and sterility testing of moulds and bacteria.

Preparation of growth media

I prepared the growth medium from powder a Tryptone Soya Broth (Soyabean Casein Digest Medium, HIMEDIA, Czech Republic) according to instruction of the producer. I weighed 9 g of powder into a glass flask and added 300 ml of distilled water. I dissolved the medium completely. Then the solution was sterilized by autoclaving at temperature 121°C for 15 minutes.

3.3 Plate Count Agar

I used a microbiological growth medium Plate Count Agar (without Dextrose) (BIO-RAD, France) for cultivation of bacteria on Petri dishes. It is commonly used to assess or to monitor growth of cells (number of colonies).

Preparation of agar solution

I weighed 6.3 g of PCA in a glass flask and dissolved it in 300 ml of distilled water. I waited for 5 minutes and mixed it well to get a homogeneous solution. Then it was sterilized by autoclaving for 15 minutes at 121°C. Final pH was approximately 7.2.

Then I kept it closed in a dry cool place. And before using I warmed it slowly in microwave and brought to the boil to complete dissolution.

3.4 Bacterial culture

As model organism, I used the bacterium *Pseudomonas putida* (Czech Collection of Microorganisms, CCM Brno). I used a medium Tryptone Soya Broth (Soyabean Casein Digest, Himedia) for cultivation of bacteria.

First, I put approximately 15 ml of medium Soya Broth to a flask. Then I added bacterial culture from a petri dish using a pipette,mixed it and placed it in an incubator (Thermostat Incucell, from BMT) overnight, where the temperature was maintained at around 27°C.

The second day I measured the concentration of bacteria in culture. I used a UV-Vis spectrophotometer (Hach Lange, Germany). I measured the spectrophotometric optical density (OD). I pipetted 0.5 ml of bacteria and 0.5 ml of deionized water in spectrophotometric container. I set the spectrophotometer for wavelength of 600 nm and started measurements. First I calibrated the device only for water and then I measured bacteria.

Then I prepared shake flasks. I added 10 ml of medium Soya Broth to each shake flask together with bacterial culture.

Calculation of volume that I needed to add:

I made a calculation using the equation

$$c_1V_1 = c_2V_2$$

where,

c_1 – concentration of bacterial culture, c_2 – needed concentration of bacteria

V_1 – total volume of solution, V_2 – needed volume of bacteria

For example:

Spectrophotometer measured OD_{600} = 0.964 and with a dilution 1: 1 is the correct value OD_{600} = 0.964*2 = 1.928

c_1 = 1.928, c_2 = 0.05, V_2= 10 ml, V_1 = ?

$$V_1 = \frac{c_2V_2}{c_1} = \frac{0.05 * 10}{1.928} = 0.259\ ml = 259\ \mu l$$

I added 259 μl of bacteria to each shake flask.

3.5 Tests to determine the toxicity

I used following methods for determining the toxicity of nanoparticles: a) Determination of cell viability b) Determination of Reactive Oxygen Species in cells c) Cultivation of bacteria on agar media

At the beginning of each series of tests I prepared a bacterial culture, measured the concentration by the spectrophotometer and calculated the required quantity. And I prepared fresh nanoparticles solution having an identical concentration of 0.01 g/l, 0.05 g/l and 0.5 g/l. The measurement was done after 0-2 h, 24 h and 48 hours.

I calculated the volume of nanoparticles again using the equation

$$c_1V_1 = c_2V_2$$

The prepared nanoparticle suspension had a concentration of 10 g/l and calculations are as follows:

$$V_{0.01} = \frac{c_2V_2}{c_1} = \frac{0.01 * 10}{10} = 0.01\ ml = 10\ \mu l$$

$$V_{0.05} = \frac{c_2 V_2}{c_1} = \frac{0.05 * 10}{10} = 0.05\ ml = 50\ \mu l$$

$$V_{0.5} = \frac{c_2 V_2}{c_1} = \frac{0.5 * 10}{10} = 0.5\ ml = 500\ \mu l$$

I added the medium Soy Broth, bacteria *Pseudomonas putida* and nanoparticles into each shake flask. And between each measurement, I put the samples into a shaking incubator (NB-205, N-BIOTEK, Korea) which was set at temperature 25°C and 100 rpm.

3.5.1 Determination of cell viability (Live/Dead staining)

The principle of this method is simple. Healthy bacteria with intact cell membranes are fluorescently labelled green, whereas bacteria with damaged membranes are stained fluorescently red, visible in fluorescence microscope. I took the images of cells using a fluorescence microscope (AxioImager, ZEISS, Germany) and then calculated how many cells were healthy or damaged.

I used LIVE/DEAD® *Bac*Light (Bacterial Viability Kit, from Life Technologies). It was mixtures of SYTO® 9 green-fluorescent nucleic acid dye and the red-fluorescent nucleic acid dye (propidium iodide). These stains were different ability to penetrate healthy and damaged bacterial cells.

Measuring procedure

I made three bacterial suspensions with different nanoparticles concentrations (0.01 g/l, 0.05 g/l and 0.5 g/l) and two control solutions (positive and negative) where I added only bacteria and growth medium, no nanoparticles. I mixed each sample with a shaker (Vortex, IKA Germany) before every sampling.

. I pipetted 1 ml of each samples into small test tubes and centrifuged for 5 minutes with 5000 rpm using a centrifuge (MiniSpin plus, Eppendorf). The bacteria settled down. I pipetted off a solution and I got a pellet. After that I added 1 ml of NaCl (0.85%) and resuspended it by Vortex shaker. Then I took 5 µl of prepared sample and 2.5 µl of live/dead staining and mixed it in smaller test tubes. I waited 15 minutes for staining in the dark and pipetted 1-2 µl on glass covered by cover glass. Next step was inspecting the samples in fluorescent microscope. I used two filter sets on microscope, 44 FITC to see the green fluorescence and 09 AF 488 to see the red fluorescence.

I also prepared a positive control, when I put the sample to boiling water for 5 minutes to damage all cells.

3.5.2 Determination of ROS (Reactive Oxygen Species) in cells

The aim of the method is to observe the influence nanoparticles on the formation of Reactive Oxygen Species inside the cells. I used The Oxidative Stress Detection Reagent which is non-fluorescent, cell-permeable dye. It reacts directly with reactive species such as hydrogen peroxide, peroxynitrite or hydroxyl radicals and we get a green fluorescent product indicative of ROS production. . I observed the marks of oxidative stress using a fluorescence microscope (AxioImager, ZEISS, Germany).

Measuring procedure

At the beginning I prepared the same solutions as for Determination of cell viability, it was 5 samples: with nanoparticles concentrations 0.01 g/l, 0.05 g/l and 0.5 g/l and positive and negative control solutions. I mixed each sample with a shaker.

I pipetted 1 ml of each samples into small test tubes and centrifuged for 5 minutes with 5000 rpm using a centrifuge (MiniSpin plus, Eppendorf). I pipetted off a solution and I got a pellet. Afterwards, I added 1 ml NaCl (0.85%) and resuspended it by Vortex shaker. Then I again used a centrifuge for 5 minutes with 5000 rpm and got a pellet removing the liquid. Subsequently I added 1 ml of NaCl 0.85%, resuspended it by shaker and pipetted 50 µl of prepared suspension and 50 µl OSDR solution (Oxidative Stress Detection Reagent, Enzo Life Sciences) in smaller small test tubes. For positive control it is necessary to add inducer of ROS and therefore I used 2.1 µl H_2O_2 and 5.3 µl $FeCl_2$. The samples were then shaken in dark for 1 hour with 30 s intervals and with 450 rpm in shaker (Thermomixer comfort, Eppendorf). After 1 hour I pipetted 1 µl on glass covered by cover glass. Next step was inspecting the sample in fluorescent microscope.

Positive control should show maximum of the ROS production and proof that the stain is working properly.

Total ROS Detection Kit

I used The Total ROS Detection Kit. It is a product of Enzo Life Sciences, Inc. This kit is for monitoring real time reactive oxygen (or nitrogen) species production in living cells using fluorescence microscopy. The kit includes Oxidative Stress Detection Reagent, ROS Inducer (Pyocyanin) and ROS Inhibitor (N-acetyl-L-cysteine), I used only Oxidative Stress Detection Reagent. I used H_2O_2 and $AlCl_3$ for positive control.

3.5.3 Cultivation of bacteria on agar media

Plate count assay or colony forming unit (CFU) is a standard method for counting cell colonies growing on agar Petri dish.

This method is based on estimation of the number of viable bacteria in a sample. Method requires culturing the microbes and counts only viable cells in contrast with microscopic examination.

Measuring procedure

The first day of measurement (0-2 hours) I worked only with negative control. I took test-tube and pipetted inside 4.5 ml of physiological solution (NaCl, 0.85%) and 0.5 ml of cells from negative control shake flask. I mixed it with Vortex shaker. Subsequently I pipetted 0.5 ml of this stock and added it into next test-tube with 4.5 ml of NaCl (0.85%) to prepared cell dilution of 10^{-2}. I continued up to the dilution 10^{-5}.

In the second and third measurements I used all concentrations of nanoparticles (0.01 g/l, 0.05 g/l and 0.5 g/l) and negative control without nanoparticles. And because after 24 hours the cells divide, I prepared higher dilution of 10^{-6}.

Afterwards, I prepared Petri dishes marked by samples and concentrations. Into each petri dish I gave 0.5 ml of solution from each test-tube described above. I prepared all samples in triplicates. During that I warmed up prepared plate count agar in the microwave to became a liquid solution and let it cool to body temperature. It must not be too hot to avoid cell damage. I poured agar on prepared petri dishes. I gently mixed it and put it in the incubator (Thermostat Incucell, BMT) with temperature set up to approximately 27°C.

After a few days when colonies of bacteria grew up I counted the number of colonies. Only values from 30 to 300 of KTJ on Petri dish were included in results.

3.6 Characterization of nanomaterials

Nanoparticles were characterized using CPS Disc centrifuge and Zeta Sizer.

3.6.1 CPS Disc centrifuge

The CPS Disk centrifuge (model DC24000 UHR, CPS Instruments, UK) is particle size analyzer in the range of 0.005 μm to 75 μm according to density of measured particles, it is shown in *Figure 3.2*. With high density (6 times the density of water) the range is from 10 μm to 0.005 μm. With low density it is from 75 μm to 0.02 μm.

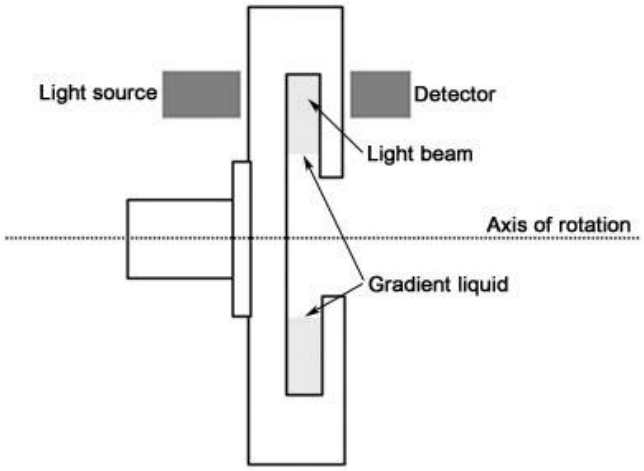


*Figure 3.1: A side cross-sectional of the disc (*CPS Disc Centrifuge *2013)*

The method is based on centrifugal sedimentation in a medium sucrose. Particles are separated by size. The density gradient ensures stability of the sedimentation. The particles gradually sediment due to the rotation. On the edge of rotating disc is a light beam passing through the disc (*Figure 3.1*). When the particles approach the edge, they block the light beam. The change in light intensity is converted into a particle size distribution.

*Figure 3.2: CPS Disk centrifuge (*CPS Disc Centrifuge *2013)*

Measuring procedure

I prepared three samples with different concentration of nanoparticles (0.01 g/l, 0.05 g/l and 0.5 g/l) resuspended it in distilled water and in medium Soya Broth which I used for bacteria cultivation. I checked out the settings and cleanliness of the disc of the centrifuge. I set the rotational speed at 8000 rpm and launched the centrifuge. Then I build up the density gradient using 24% and 8% solution of pure sucrose. The last layer was formed by 0.5 ml of Dodecane to prevent evaporation. I waited 20 minutes for stabiliza-

tion of the density gradient. I chose a procedure and changed density, absorption and refractive index (RI) based on nanoparticle type.

Table 3.1: Density, absorption and refractive index (RI) for different nanoparticles

	nZVI	diamond	magnetite
density [g/ml]	5,24	3,515	5,24
absorption [K]	0,5	1	0,5
refractive index	1,7	2,42	1,7

I checked out the gradient stability with a poly(vinyl chloride) standard 476 (a mean weight particle diameter of 476 nm). Then I started a measurement. I put 0.1 ml of standard and then 0.1 ml of sample. I utilized a mean weight of nanoparticles diameter and the polydispersity index of nanoparticle. In the end of measurement I again checked out the gradient stability and cleaned of the centrifuge.

3.6.2 Zeta Sizer Nano ZS

Zeta Sizer (Nano Series, Malvern Instruments) is used for measuring the size and zeta potential of nanoparticles, is shown in *Figure 3.3*. High power allows the measurement of molecular weight and virial coefficient. Measurable particle size is 0.3 nm - 10 μm (Malvern 2015).

The method is based on Dynamic Light Scattering (DLS) for particle size characterization. Sample is irradiated with laser beam which is scattered and scanned using the detector, which is in specific angle 173°. On particles, it is applied a Brownian motion and converts this to size distribution using the Stokes-Einstein equation.

Figure 3.3: Zeta Sizer Nano ZS (Malvern 2015)

Measuring procedure

I prepared the same suspension of nanoparticles as for CPS Disk centrifuge. I set a Zeta Sizer before measurement. I changed a different refractive index: nZVI – 2.7, nanodiamond – 2.418, magnetite – 2.420 and absorption of nanoparticles 0.01. I chose water as dispersant with viscosity 0.8872 cP, refractive index 1.33 and heating to 25 °C. Equilibration time was 60s. Detection angle was 178° Backscatter (NIBS). I pipetted 200 µl of samples into disposable cuvettes (ZEN0040) and set measurement for 10 runs after 5 s. Zeta Sizer advantage is triplicate measurements simultaneously in contrast with disc centrifuge.

4 Results and discussion

The following chapter summarizes the results of the measurements. All measurements were performed thoroughly. The samples were prepared in triplicates, in order to prevent deviations. The aim was to get acquainted with the issue of determining the toxicity of nanoparticles using prokaryotic microorganism – *Pseudomonas putida* and to compare the methods used.

4.1 Toxicity tests

Toxicity of nanoparticles was studied using two analytical methods (Determination of cell viability and Determination of Reactive Oxygen Species in cells) and results were compared with cultivation of bacteria on agar media. Nanoparticles were characterized using the DCS and DLS methods (CPS Disc centrifuge and Zeta Sizer).

4.1.1 Determination of cell viability

The first method was Determination of live and dead cells. I used fluorescent microscope (AxioImager, ZEISS, Germany) where I took images of the bacterial cells and I counted fluorescently labeled cells.

The *Figure 4.1* shows influence of nanodiamond particle concentration on percentage of living cells of *Pseudomonas putida* . Number 0 marks negative control without any nanoparticles, to compare the behavior of solutions with different particle concentrations. The positive control is not shown in the graph, because all cells were damaged after placed to hot water. Positive control was to confirm the functionality of the method; all affected cells were labeled with red fluorescence.

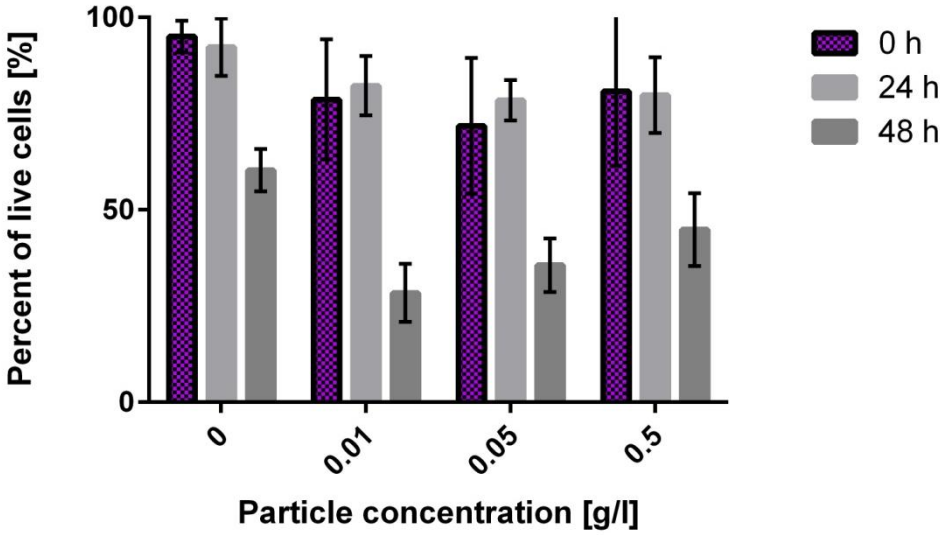


Figure 4.1: Influence of nanodiamond particle concentration on cell viability of Pseudomonas putida. Error bars - SD.

We can see a slight effect with the addition of nanoparticles in comparison with negative control. Nanodiamond concentrations of 0.01 g/l most affected the number of viable cells after 48 hours where is the biggest difference against negative control. After 24 hours there was a large multiplication of cells and *Figure 4.1* shows even higher percentage of viable cells than the first day. But after 48 hours it is much lower, because a lot of the cells died died during incubation even in control samples. It could be caused by rapid growth of *P. putida* culture and exhaustion of nutrients in the samples.

There are rather large error bars in *Figure 4.1*. There could easily occur deviations due to human factor in counting cells, because the bacteria multiply rapidly. The error might arise during a longer time interval between microscopic observations or slower time of taking images as well. The cells were rapidly dying in the fluorescent light, therefore measuring had to be done quickly. However, it was not always possible, for example when cells moved quickly.

The *Figure 4.2* is showing the same experiment, but with different graphical expression: in dependence on exposure time for better demonstration of the cell viability development.

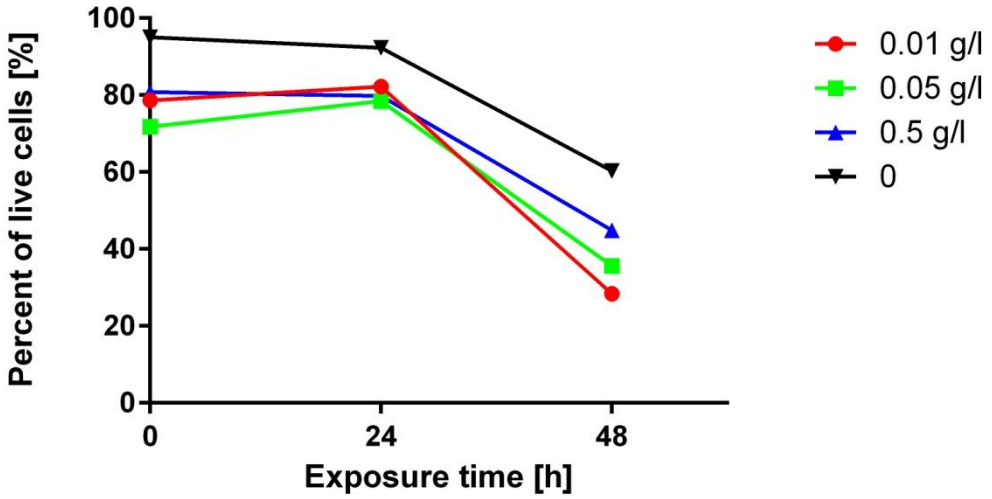


Figure 4.2: Influence of nanodiamond particle concentration on percent of live cells of Pseudomonas putida in dependence on exposure time

The scientists from the University of Bremen identified the strong antibacterial properties of nanodiamonds (Wehling et al., 2014). They stated that nanodiamonds could be used against bacterial contaminations and infections and it is nontoxic for human cells. For the antibacterial effect are responsible oxygen-containing groups on the surface (e.g. acid anhydride). But they worked with a diameter of 5 nm and my nanodiamonds formed aggregates, which can be found in chapter 4.2, it could be reason that there is not higher toxic effect

The results of the experiment with magnetite are shown in *Figure 4.3.* and in *Figure 4.4* where is dependence on exposure time. The first day was the lowest percentage of alive cells in negative control, but it seems more like an incorrect measurement due to large error bars. The second day there was recorded the biggest influence on *P. putida* with magnetite concentration of 0.5 g/l as well as after 48 hours.

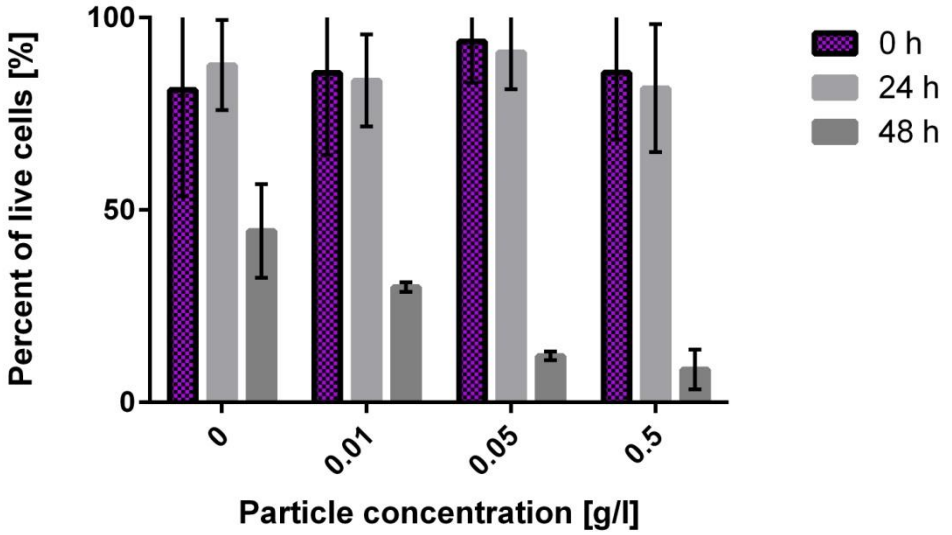


Figure 4.3: Influence of magnetite particle concentration on cell viability of Pseudomonas putida. Error bars - SD.

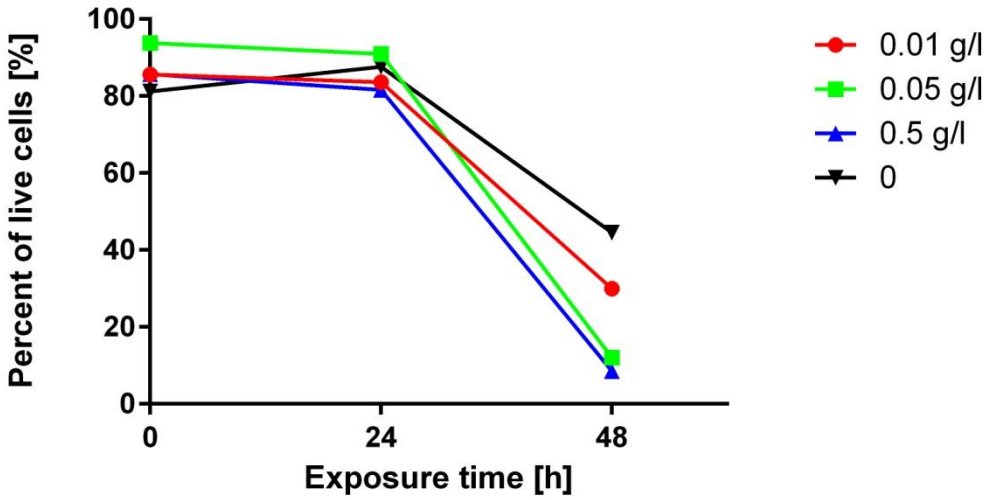


Figure 4.4: Influence of magnetite particle concentration on percent of living cells of Pseudomonas putida in dependence on exposure time

The magnetite nanoparticles showed anti-bacterial activity against some of the bacteria. Their toxicity has not been reported. They were nontoxic for *Pseudomonas stutzeri* (Mirhendi et al., 2013) or *Vibrio fischeri* (Heinlaan et al., 2008).

The results of the experiment with nZVI are pictured on *Figure 4.5*. It could be seen that nanoparticles reacted with cells immediately after addition 0.01 g/l and 0.05 g/l of nZVI. Nevertheless, higher percentages of damaged cells were observed for concentration 0.05 g/l and 0.5 g/l nZVI after 48 hours. On *Figure 4.6* is illustrated the experiment with nZVI in dependence on exposure time for clearer comparison of the time dependence of a variation in concentrations of nanoparticles.

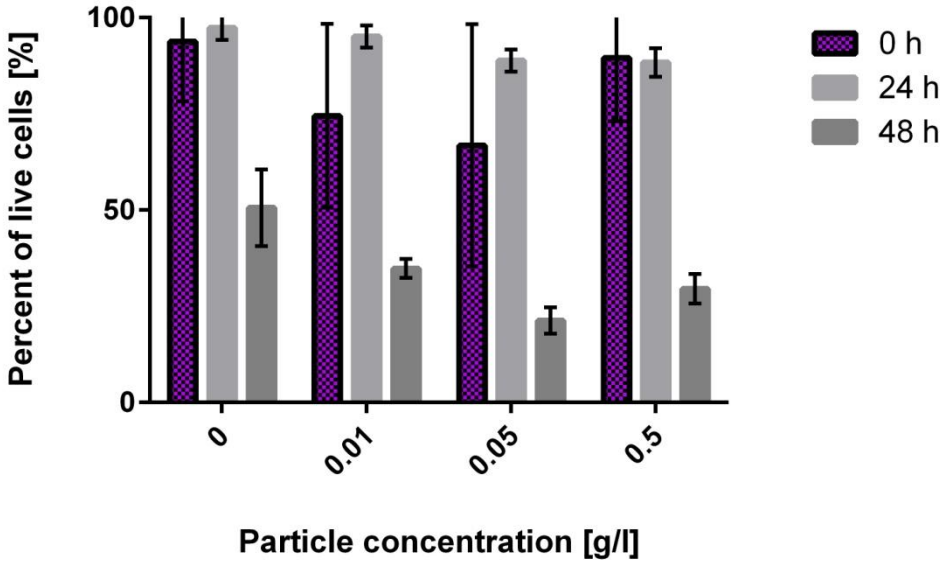


Figure 4.5: Influence of nZVI particle concentration on cell viability of Pseudomonas putida. Error bars - SD.

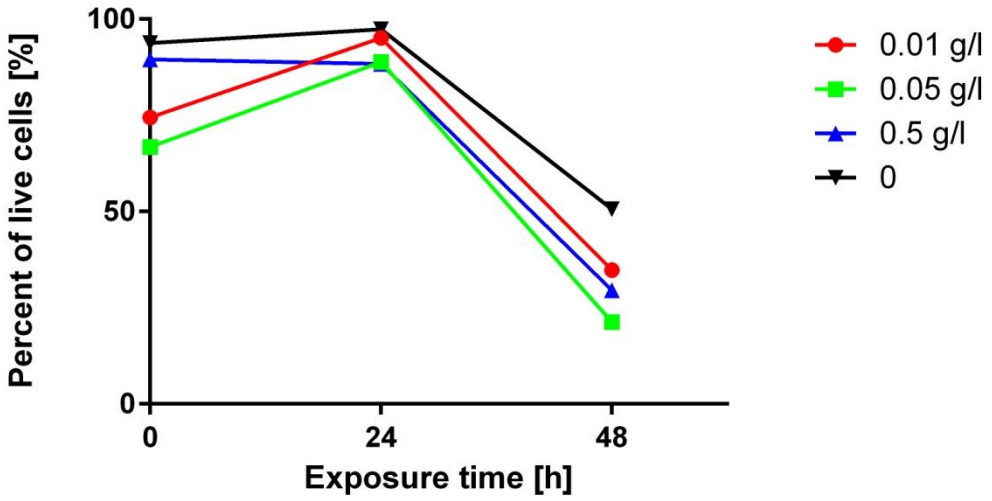


Figure 4.6: Influence of nZVI particle concentration on percent of live cells of Pseudomonas putida in dependence on exposure time

The zero-valent iron (nZVI) is used in various fields of environmental contamination. Its toxicity depends on the composition, concentration, size and surface properties (Jang et al., 2014), (Ševců et al., 2011). The high reduction potential of nZVI can cause toxicity to bacteria. For example the negative charge of *Bacillus subtilis* cell surface relieved to reduced toxicity. In comparison with *Escherichia coli* which gram-positive cell wall confers protection (Chen et al., 2010).

In all three experiments with different nanoparticles we can see their influence mainly after 48 hours of exposure. In shorter exposure times the impact varied only slightly. The method is not completely accurate, because of the higher measurement errors caused by manual cell counting, but this could be overcome by more experienced person.

The membrane damages due to visible light could cause that some viable cells might be counted as dead. This method could give confusing results which is in accordance with findings of Ivanova et al. who had the same problem with determination of *Pseudomonas Putida* live cells at Sofia University (Ivanova at al., 2014).

Examples of fluorescently labeled bacteria are shown in following *Figure 4.7, Figure 4.8* and *Figure 4.9*

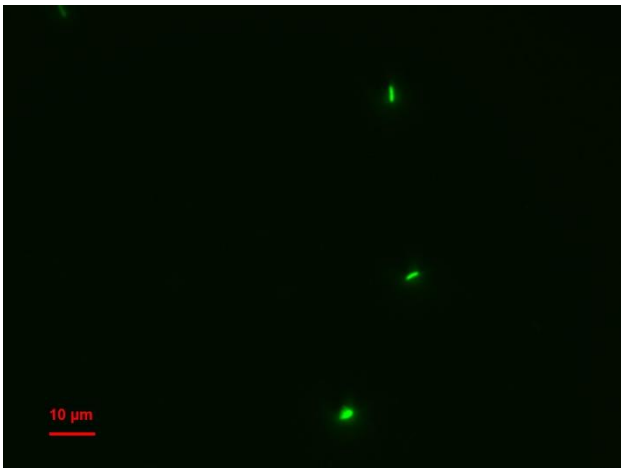


Figure 4.7: Pseudomonas putida exposed to 0,05 g/l nZVI – 0 hours. Healthy cells are labeled by green fluorescence.

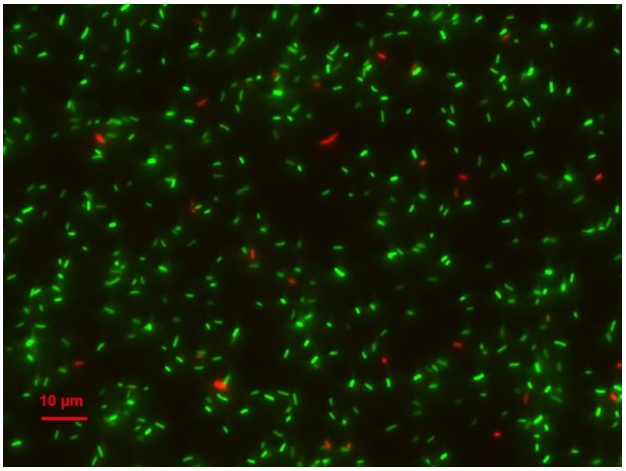


Figure 4.8: Pseudomonas putida with 0,05 g/l nZVI – 24 hours. Healthy cells are labeled by green fluorescence. Damaged cells are labeled by red fluorescence.

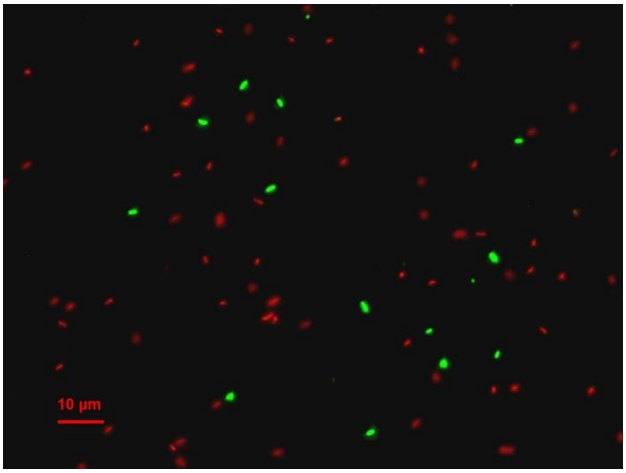


Figure 4.9: Pseudomonas putida with 0,05 g/l nZVI – 48 hours. Healthy cells are labeled by green fluorescence. Damaged cells are labeled by red fluorescence.

4.1.2 Determination of Reactive Oxygen Species (ROS) in cells

The second method was determination of reactive oxygen species in cells exposed to nanoparticles using the fluorescence microscope (AxioImager, ZEISS, Germany).

All graphs show the percentage of labeled cells that means the cells of increased concentrations of ROS. The experiments were carried out 3 days and samples taken at time zero, after 24 and 48 hours, but after 48 hours the data were immeasurable due to high number of cells. The positive control was the maximum number of labeled cells. The negative control was sample without nanoparticles; it should be almost zero percent of labeled cells, which did not work as you can see in graphs *(Figure 4.10 – 4.11)*.

The *Figure 4.100* shows results of testing the influence of nanodiamond on production of reactive oxygen species in *Pseudomonas putida*. The first day the lowest influence had the concentration 0.05 g/l. The second day there was low influence of nanodiamond and similar intensity for each concentration. The negative control had high percentage of affected cells, the reason could be longer exposure time for effect of staining than it should be according to instructions.

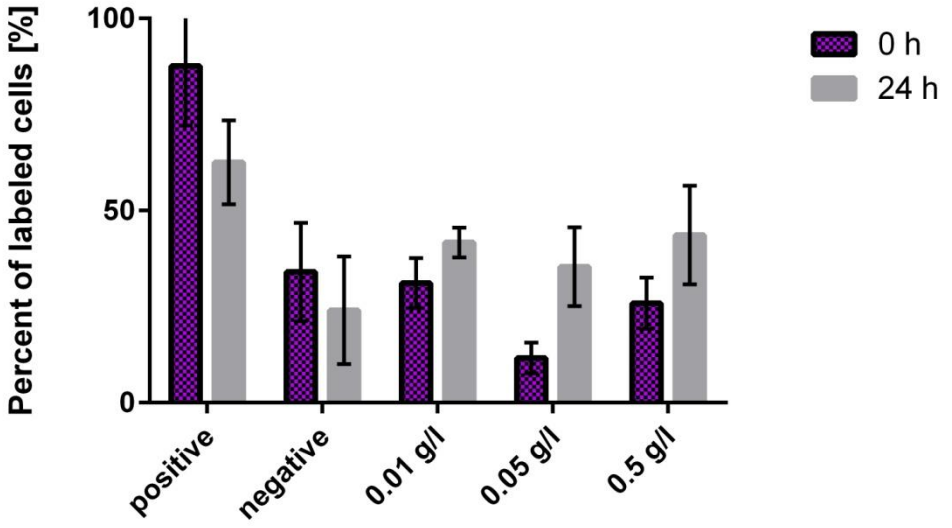


Figure 4.10: Influence of nanodiamond particle concentration on production of reactive oxygen species in bacteria Pseudomonas putida. Positive – cells with induced ROS, Negative –cells without nanoparticles. Error bars - SD.

Test with magnetite nanoparticle showed big formation of reactive oxygen species for concentration 0.01 g/l and 0.05 g/l in range 0-2 hours. After 24 hours, there is again the same effect as with nanodiamond. The results of the experiment are shown in the *Figure 4.111*.

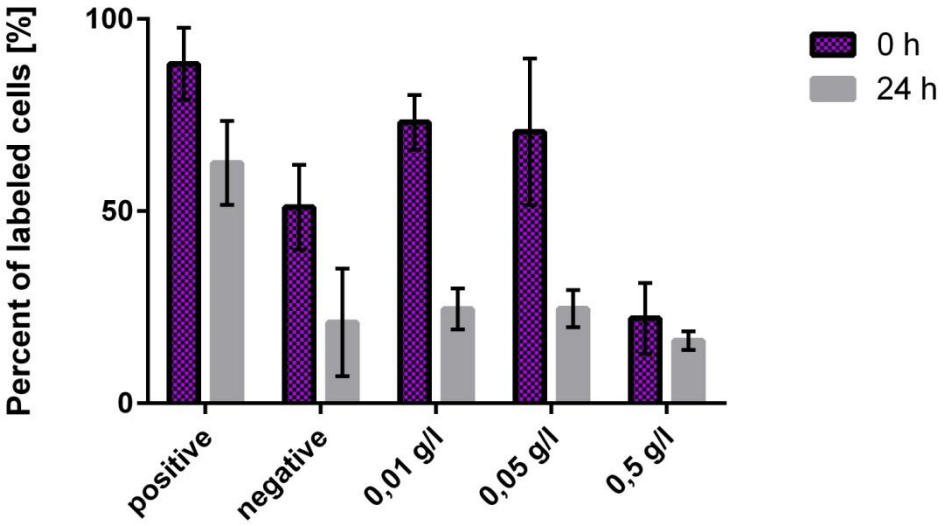


Figure 4.11: Influence of magnetite particle concentration on production of reactive oxygen species in bacteria Pseudomonas putida. Positive – cells with induced ROS, Negative –cells without nanoparticles. Error bars - SD.

The magnetite nanoparticles showed some negative effect on *S. aureus* (Darwish et al., 2015). Gram-positive bacteria can form a biofilm to protect from stressful conditions. Nevertheless, the magnetite nanoparticles can penetrate the biofilms and generate reactive oxidation species.

The results from the experiment with nZVI are shown in *Figure 4.122*Figure 4.1. The first day the highest concentration 0.5 g/l of nZVI caused increased production of free radicals. The percentage of labeled cells was similar to negative control after 24 hours. It could mean that the nanoparticles do not affect the bacteria due to aggregation or oxidation after 24 hours. On the other hand, the nZVI clearly affected the cells in the beginning of the experiment which could be due to its higher initial reactivity and quick action. Moreover, the cells can employ defensive strategies, such as production of antioxidant enzymes. It might be interesting to test the impact on the cells in shorter intervals to see the development of the effect.

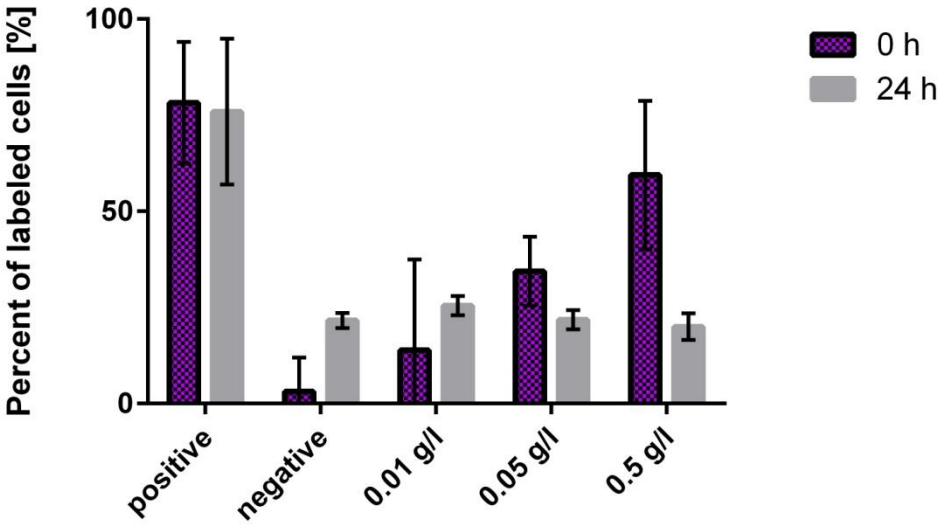


Figure 4.12: Influence of nZVI particle concentration on production of reactive oxygen species in bacteria Pseudomonas putida. Positive – cells with induced ROS, Negative –cells without nanoparticles. Error bars - SD.

This method had inaccurate and too variable results and in my opinion it is not very informative. In comparison with determination of cell viability is ROS detection unclear. In most measurements the negative control gave too high numbers. This could be due to wrong preparation of the sample or a longer staining procedure, because I let the dye have an effect for longer period than was recommended by producer.

Determination of Reactive Oxygen Species (ROS) in bacteria can be used to determine size-dependent inhibition by Ag nanoparticles in the presence and absence of the bacteria (Choi et al., 2008).

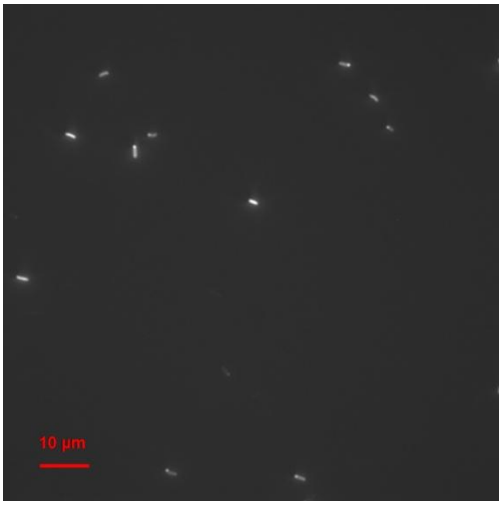
The example images of the *P. putida* cells are in the *Figure 4.13, Figure 4.14, Figure 4.15 and* 

Figure 4.16

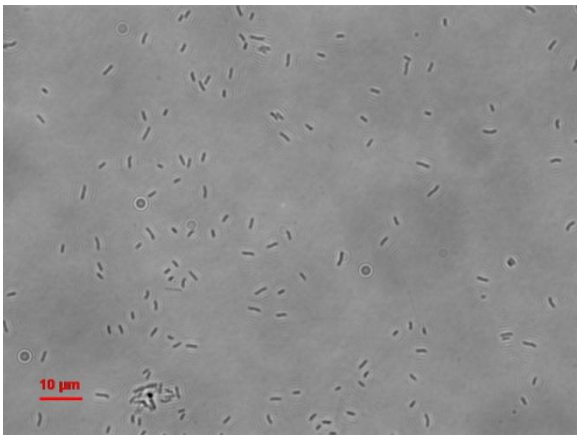


Figure 4.13: The positive control of nanodiamond after 24 hours. Image from light microscopy.

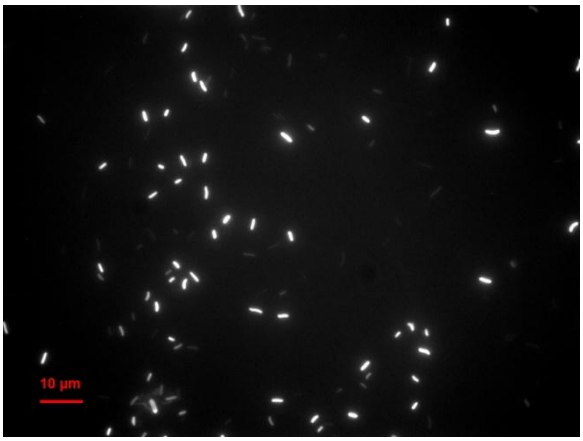


Figure 4.14: The positive control of nanodiamond after 24 hours. Labeled cells are brighter than non-labeled cells.

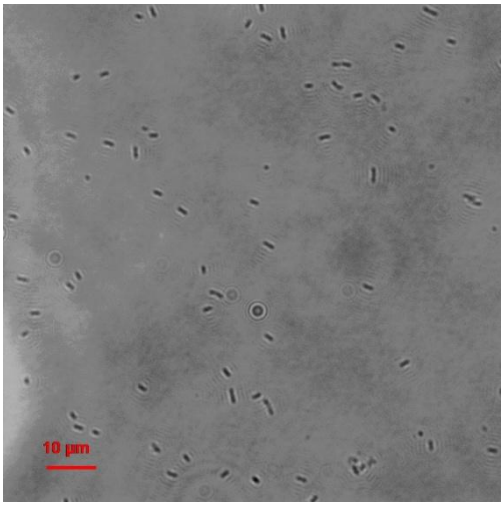


Figure 4.15: nZVI with concentration of 0.05 g/l after 24 hours

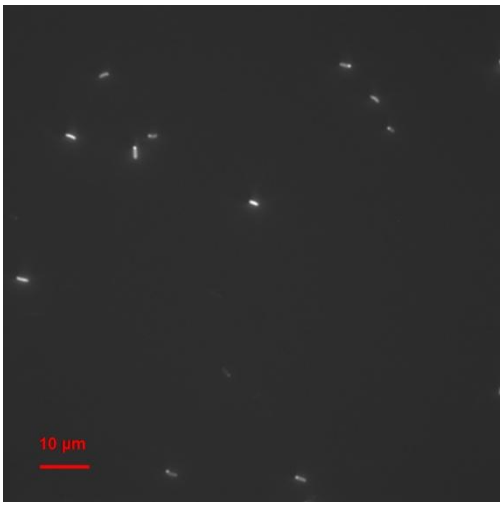


Figure 4.16: nZVI with concentration of 0.05 g/l after 24 hours

4.1.3 Cultivation of bacteria on agar media

The last method for testing of toxicity of nanoparticles was the cultivation of bacteria P. putida on agar medium. This method was chosen for comparison of the previous two analytical methods.

In *Figure 4.17* are the number of colony forming unit (CFU) detected in 1 ml in dependence on the concentration of nanodiamond. The zero is again control without nanoparticles. The graph is showing that nanodiamond in concentration of 0.05 g/l had little toxicity effect on the bacteria, whereas the CFU of bacteria almost multiplied in concentration 0.5 g/l of nanodiamond.

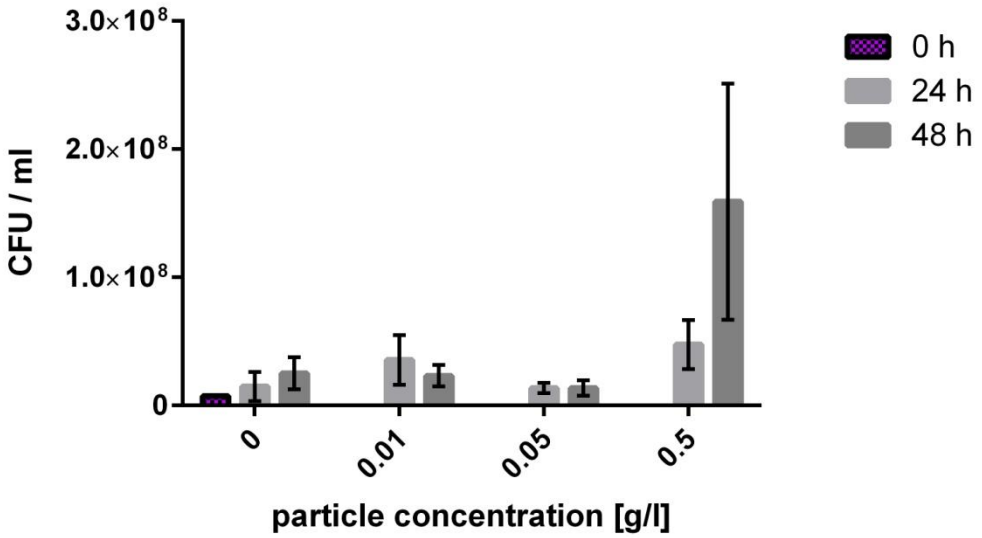


Figure 4.17: CFU dependence on the concentration of nanodiamond after 24 and 48-hour exposure. Error bars - SD.

Magnetite caused some toxicity effect when the nanoparticle concentration was 0.01 g/l (*Figure 4.18*). Concentration of 0.5 g/l caused the same increase of colonies like nanodiamond .

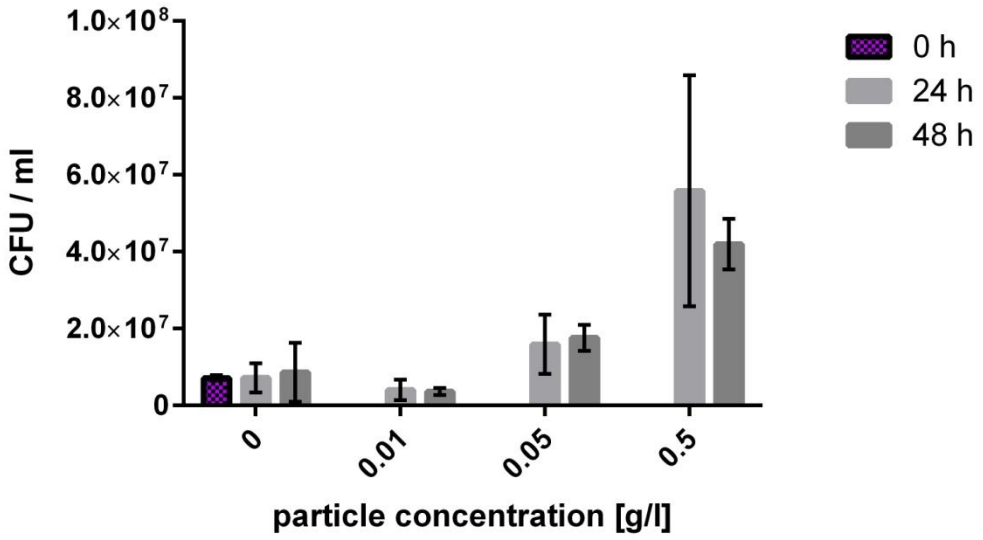


Figure 4.18: CFU dependence on the concentration of magnetite after 24 and 48-hour exposure. Error bars - SD.

In *Figure 4.19* is shown different effect. Number of CFU was growing after 24-hour exposure, but after 48 hours it sharply decreased.

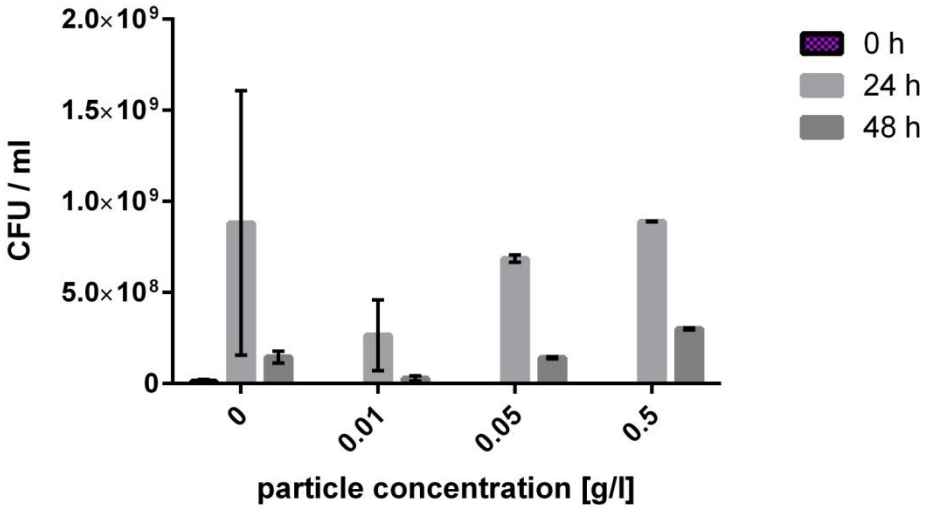


Figure 4.19: CFU dependence on the concentration of nZVI after 24 and 48-hour exposure. Error bars - SD.

Cultivation of bacteria on agar media gives us different results than determination of cell viability. Some cells had a color between green and red in fluorescence microscope, which could be a problem. It could be supposed, that fast growing cells have higher membrane permeability to live/dead staining which enter into the dividing cells and stain them red. Same problem was recorded with zinc oxide and *Pseudomonas putida* (Ivanova et al., 2014).

The following photo was taken during measurement (*Figure 4.20*). We can see the differences of CFU numbers in different dilution of the samples.

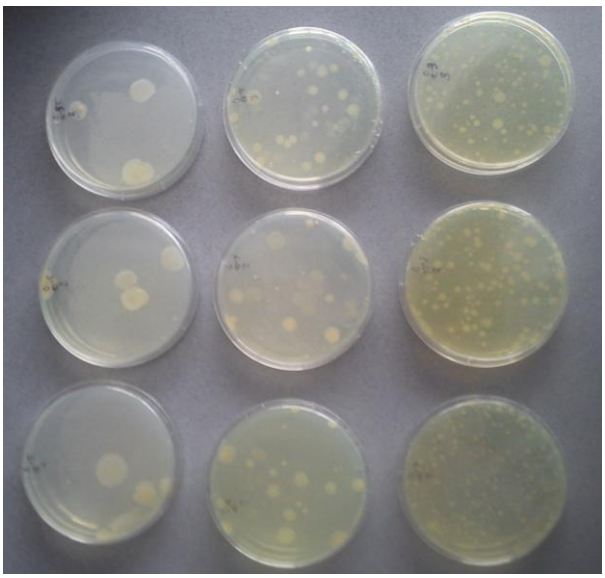


Figure 4.20: Examples of CFU on agar Petri dishes. There are triplicates of samples for control without nanoparticles after 0-2 hours, on the left is the highest dilution and on the right id the lowest dilution.

In my opinion, the most useful method for assessing the toxicity is determination of cell viability or cultivation of bacterial cells on agar media. However, we must be aware that much more measurements have to be done for reducing deviations caused by counting.

4.2 Characterization of nanoparticles

The used nanoparticles were characterized by Disc Centrifuge and Zeta Sizer. The results are shown in graphs in the

Apendix 1 (CPS Disc centrifuge) and *Apendix B* (Zeta sizer).The measurements were made in two environments: deionized water (DI) and bacterial growth medium Soya Broth. The data are processed from triplicates and the graphs contain standard deviations.

Characterization of nanomaterialsI had to do in order to see if the particles do not form aggregates or agglomerates and thus have different behavior and toxicity. Used medium may also have a different effect.

4.2.1 CPS Disc centrifuge

I measured a mean weight and polydispersity index (graphs in the

Apendix 1). Mean weight particle expects that particles are of spherical shape with known density. The measured particle has the same weight as the diameter of the sphere. The polydispersity index is influenced by broad size distribution.

The disc centrifuge hardly analyzed the lowest nanoparticle concentration of 0.01 g/l. For nanodiamond was the mean weight the same for both environments. But nZVI according to *Table 4.1* formed aggregates which could influence the toxicity. And, notably, magnetite nanoparticles had lower mean weight in soya broth than in DI water.

Table 4.1: Mean weight particle size distribution for different nanoparticles (0.5 g/l) in DI water or Soya Broth

Nanoparticles	**Environment**	**Mean weight [nm]**
Nanodiamond	DI water	(560 ± 7)
	Soya Broth	(570 ± 60)
Magnetite	DI water	(650 ± 20)
	Soya Broth	(570 ± 50)
nZVI	DI water	(810 ± 70)
	Soya Broth	(950 ± 50)

4.2.2 Zeta sizer

I recorded Z-average size and polydispersity index by Zeta Sizer. The *Table 4.2* shows that nanodiamond and nZVI aggregated in Soya Broth. In contrast to the magnetite nanoparticles which do not differ so much in water and Soya Broth.

Table 4.2: Z-average size distribution for different nanoparticles (0.5 g/l) in DI water or Soya Broth

NPs	**Environment**	**Z-average size [nm]**
Nanodiamond	DI water	(490 ± 20)
	Soya Broth	(1200 ± 300)
Magnetite	DI water	(1120 ± 40)
	Soya Broth	(1000 ± 100)
nZVI	DI water	(490 ± 20)
	Soya Broth	(3800 ± 700)

The data are influenced by the polydispersity index, when index is higher than 0.7, the sample has a wide size distribution. The results fluctuate more than for disc centrifuge. It could be due a bad setting of the instrument.

6 Conclusion

In this thesis I studied the toxicity of nanoparticles using prokaryotic microorganism. The thesis includes basic information about the toxicity of nanoparticles and description for various methods of measurement. This bachelor thesis also provides information about characterization of nanoparticles. The results are processed in chapter 4. For better results, I would recommend more repetition. The used nanoparticles seemed to be environmentally friendly.

Used methods provided us the results with a similar trend differing by displacements. For example nZVI by the determination of cell viability showed decreased number of viable cells after 48 hours. Also for cultivation of bacteria on agar media we got same results with reducing the amount of colonies. Determination of Reactive Oxygen Species in cells gives fluctuating values for nZVI for the first and second day.

In my opinion the thesis reached all goals and demonstrated the new results in toxicology and characterization of nanoparticles.

7 References

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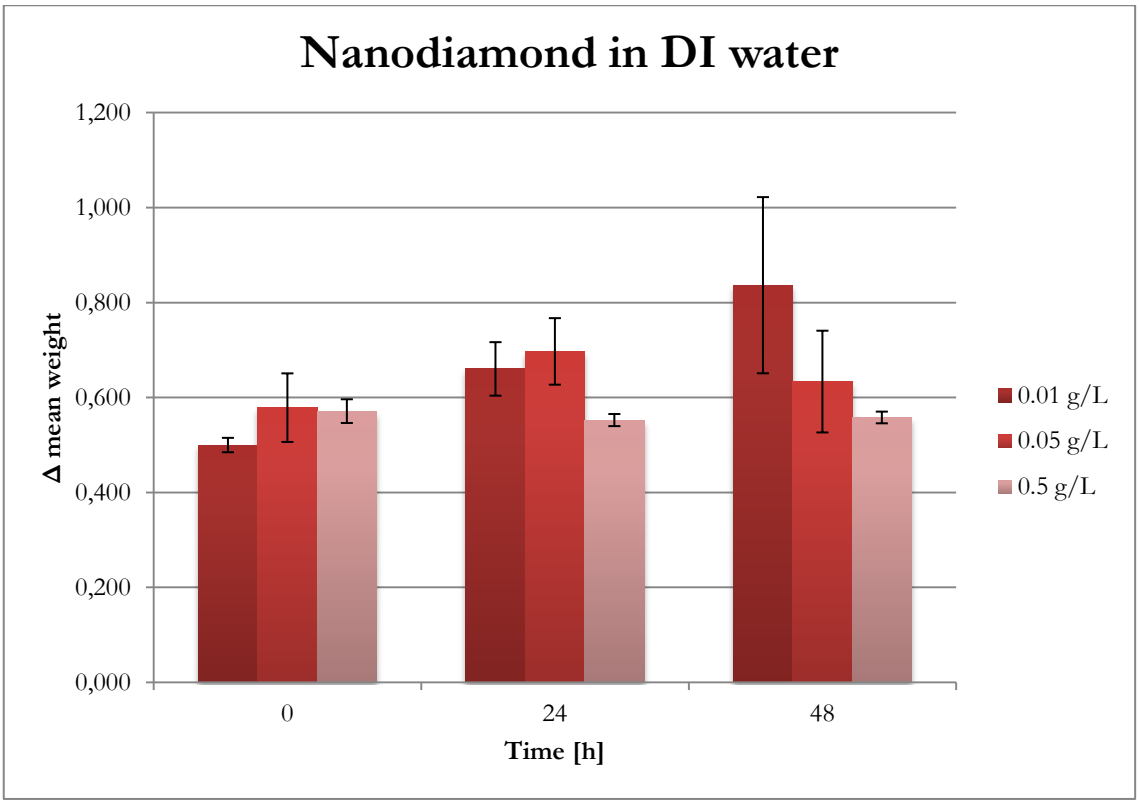
Apendix 1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1,000
0,800
0,600
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0,000
Δ mean weight
0
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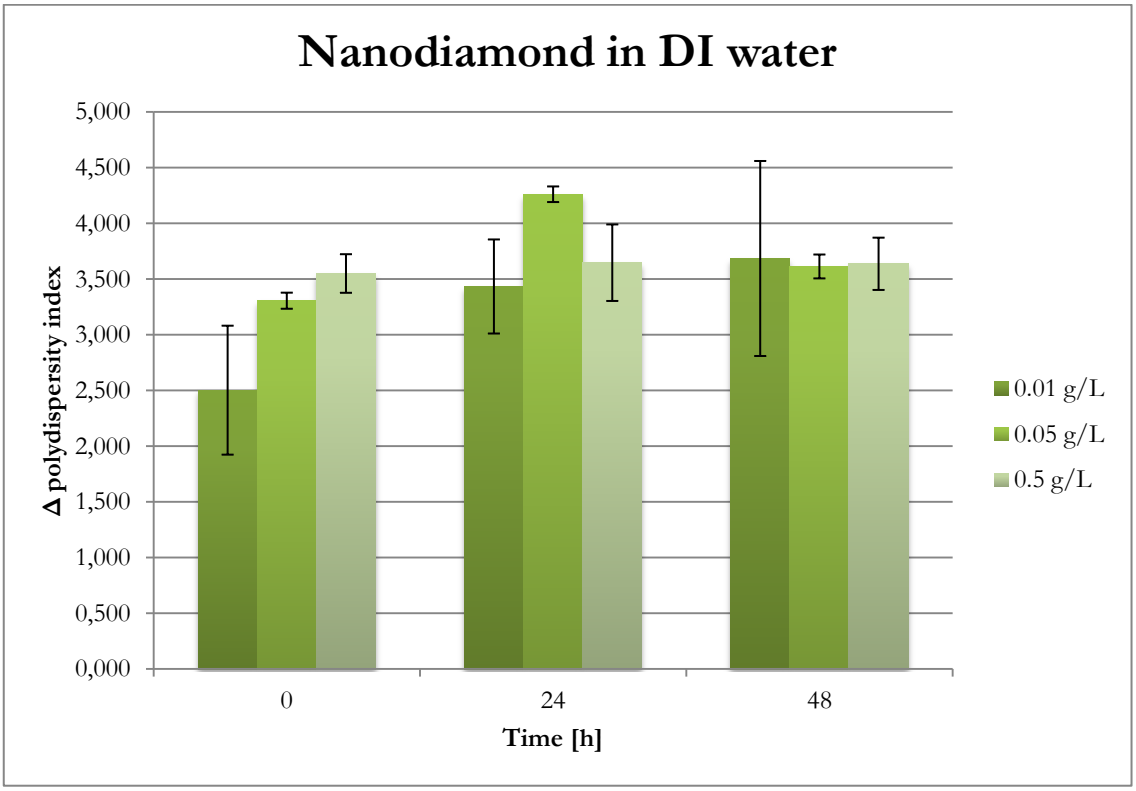
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L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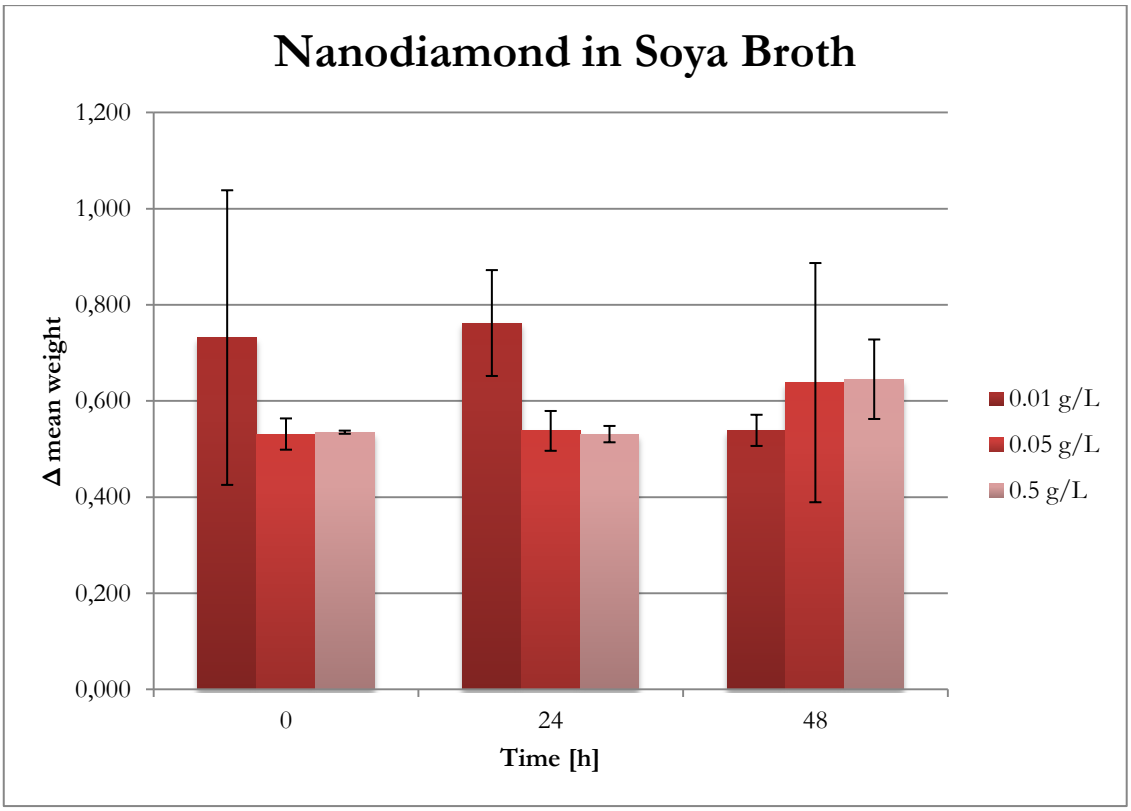
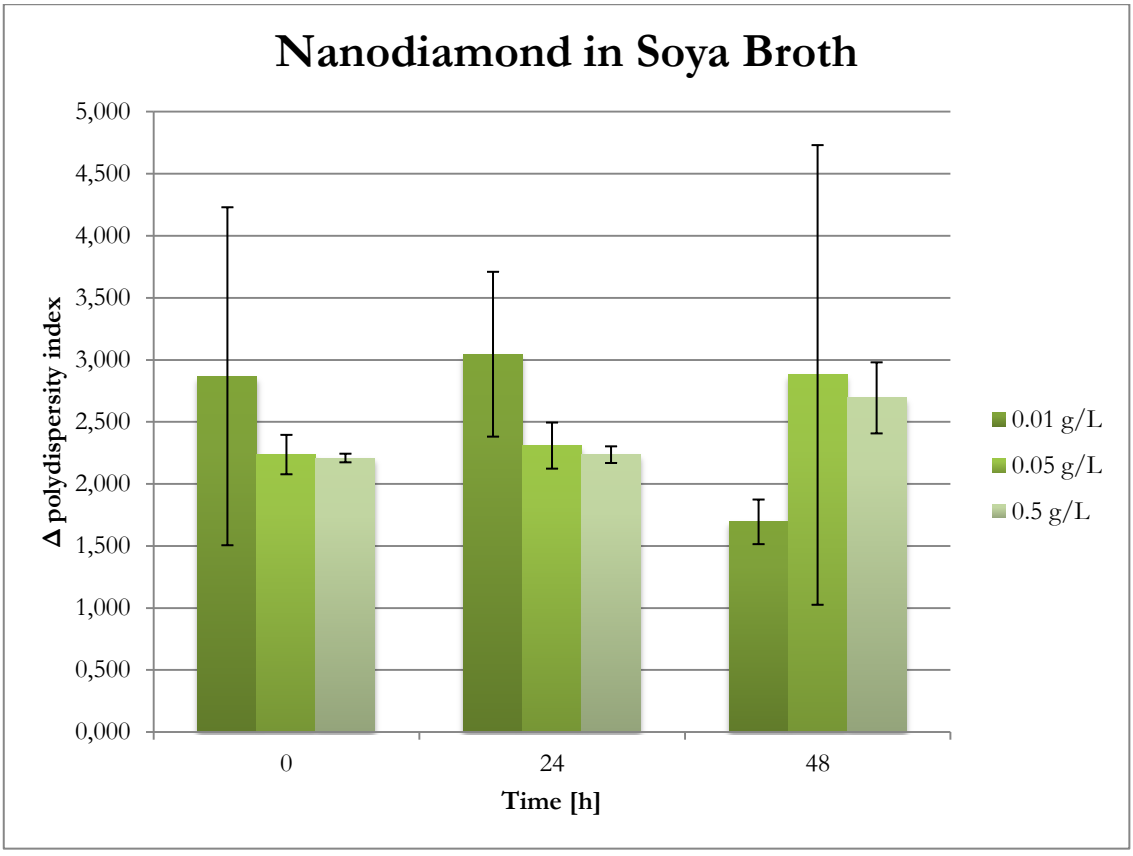
5,000
4,500
4,000
3,500
3,000
2,500
2,000
1,500
1,000
0,500
0,000
Δ polydispersity index
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L


Nanodiamond in Soya Broth
1,200
1,000
0,800
0,600
0,400
0,200
0,000
Δ mean weight
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L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4,500
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3,000
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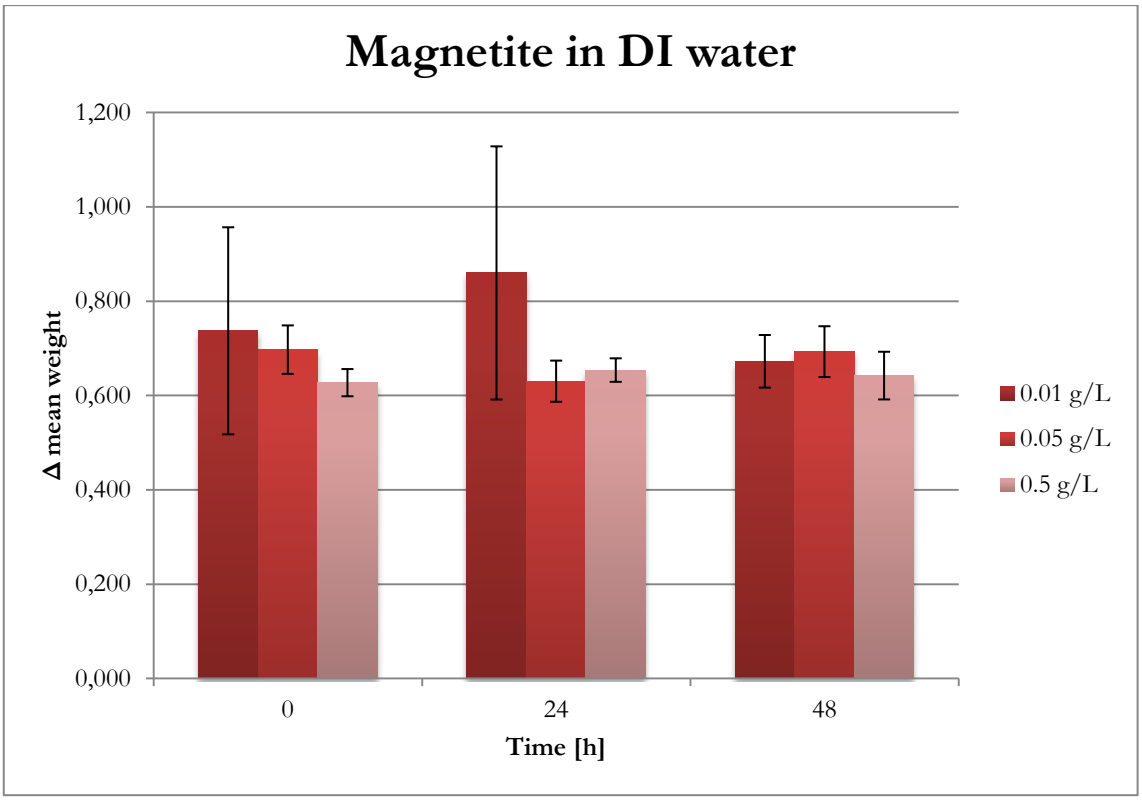
0,000
Δ polydispersity index
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L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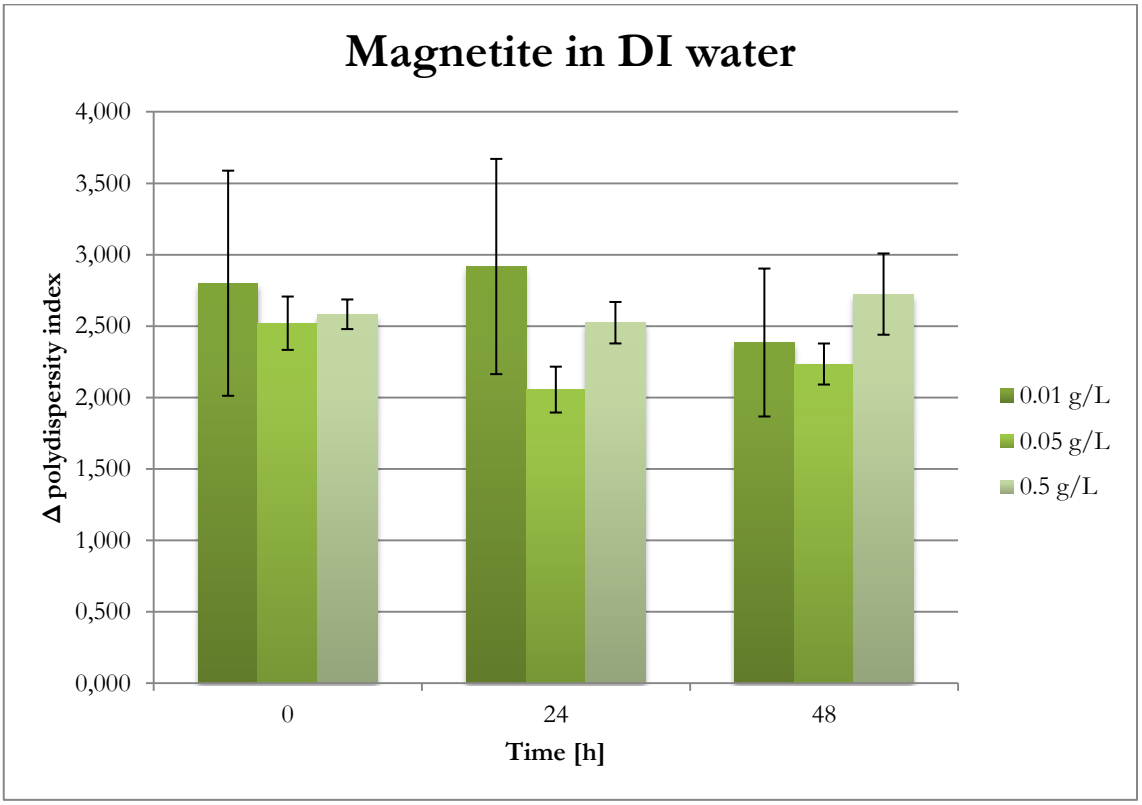
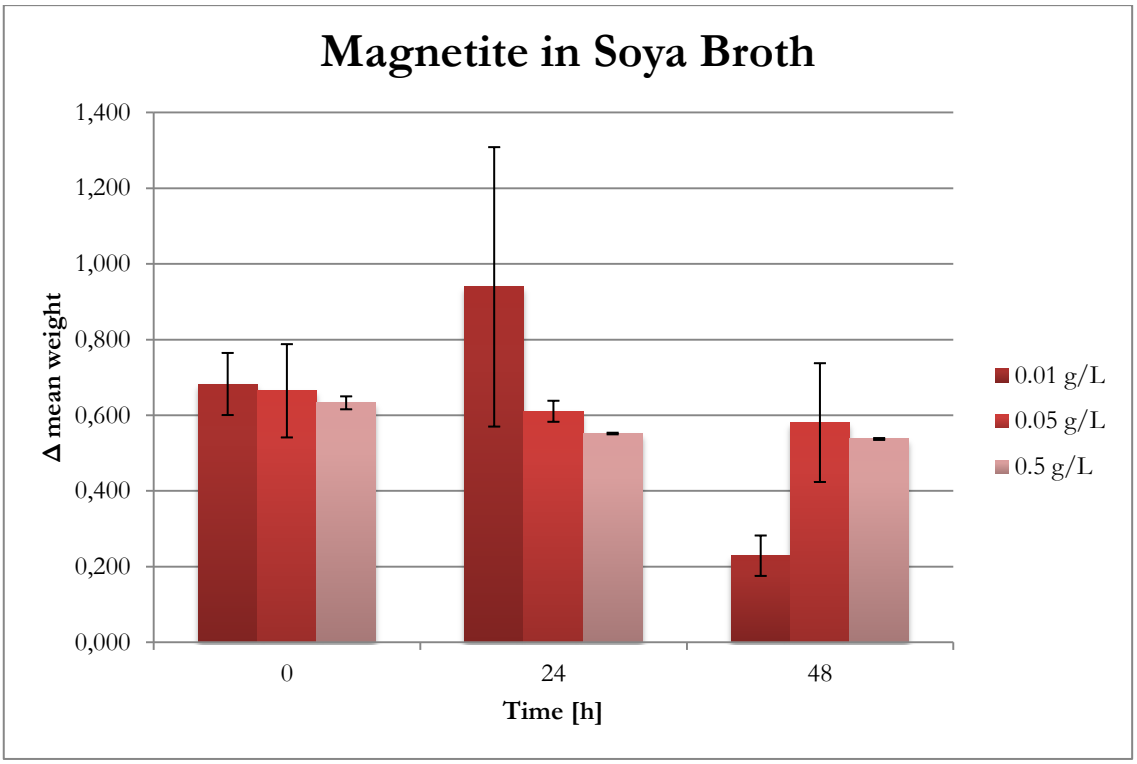
0,000
Δ mean weight
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L


Magnetite in DI water
4,000
3,500
3,000
2,500
2,000
1,500
1,000
0,500
0,000
Δ polydispersity index
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L


Magnetite in Soya Broth
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0,800
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0,000
Δ mean weight
0
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48
Time [h]
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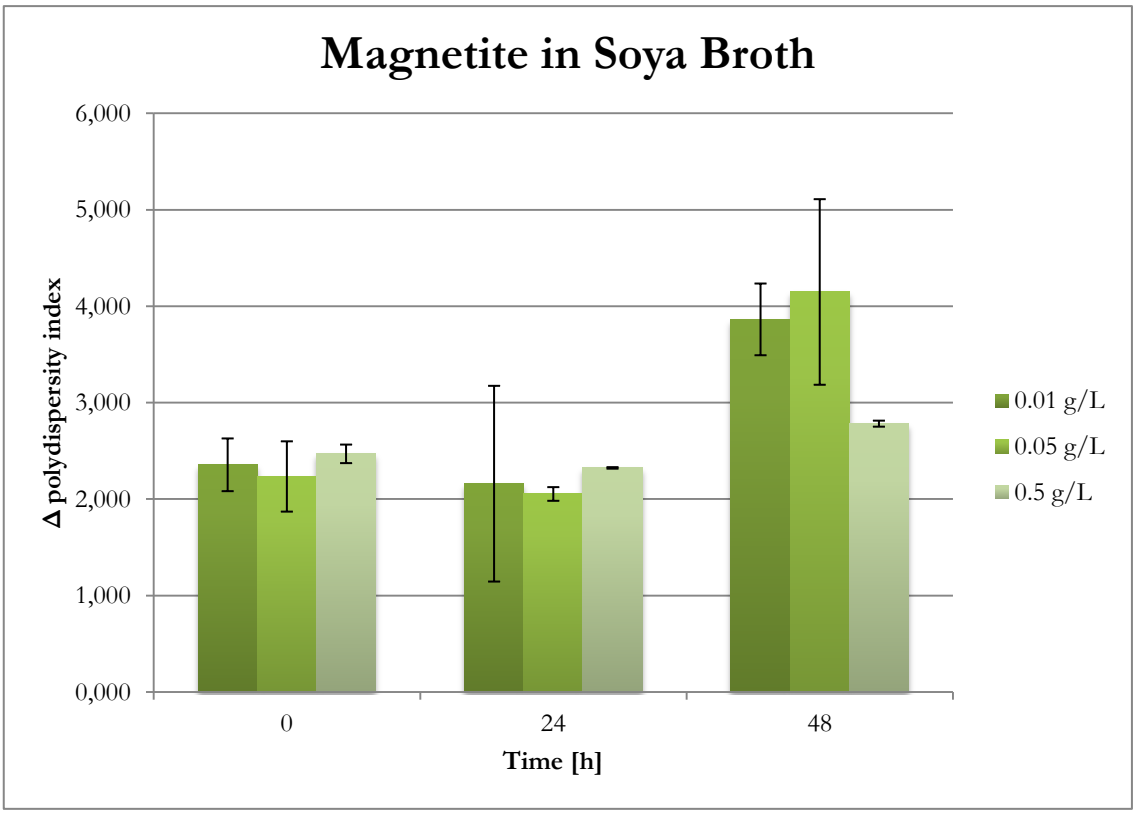
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4,000
3,000
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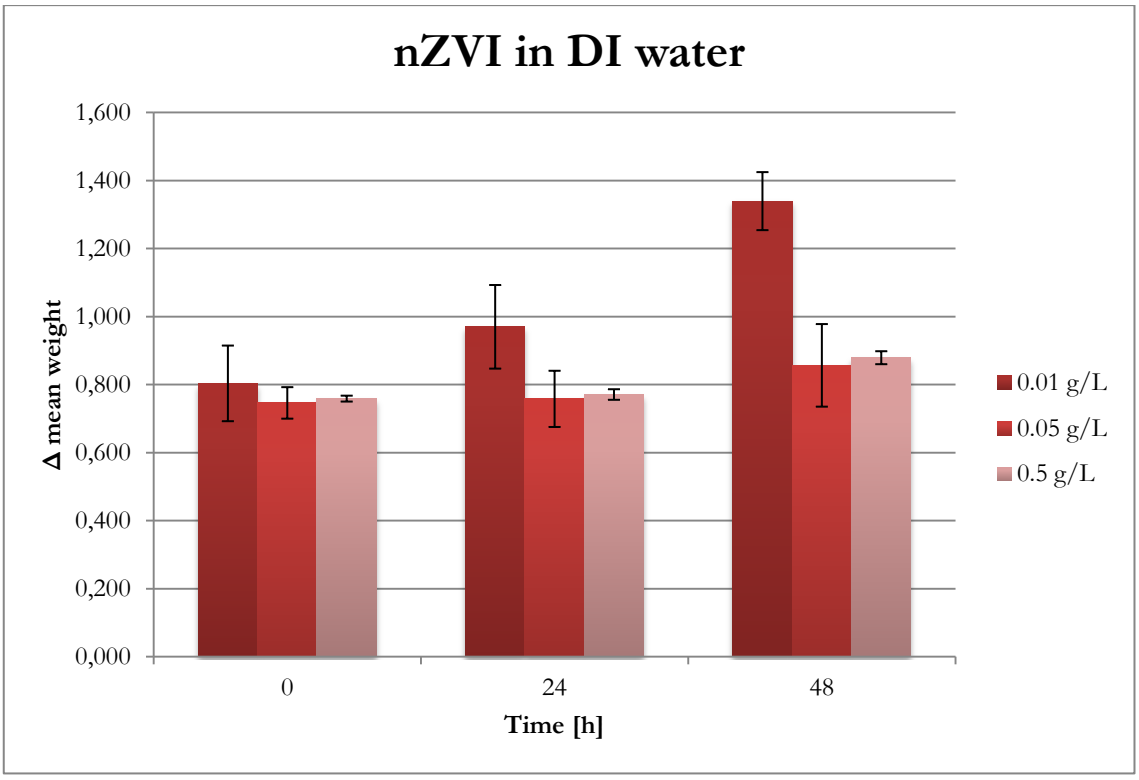
Δ polydispersity index
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L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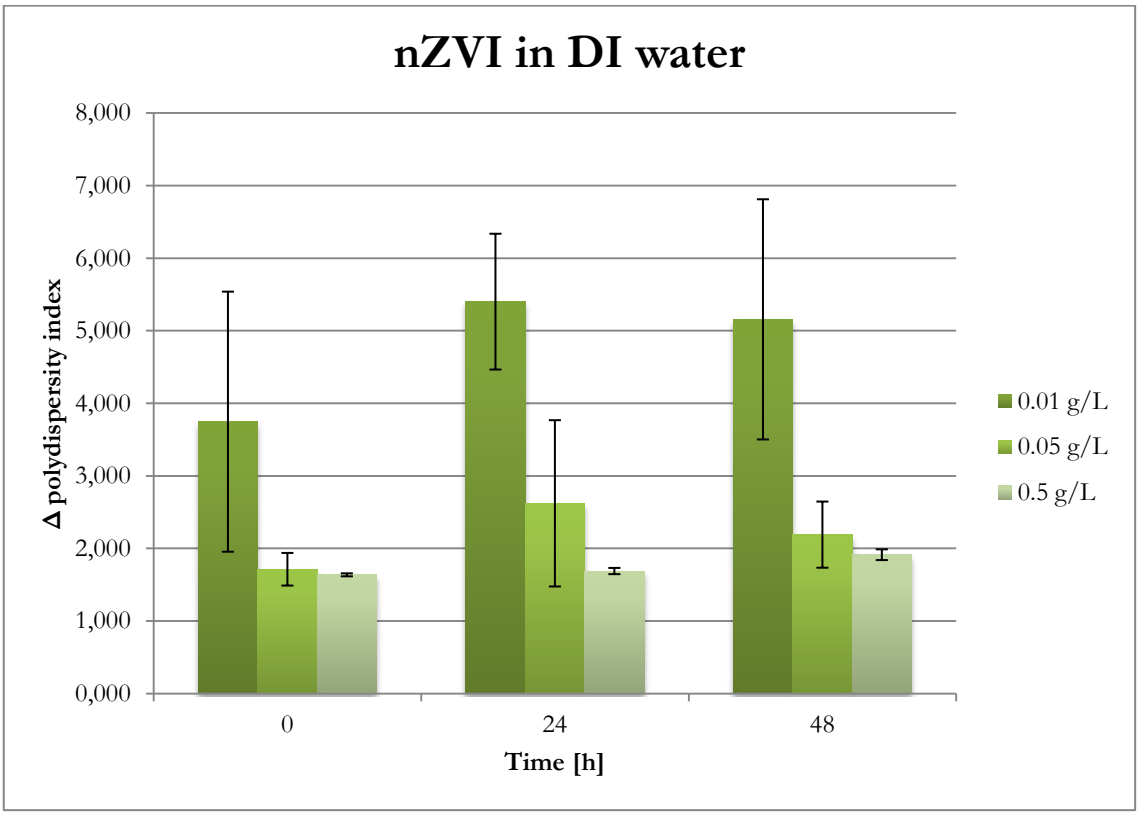
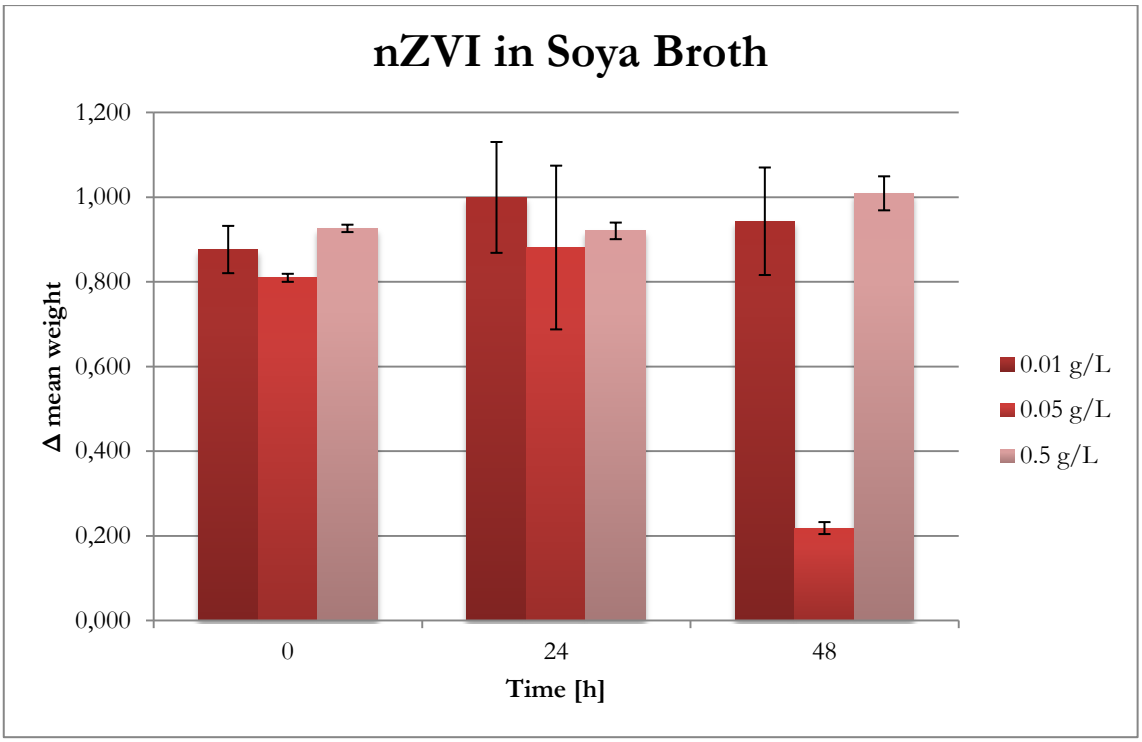
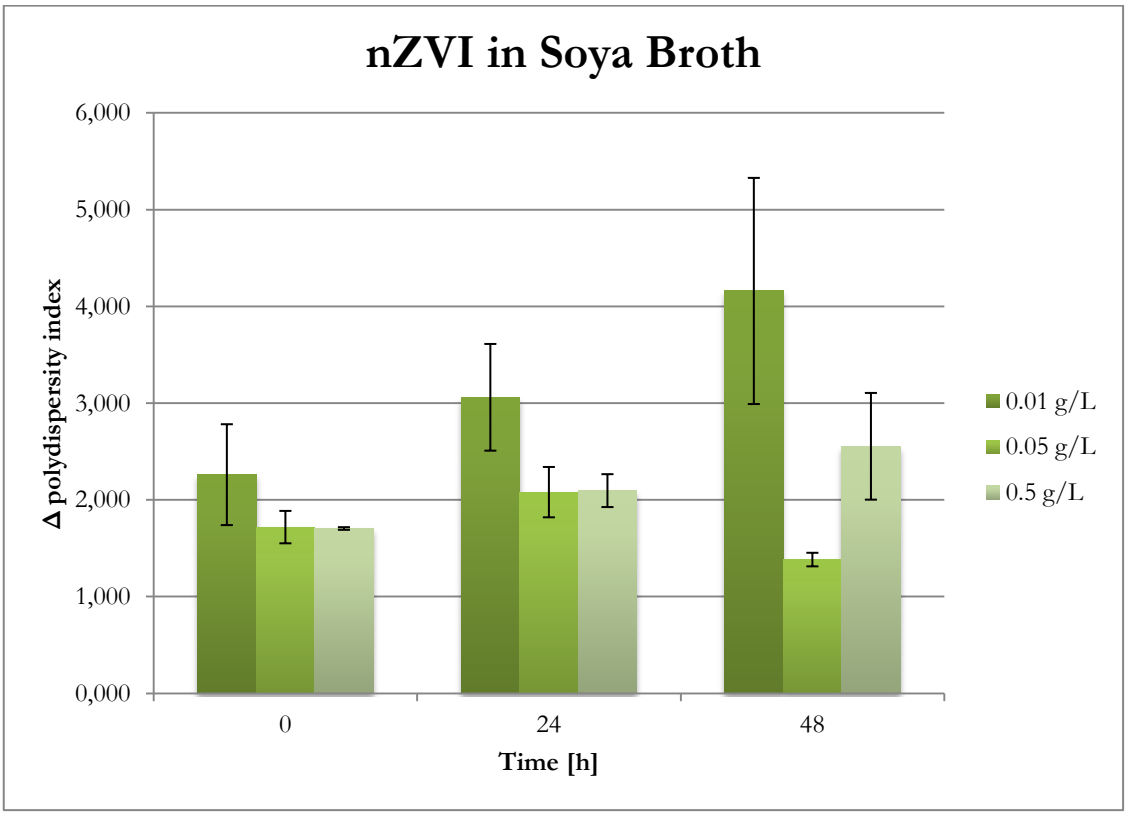
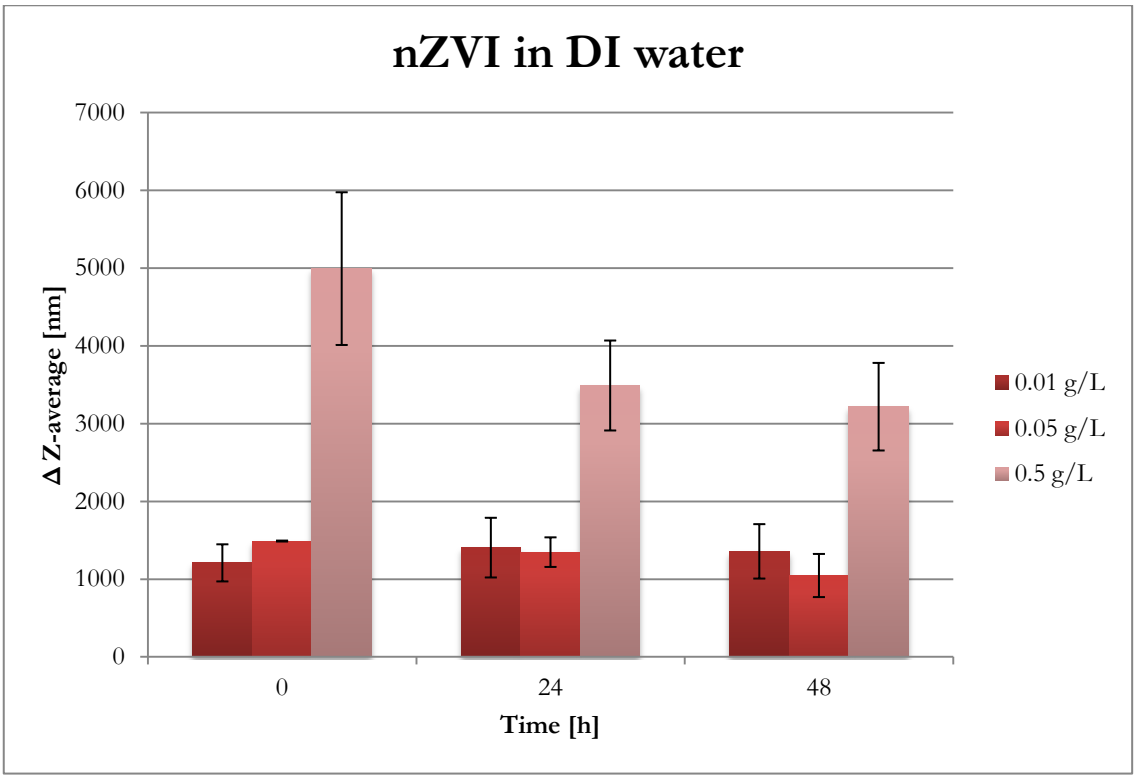
1,400
1,200
1,000
0,800
0,600
0,400
0,200
0,000
Δ mean weight
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

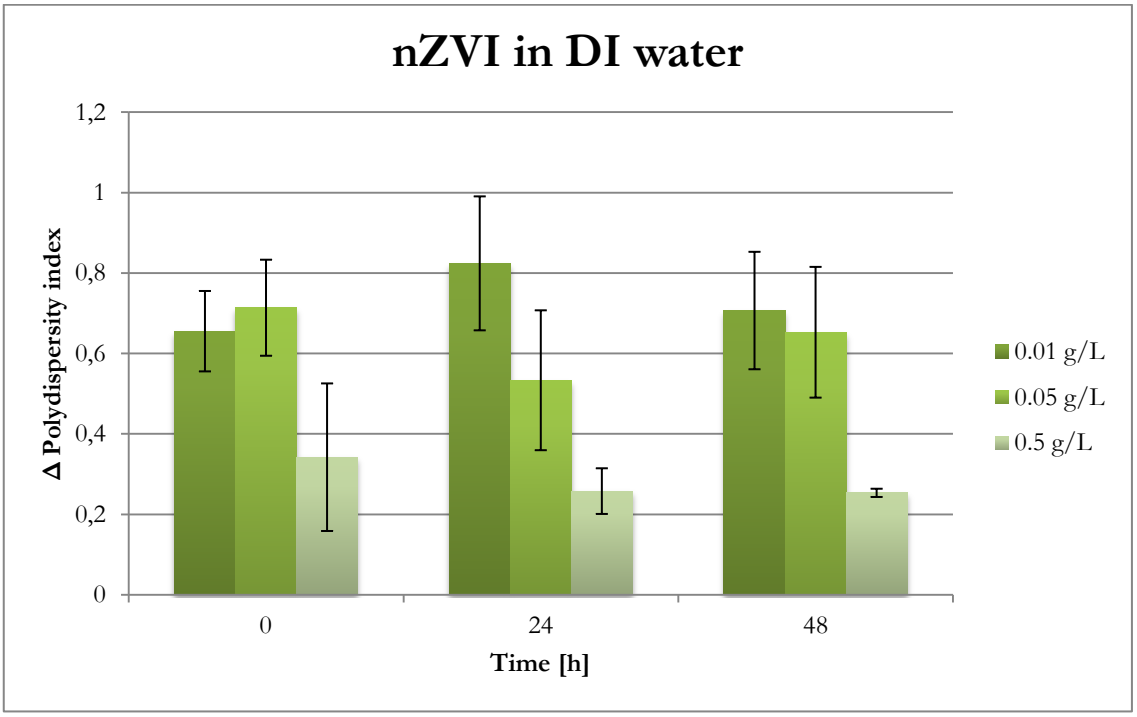

nZVI in DI water
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0,000
Δ polydispersity index
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

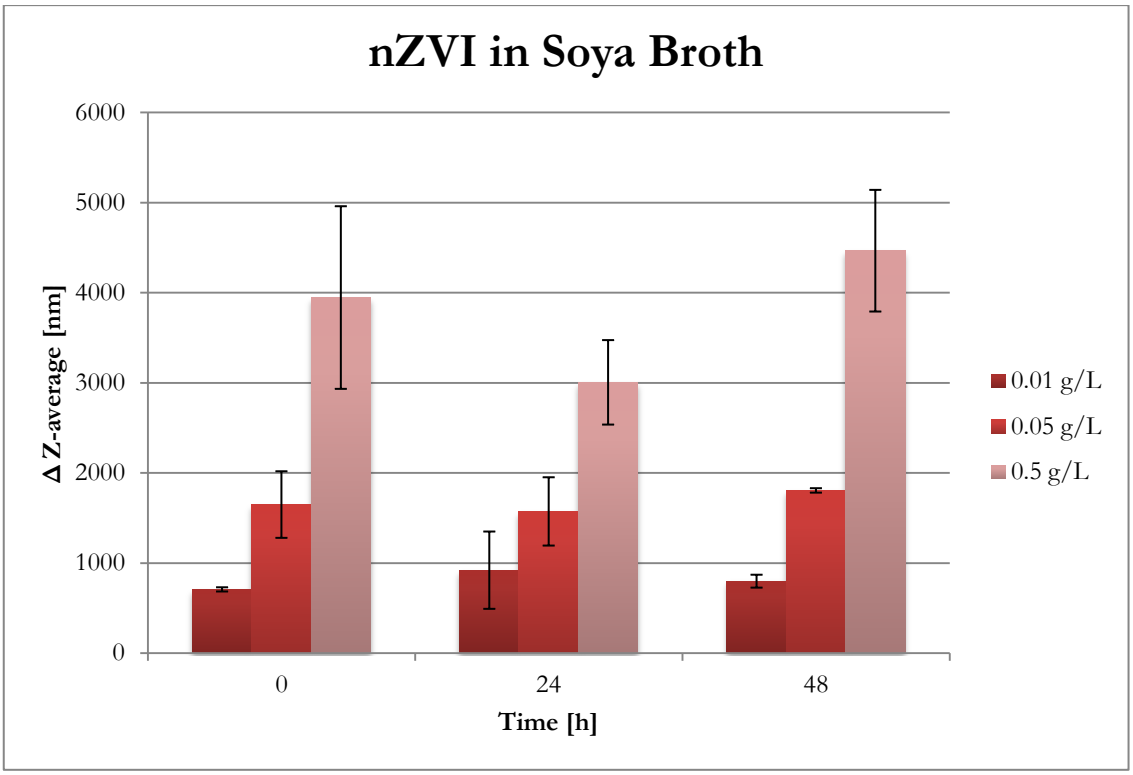

nZVI in Soya Broth
1,200
1,000
0,800
0,600
0,400
0,200
0,000
Δ mean weight
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

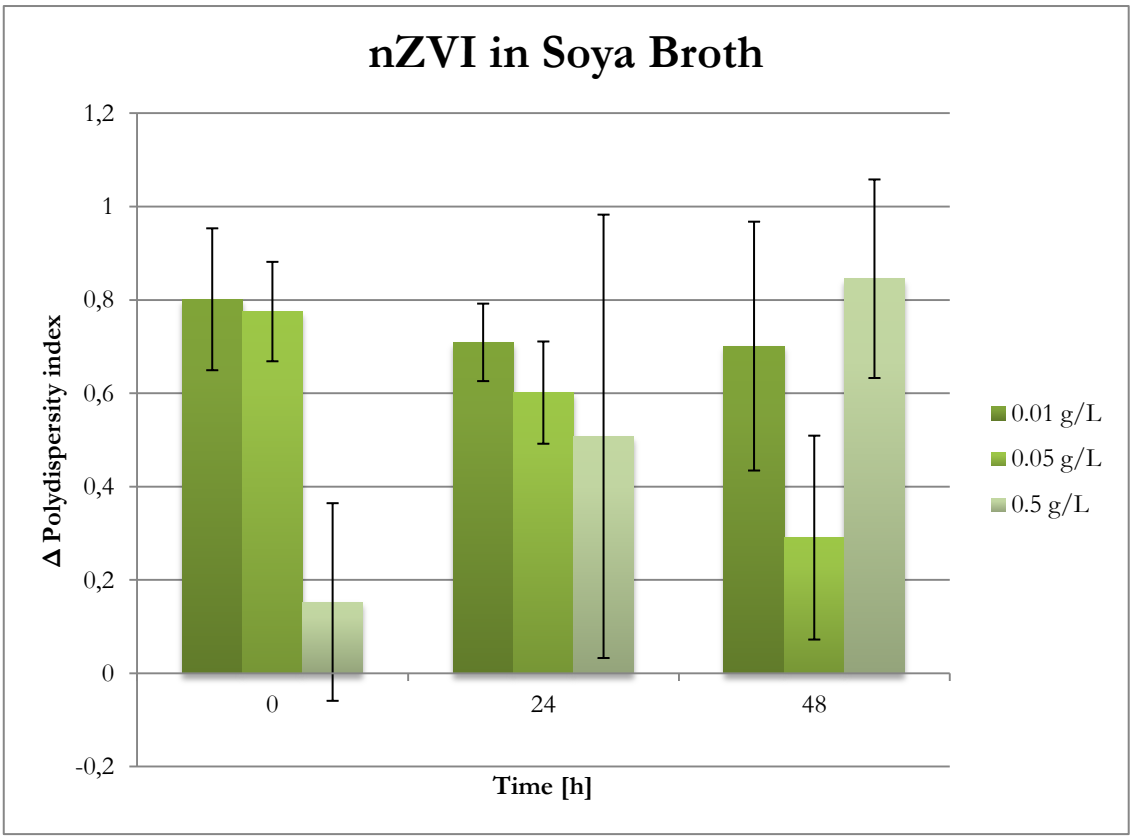

nZVI in Soya Broth
6,000
5,000
4,000
3,000
2,000
1,000
0,000
Δ polydispersity index
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

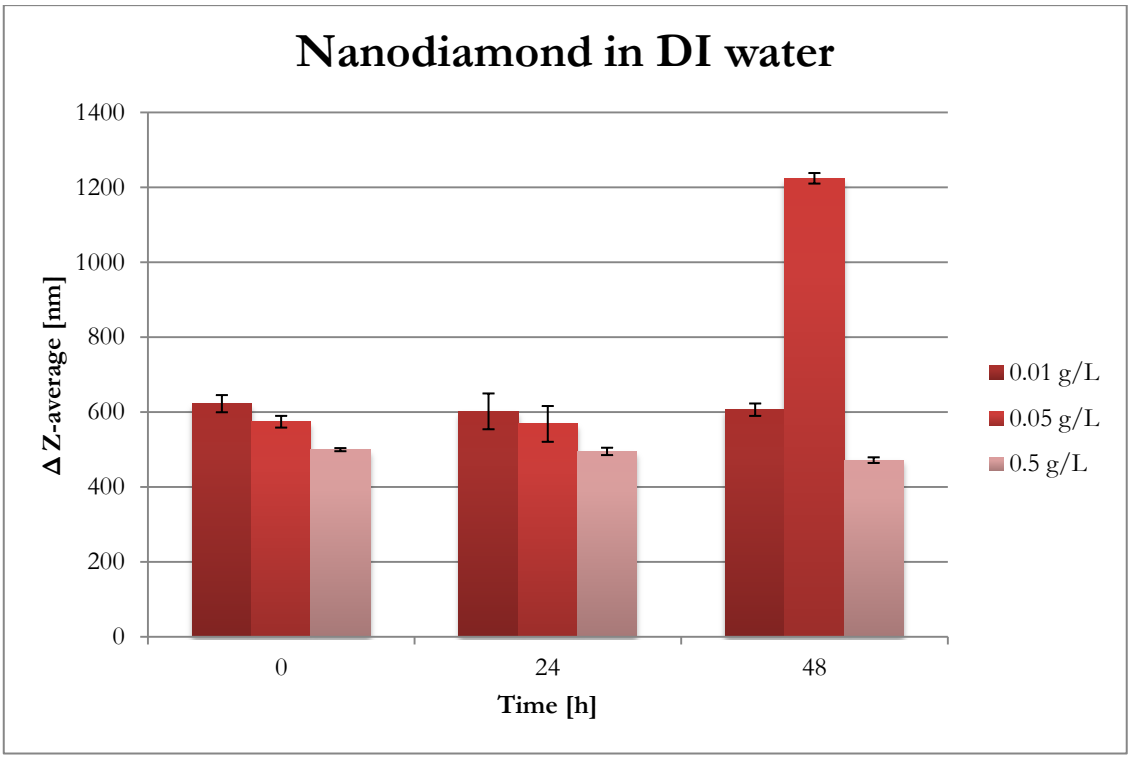
Apendix B

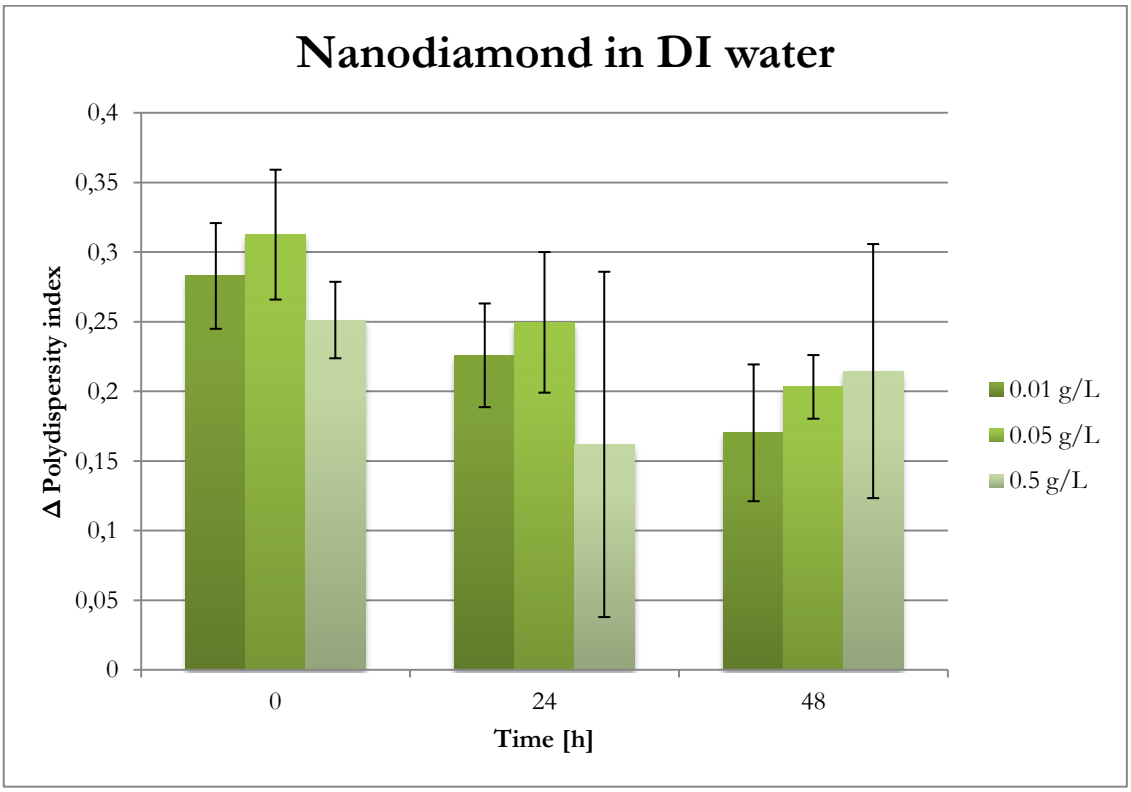

nZVI in DI water
7000
6000
5000
4000
3000
2000
1000
0
Δ Z-average [nm]
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

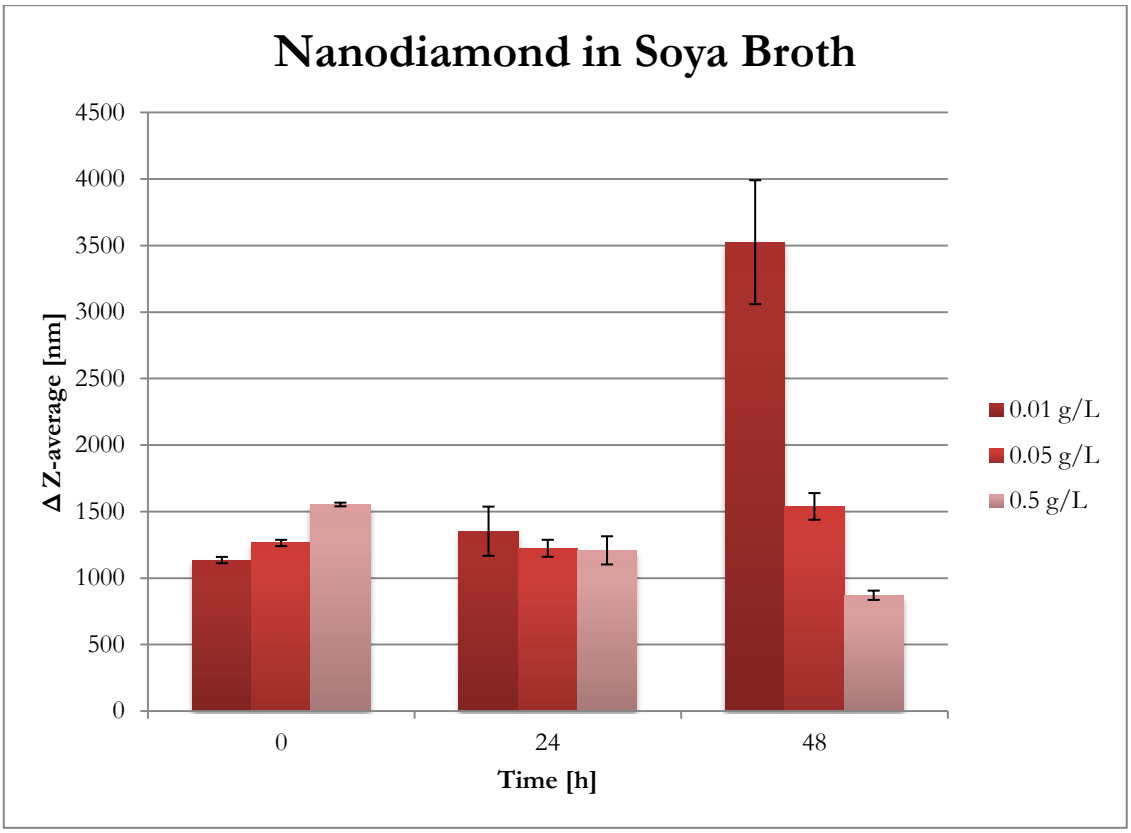

nZVI in DI water
1,2
1
0,8
0,6
0,4
0,2
0
Δ Polydispersity index
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

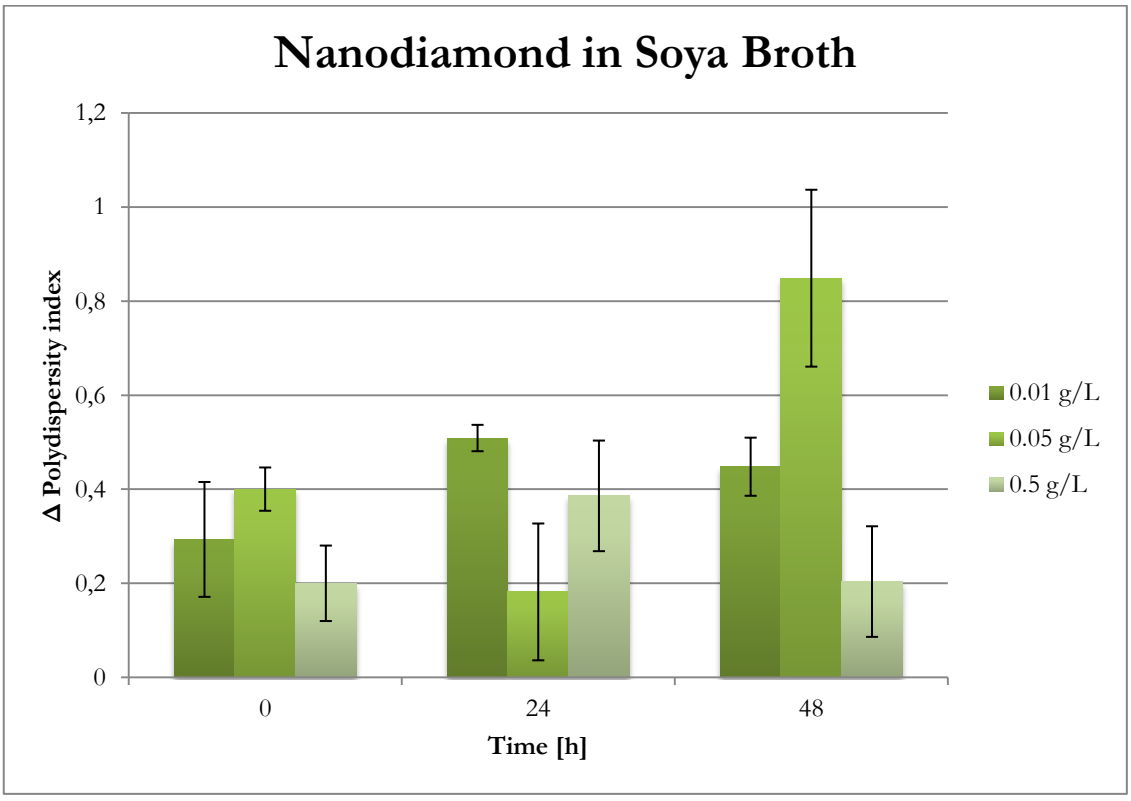

nZVI in Soya Broth
6000
5000
4000
3000
2000
1000
0
Δ Z-average [nm]
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

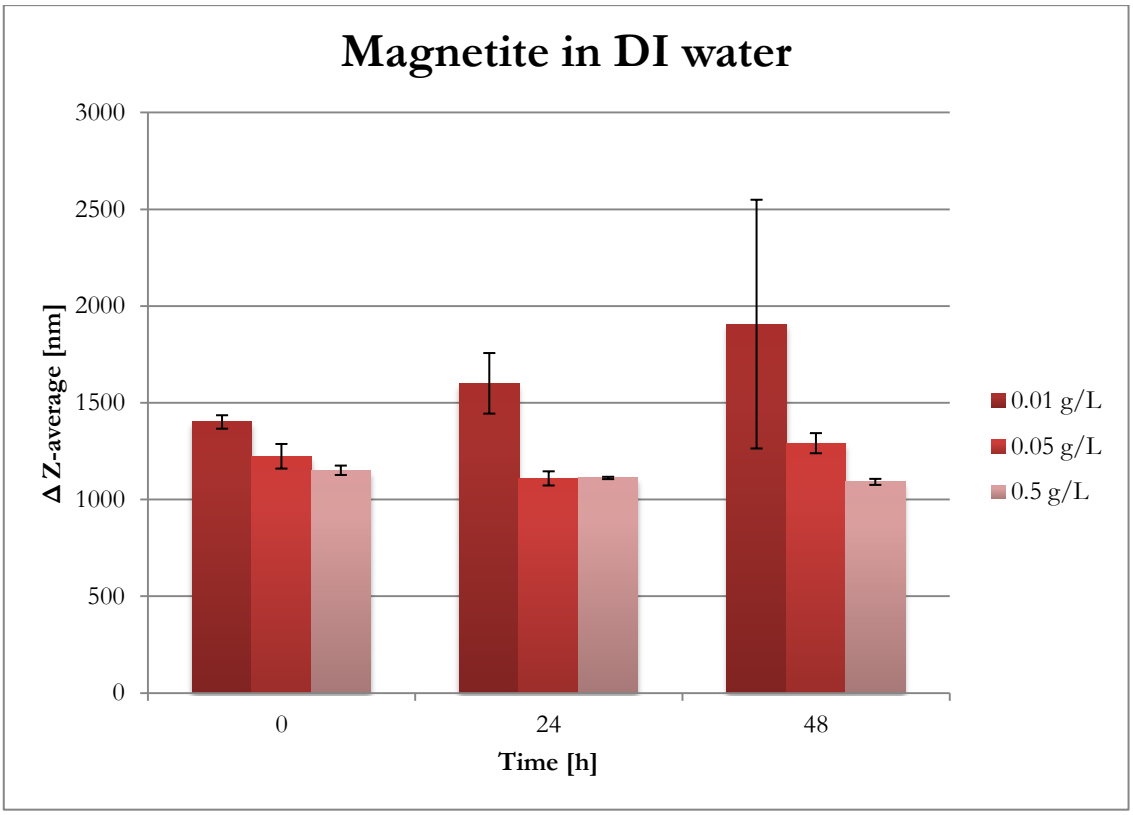

nZVI in Soya Broth
1,2
1
0,8
0,6
0,4
0,2
0
-0,2
Δ Polydispersity index
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

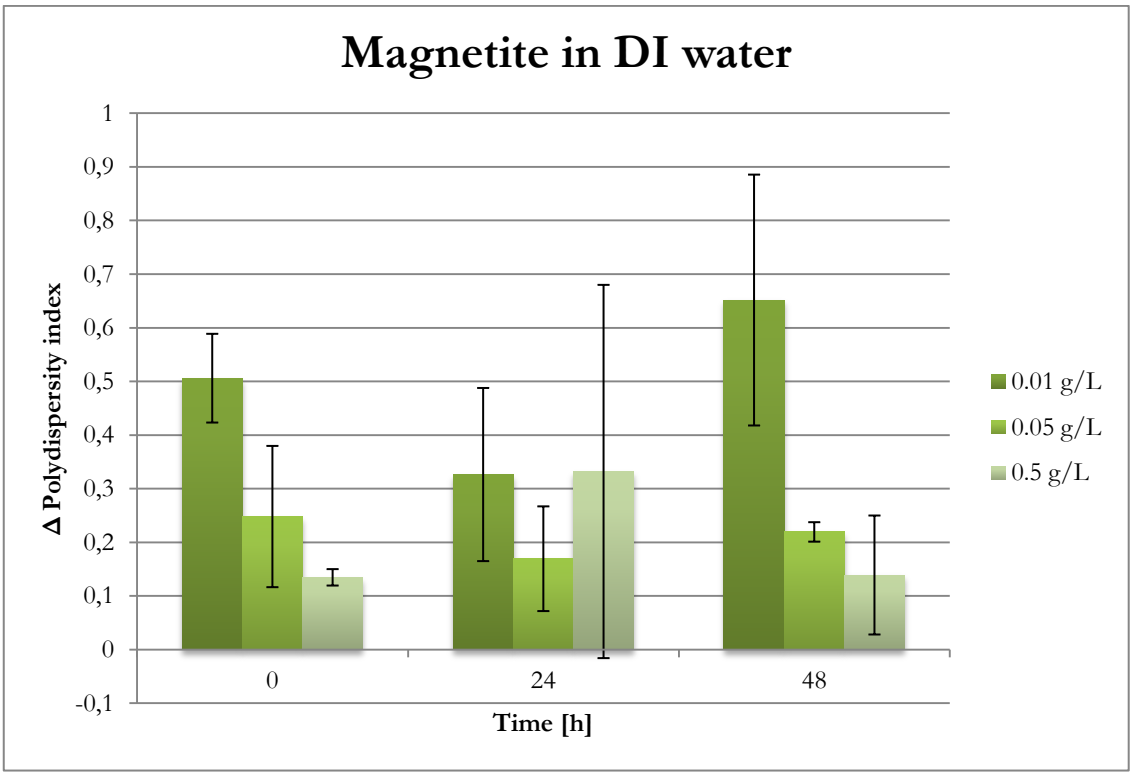

Nanodiamond in DI water
Δ Z-average [nm]
1400
1200
1000
800
600
400
200
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

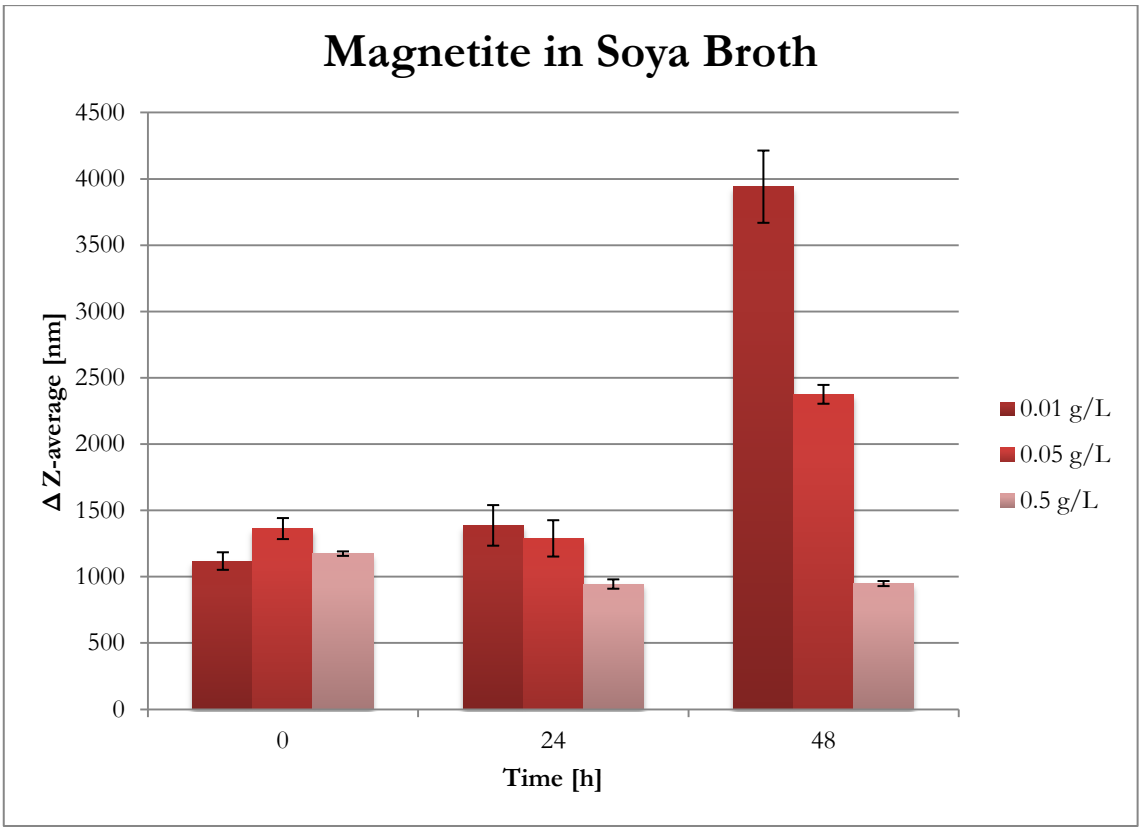

Nanodiamond in DI water
Δ Polydispersity index
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

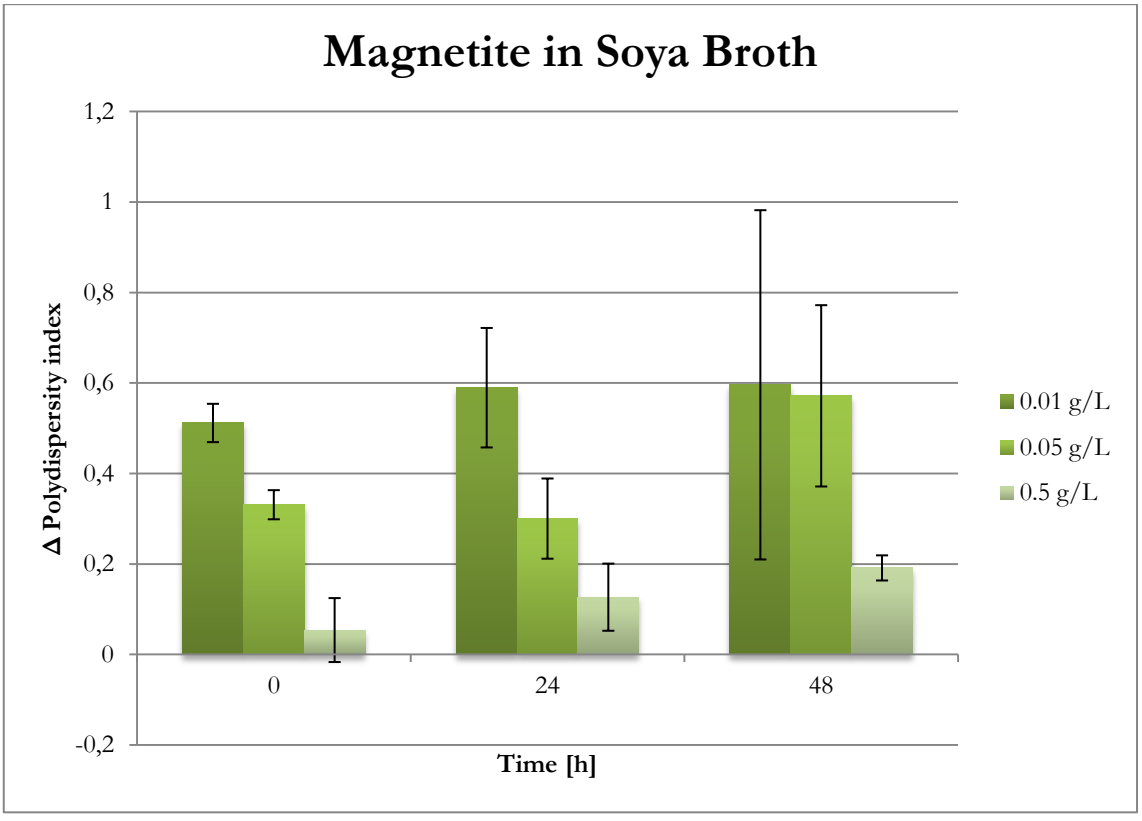

Nanodiamond in Soya Broth
Δ Z-average [nm]
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L


Nanodiamond in Soya Broth
Δ Polydispersity index
1,2
1
0,8
0,6
0,4
0,2
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L


Magnetite in DI water
3000
2500
2000
1500
1000
500
0
Δ Z-average [nm]
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L


Magnetite in DI water
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
-0,1
Δ Polydispersity index
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L


Magnetite in Soya Broth
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Δ Z-average [nm]
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L


Magnetite in Soya Broth
1,2
1
0,8
0,6
0,4
0,2
0
-0,2
Δ Polydispersity index
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L