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Fakulta mechatroniky, informatiky
a mezioborových studií

Impacts of Cerium Dioxide Nanoparticles on Freshwater Microorganisms

Bakalářská práce

Studijní program: B0719A130001 Nanotechnologie
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3. Dose effects of CeO_2 on microbial community nanoparticles.

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Abstrakt

S rostoucím používáním nanomateriálů vyvstává i otázka jejich bezpečnosti pro životní prostředí. Jedním z materiálů, kterým je v poslední době věnována pozornost, jsou nanočástice oxidu ceričitého (CeO_2). Kromě výhod nanočástic CeO_2 se ovšem v literatuře setkáváme i s možnou toxicitou vůči mikroorganismům.

Tato práce si klade za cíl zjistit účinky nanočástic CeO_2 na říční mikroorganismy – bakterie a řasy, a chování CeO_2 v různých typech vodních biotopů (dle pH). Za tímto účelem byly mikroorganismy z řeky exponovány CeO_2 nanočásticím bez úpravy (naked CeO_2 4 nm a 25 nm), a dispergovaným v collyriu (collyrium CeO_2 4 nm). Byly aplikovány různé koncentrace od 0.001 do 0.05 g/L a měření proběhla po 5, 14 a 28 dnech. Pro hodnocení účinků CeO_2 na mikroorganismy byl použit epifluorescenční mikroskop (morfologie buněk), a především molekulárně biologické analýzy (změny v biomase řas a bakterií a zastoupení bakteriálních populací). Pro studium chování CeO_2 při různém pH byly měřeny velikosti a náboj pomocí dynamického rozptylu světla.

Celkově bylo relativní množství řas pouze mírně ovlivněno menšími nanočásticemi CeO_2 (4 nm naked a 4 nm collyrium), nicméně celkově řasy ve vzorcích narostly a až ke konci studie začaly odumírat pravděpodobně kvůli vyčerpání dostupných živin. Relativní množství bakterií pokleslo ve všech vzorcích oproti původnímu stavu po dobu 14 dnů a mírně se zvýšilo po 28 dnech. Bakteriální populace měli nižší biodiversitu po expozici 4 nm CeO_2. Zastoupení *Sediminibacterium* bylo po celou dobu vyšší a nárůst *Cyanobaterium* PCC-6307 bylo možné pozorovat po 28 dnech. Ve vzorcích naked CeO_2 4 nm *Verrucomicrobiae* narostly po 5 dnech, *Candidatus Aquirestis* po 14 dnech a *Limnobacter* po 28 dnech. *Niveispirillum* se objevilo v obou vzorcích s CeO_2 nanočásticemi po 28 dnech. Všechny $Ce0_2$ nanočástice byly záporně nabité v rozsahu -5 až -25 mV ve všech pH 6.6, 7.6 a 8.3, měly proto tendenci agregovat při všech pH, kromě obou velikostí naked CeO_2, ty byly stabilní při pH 6.6. Nanočástice CeO_2 s collyriem byly stabilní při pH 8.3.

Klíčová slova

Nanočástice, oxid ceričitý, sladkovodní mikroorganismy, toxicita

Abstract

With the increasing use of nanomaterials, the question of their safety for the environment also arises. The toxicity of these substances could affect organisms in the environment, including microorganisms. Cerium oxide (CeO_2) nanoparticles (NPs) are one of the materials that have recently received attention. In addition to the benefits, CeO_2 NPs have contradictory possible toxicity to microorganisms.

This thesis aims to determine the effects of CeO_2 NPs on river microorganisms – bacteria and algae and the behavior of CeO_2 in different types of aquatic habitats (according to pH). For this purpose, microorganisms from the river were exposed to CeO_2 nanoparticles without treatment (naked CeO_2 4 nm and 25 nm), and dispersed in collyrium (collyrium CeO_2 4 nm). Different concentrations were applied, from 0.001 to 0.05 g/L, and the exposure was examined after 5, 14, and 28 days. To evaluate the effects of CeO_2 on microorganisms, an epifluorescence microscope (cell morphology) was used, and especially molecular biological analyses (changes in the biomass of algae and bacteria and the representation of bacterial populations). To study the behaviors of CeO_2 NPs in different pH, sizes and charge were measured by a dynamic light scattering.

Overall, the relative abundance of algae was only slightly affected by the smaller CeO_2 NPs (4 nm naked and 4 nm collyrium). However, overall algae in the samples increased and began to die by the end of the study, probably due to the depletion of available nutrients. The relative abundance of bacteria was more stable than of algae over 14 days, and increased after 28 days. The composition of bacterial communities changed but diversity remained relatively high in the presence of 4 nm CeO_2. *Sediminibacterium* increased over the whole time and *Cyanobaterium* PCC-6307 was found after 28 days. In naked CeO_2 4 nm samples, *Verrucomicrobiae* developed after 5 days, *Candidatus Aquiresis* was found after 14 days and *Limnobacter* found after 28 days. *Niveispirillum* showed in one sample of both CeO_2 after 28 days. All CeO_2 NPs were negatively charged in a range of -5 to -25 mV in all pH 6.6, 7.6, and 8.3. They tended to aggregate during 28 days in all pH, except both sizes of naked CeO_2 were stable in pH 6.6, and collyrium CeO_2 was stable in pH 8.3.

Keywords

Nanoparticles, cerium oxide, freshwater microorganisms, toxicity

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List of Abbreviations

BR	broad range
Ct	threshold cycle
DOC	dissolved oxygen species
NGS	next–generation sequencing
NPs	nanoparticles
NTC	non-template control
OTUs	operational taxonomic units
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
SC	sodium citrate
SEM	scanning electron microscope
SybrM	SYBR Green I Master mix
WWT	wastewater treatment
ZP	zeta potential

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1 Introduction

Nanoscience is one of the modern and rapidly evolving scientific disciplines. There are many types of materials in the nano dimension with specific functional properties. Different types of these nanomaterials are used in different areas and spheres, from industry to medicine. However, the use of these materials might have a negative impact on microorganisms in the environment. Therefore, in handling and minimizing the contact of substances with the environment, we should proactively investigate how these substances behave in the environment and how can affect they various organisms.

Nanoparticles (NPs) have been fully paid attention to in many applications. Especially metal oxide NPs (e.g. CuO, ZnO, SnO_2, Al_2O_3, MgO, ZrO_2, AgO, TiO_2, Fe_3O_4) have potential technological application in material chemistry, medicine, agriculture, information technology, optics, electronics, catalysis, environment, energy, and sensing (Baek et al. 2011; Chavali et al. 2019; Nguyen et al. 2018). Cerium oxide (CeO_2) NPs are widely used in chemical mechanical polishing as anticorrosion agents in solar cells, fuel oxidation catalysis, and automotives and are expected to be used more in the future (Campbell et al. 2005; Dahle et al. 2015; Das et al. 2007; Dhall et al. 2018; Karakoti et al. 2008). The CeO_2 NPs properties, advantages, and disadvantages of their applications have been summarized in several reviews (Dhallet al. 2018; Prajitha et al. 2019; Singh et al. 2020; Xu et al. 2014). Yet, there is still a need for deeper understanding of the fate of CeO_2 NPs in the environment and toxicity.

This thesis aims to investigate the impact of CeO_2 NPs on freshwater microorganisms, particularly bacterial and algal communities. The study consists of exposure of freshwater microorganisms to two different sizes of naked CeO_2 and CeO_2 NPs with collyrium for one month. Various methods were employed to evaluate the endpoints such as visualization of cell morphology by epifluorescence microscopy, quantification of microbial biomass by a polymerase chain reaction (PCR), and classification of bacterial taxonomy by next-generation sequencing (NGS). Additionally, the behavior of CeO_2 NPs in exposure conditions was characterized using a dynamic light scattering (DLS) and measured pH.

2 Literature Overview

2.1 Definition of NPs

Generally, NPs are defined as very tiny particles with at least one dimension in the range of 1 to 100 nm (Khan et al. 2019), (Fig. 2.1). All NPs share the exact size range definition but differ based on physical and chemical properties. They have been used in different industries such as medicine, chemistry, physics, biology and engineering (Zahmakıran et al. 2011). NPs are classified according to their chemical nature, e.g. metal, carbon, ceramic, polymer or lipid NPs (Khan et al. 2019). NPs can be synthesized by two main approaches: top-down and bottom-up (Iravani 2011).

The top-down method starts with a larger molecule and uses various ways to decompose it into NPs. Several methods include mechanical milling, chemical etching, electro-explosion, sputtering or laser ablation (Iravani 2011).

The bottom-up method is the reverse. NPs are prepared from smaller and simpler units to build up to desired NP sizes. Several methods are involved, such as spinning, template support synthesis, plasma or flame spraying synthesis, laser pyrolysis, atomic or molecular condensation and green synthesis, including different biological methods (Iravani 2011; Khan et al. 2019).

Prepared NPs can be characterized from different points of view, including morphology, structure, size, and surface. These characteristics are critical and reflect both the chemical and physical properties of NPs (Khan et al. 2019).

2.2 NP properties

In general, the properties of NPs are the main reasons for their applications and further research (Rastar et al. 2013). The properties in which NPs excel may be of a character: optical, magnetic, electronic, thermal and mechanical (Khan et al. 2019), photocatalytic (Stefan et al. 2019) and antibacterial (Hajipour et al. 2012). To define NPs, they should have some essential properties: size, shape, surface (functional groups, coating, charge) and materials (carbon, metal, polymer, etc.) (Fig. 2.1).

The size of NPs is regarded as the first parameter to measure NPs. Smaller NPs should provide more reactivity and lead to higher toxicity (Yokel et al. 2014). The impacts of NPs on cells may depend on NP sizes and cell types and exposed conditions, but in the same terms, tiny NPs are more likely to be toxic (Shang et al. 2014). Additionally, the size relates to cellular absorption. Small sized NPs can easily get through cell barriers, leading to better distribution in living microorganisms and larger affected areas (Sajid et al. 2015).

For example, according to Ma et al. (2021), CeO_2 NPs of 15 nm in size showed more significant cytotoxicity against human retinal pigment epithelial cells than larger CeO_2 NPs (30 and 45 nm).

The shape of NPs is directly connected with their sizes. Methods for preparing NPs of various shapes can be described as shape-selective. It is possible to prepare NPs of various shapes: nanocubes, nanospheres, nanorods, nanostars and nanocarrots, etc. (Liang et al. 2016; Sun et al. 2014). Various shapes can modify the largeness of the surface. This can then lead to higher reactivity and toxicity. Different shapes of NPs can also lead to an easier way for NPs to get into the biological system (Sajid et al. 2015). According to Naganuma (2017), different shapes of CeO_2 NPs (nanocubes, irregular CeO_2, nanorods, nanopolyhedra, etc.) can affect, for example, the oxygen storage capacity, catalytic properties, or antioxidant activity of microorganisms.

The surface of NPs plays a significant role in the overall functioning. There are different ways to functionalize the surface of NPs based on applications. These different types subsequently lead to properties that can be used in many areas. For example, Au NPs are attached doxorubicin to be more effective in cancer treatment. Also, stabilizers of NPs are often used to prevent NP aggregation, such as phosphates, polymers, or inorganic substances (silica and gold) (Brennan et al. 2006; Sapsford et al. 2013; Yeh et al. 2014). For example, CeO_2 NPs have been modified with polymers: polyacrylic acid, polyethylene glycol, dextran, polyethyleneimine, cyclodextrin, glucose, and folic acid (Dhall et al. 2018). However, functionalized groups or stabilizers may decrease or increase toxicity. For example, NPs coating with a biocompatible group leads to being more stable, having a longer retention time and lower toxicity due to reduced interaction (Dhall et al. 2018).

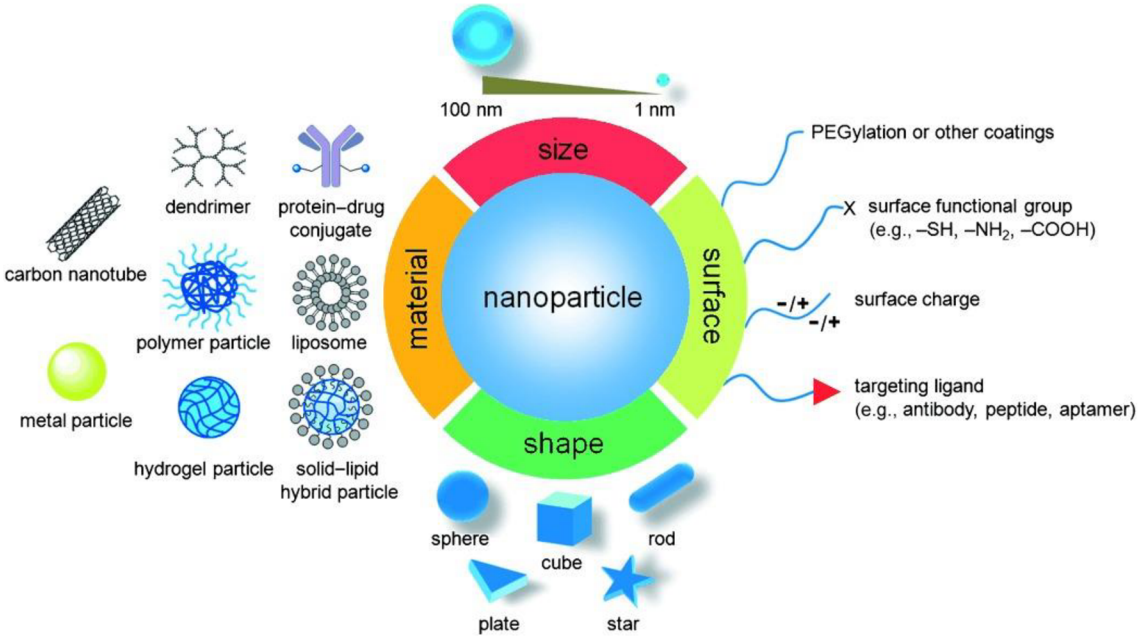

Figure 2.1: Illustration of various biophysiochemical properties of NPs in general (Sun et al. 2014).

2.3 Behavior of NPs in the environment

Many of those properties are linked with the behavior of NPs in the environment. As stated by Khan et al. (2019), NPs can get into the environment through various human activities by accident or on purpose and enter the environment through water, soil, or air. NP behaviors depend on geography and environmental factors such as temperature, sunlight, pH, inorganic and organic compounds, and living microorganisms. In the case of NPs getting into the ground or an aquatic system, they can interact with the existing components and change their properties via heteroaggregation, decomposition and transformation. For example, NPs can be dissolved in acidic conditions, and sunlight can oxidate NPs. The beneficial properties of NPs (e.g. reactivity) might become harmful to organisms of a given system (Fig. 2.2) (Sajid et al. 2015).

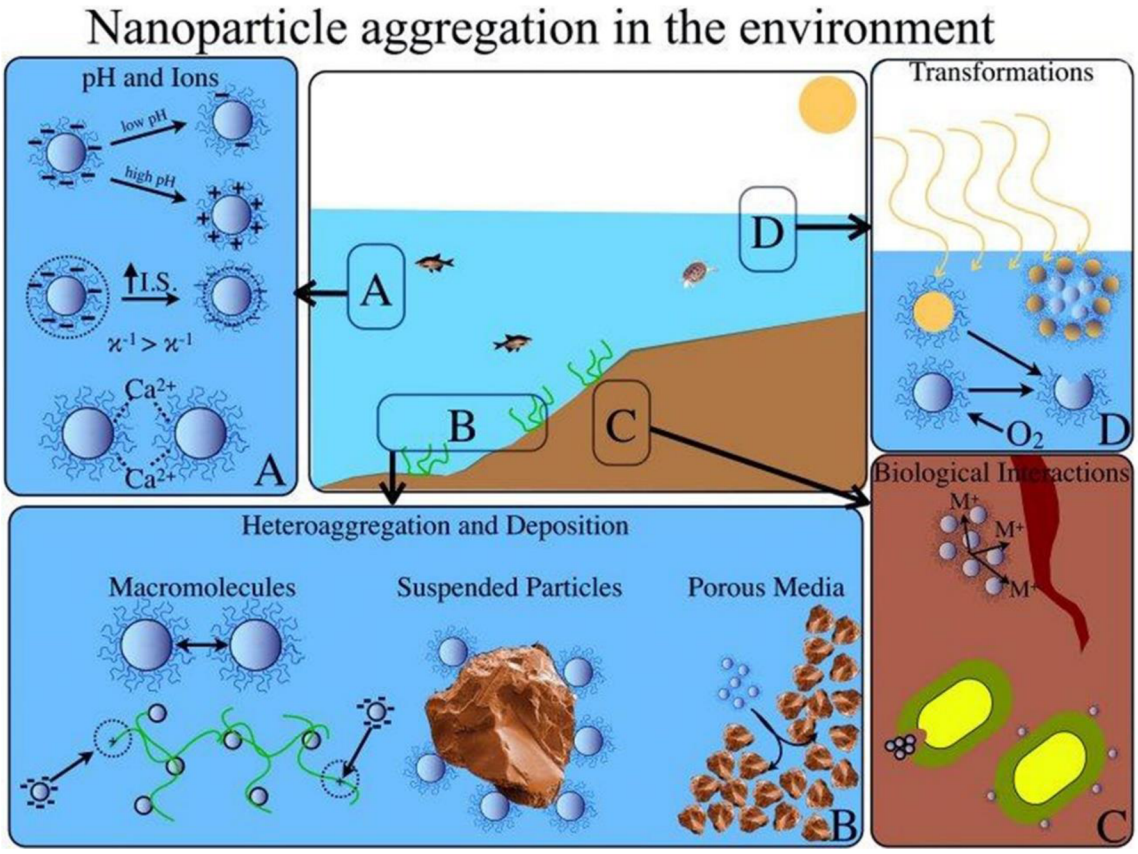

Figure 2.2: Graphical representation of NPs aggregation in the environment. Subpicture A shows the pH and ions associated with the charge. Subpicture B shows how NPs aggregate on different materials. Passage through porous materials and coating larger. Subpicture C shows the possibility of interaction with cells. Subpicture D shows the effect of aggregation on sunlight or oxidation. Adopted from (Hotze et al. 2010).

2.4 Characterization of NPs in exposure environment

The characterization of NPs is one of the essential procedures in any study. NPs can be characterized along with their properties in an exposure environment. The changes of NPs can help to explain their impacts on targeted microorganisms in exposed conditions. The morphology of NPs is one of the most frequently discussed properties. It consists of a description of sizes and shapes. The methods used to describe them are DLS, atom force microscopy, and electron microscopies (Kumar et al. 2017). The NP surface can be characterized using X-ray diffraction or the Brunauer-Emmett-Teller method. Examples of chemical characterizations can be UV-visible spectroscopy or Electron dispersive X-ray spectroscopy. Below are some examples of NP characterization that are worked on in this thesis.

DLS is, as already mentioned, a method for obtaining the sizes of NPs. It is a measurement of light interference based on Brownian motion in a suspension. The measurement is based on illuminating the sample with a laser and analyzing the intensity fluctuations in scattered

light. Brown's motion suggests that smaller NPs move more dynamically than larger particles. The moving speed is correlated to NP sizes. The correlation of velocity with the sizes of NPs are calculated using the Stokes-Einstein equation (Kumar et al. 2017).

A scanning electron microscopy (SEM) uses a beam of electrons instead of photons as in an optical microscope. Due to the wavelength of the electrons, we are able to achieve greater magnification and observe the samples even in nano-dimensions. Electrons are emitted from the cathode. Subsequently, they are focused on electromagnetic coils. The scanning microscope has a sample at the bottom of the chamber. The interaction of electrons and samples results in the formation of secondary electrons. Then the detector records it due to the interaction in different parts of the samples. An image is produced based on the contrast (difference in brightness of the parts). (Inkson 2016)

2.5 Impacts of NPs on bacterial and algal communities

According to Klaine et al. (2008), microorganisms play essential roles in the environment. They include bacteria as decomposers and producers as algae in the ecosystem, where NPs may impact the communities. The most common toxic pathway is the generation of reactive oxygen species (ROS), causing cell membrane damage, inhibiting chlorophyll synthesis, cellular NP agglomeration, DNA and organelle damage (Nguyen et al. 2018; Prajitha et al. 2019; Ševců et al. 2011).

In the case of bacteria, one of the main factors determining sensitivity toward NPs is bacterial type, Gram-negative or Gram-positive (Darwish et al. 2015; Martínez et al. 2021; Prajitha et al. 2019). Different impacts of NPs on bacteria are due to different cell compositions. The mechanisms of bacterial toxicity are not entirely understood. However, it is known that NPs affect cell walls and cause oxidative stress, metal ion release and non-oxidative mechanisms (Martínez et al. 2021). Studies suggest two types of NPs toxicity mechanisms: direct toxicity due to cellular damage, and indirect abiotic effects such as water types or nutrient components (Nguyen et al. 2018; Martínez et al. 2021; Ge et al. 2012). Examples of some metal and metal oxide NPs with their effect on bacteria are listed in Table 2.1 (including sources).

The description of the effects (or specifically the toxicity) of the substance on the organism needs to be described in some way. LC50 and EC50 values (or combined as L(E)50) are used often for description. LC50 is the concentration of toxic substant which is lethal to 50 % of tested organisms during an experiment (Kooijman 1987). EC50 is the concentration or dose which gives a 50% effect (e. g. toxicity) (Vanewijk et al. 1993). Key organisms are important in describing environmental toxicity. The following organisms are considered key for the

aquatic environment: algae, crustaceans and fish (Bondarenko et al. 2013; Sanderson et al. 2003). NPs (and their soluble salts) are toxic according to EU-Directive 93/67/EEC based on their L(E)50: specifically: <0.0001 g/L as extremely toxic to aquatic organism, 0.0001–0.001 g/L as very toxic to aquatic organism, 0.001–0.01 g/L as toxic to aquatic organisms, 0.01–0.1 g/L as harmful to aquatic organisms, <0.1 g/L as non-toxic to aquatic organisms (Bondarenko et al. 2013; Sanderson et al. 2003; Van Leeuwen 1996).

Metal oxides				
ZnO	CuO	TiO_2	Al_2O_3	**NPs**
<100 nm	20 to 30 nm	15 to 20 nm/semispherical	60 nm	**Sizes / Shapes**
1, 10, 20, 40, 80 mg/L	25, 50, 75, 100, 125 mg/L	0.5, 1.0, 2.0 mg/g	20 mg/L	**Concentration**
Deinococcus radiodurans	*E. coli*, *B. subtilis*, and *S. aureus*	Decrease of *Rhizobiales*, *Bradyrhizobiaceae*, *Bradyrhizobium*, *Methylobacteriaceae* communities Better growth of *Streptomyces or Sphingomonadaceae* communities	*B. subtilis* (57 %) *E. coli* 36 % *P. fluorescens* 70 %	**Microorganisms**
Concentration-dependant toxicity with membrane damage and significant DNA damage to bacterial cell	NPs were found toxic (Highest at concentration of 50 and 125 mg/L)	Dose and species dependant effect. Higher concentration resulted in higher effect	Toxicity test resulted in mortality (mortality rate)	**Toxic effects**
(Singh et al. 2020)	(Baek et al. 2011)	(Ge et al. 2012)	(Jiang et al. 2009)	**Source**

Table 2.1: Toxic effects of NPs on microorganisms from various sources. Note: mg/g is concentrations in soil

Metalic					
Au	Ag	SiO_2			
(15 ± 1.2) nm / spherical	(12 ± 1.2) nm / spherical	20 nm	20 to 30 nm / spheroid		
≈ (2 to 30) mg/L	≈ (1 to 16) mg/L	20 mg/L	0.05, 0.1, 0.5 mg/g		
E. coli and *Staphylococcus capitis*	*E. coli* and *Staphylococcus capitis*	*B. subtilis* 40 % *E. coli* 58 % *P. fluorescens* 70 %	Decrease of *Rhizobiales* or *Methylobacteriaceae* communities Better growth of *Streptomyces* communities		
No significant effect on bacteria	Concentration dependant toxicity	Test resulted in mortality (mortality rate)	Dose and species dependant effect. Higher concentration resulted in higher effect.		
(Amin et al. 2009)	(Amin et al. 2009)	(Jiang et al. 2009)	(Ge et al. 2012)		

2.6 CeO_2 NPs

Cerium is the first element in the lanthanide group and belongs to rare earth elements. CeO_2 NPs can be classified as metal oxide NPs. Depending on the purity, CeO_2 NPs form as a white, pale-yellow, brownish powder and cerium compound, which is in most commercial use (Dahle et al. 2015). With other NPs, CeO_2 NPs determine their size, shape, coating, agglomeration, aggregation, surface charge and dissolution. Then the properties of CeO_2 NPs have different impacts on targeted microorganisms or cells (Milani et al. 2017). All of these factors are connected with synthesis methods (Dhall et al. 2018). Interestingly, the cerium can

exist in two states, namely CeO_2 and Ce_2O_3. Decreasing the NP diameter leads to more Ce^{3+} and oxygen release (Dhall et al. 2018).

The main feature of CeO_2 is the ability to convert cerium between two oxidation states: $Ce^{4+} \leftrightarrow Ce^{3+}$ (Zhang et al. 2019). This conversion can create oxygen vacancy in the structure of CeO_2. Then, this vacancy can become occupied by oxygen from the surroundings. In fact, CeO_2 can provide oxygen by creating a vacancy and can remove oxygen by filling a vacancy. Therefore, CeO_2 NPs have both anti-oxidant and pro-oxidant properties.

Due to their properties, CeO_2 NPs can destroy free oxygen radicals and other ROS. ROS are derived from oxygen and are superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$) and peroxide ($O_2^{\cdot -2}$) (Bayr 2005). They are substances that are formed as a byproduct of oxygen metabolism and can be harmful due to inflammation and can lead to damaging cell membrane, proteins, DNA or cell organelles (Singh et al. 2020).

CeO_2 NPs can be synthesized using different methods as stated by Dhall and Self (2018). Each method results in a CeO_2 size. All methods are divided into two categories: a traditional and green synthesis. Green synthesis is a more environmentally friendly method that uses natural material, is often mediated by organisms, minimizes toxic chemicals, and leads to bio-compatible products (Dahle et al. 2015; Iravani 2011). Products of green synthesis are intended primarily for biomedical and pharmaceutical purposes.

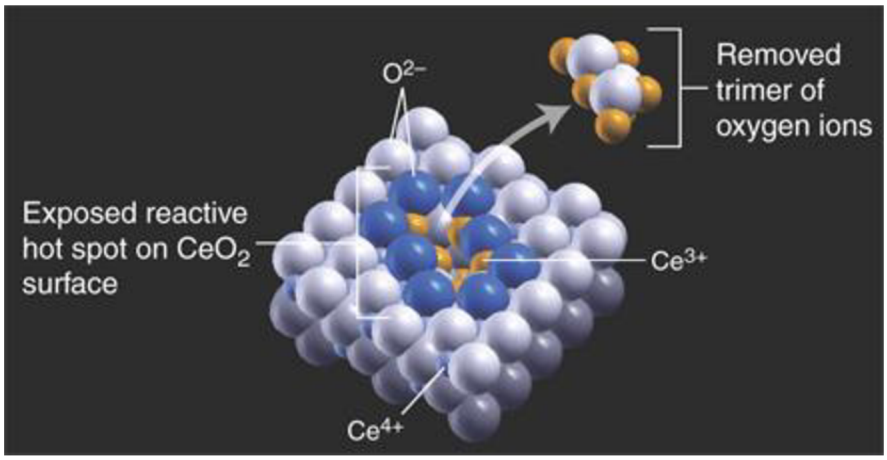

Figure 2.3: Illustration of conversion of two oxidation states of CeO_2 NPs. Ce^{4+} ions of cerium oxide NPs are reduced to Ce^{3+} by removal of oxygen (adopted from Campbell et al. (2005)).

2.7 Application of CeO_2

The properties of CeO_2 NPs make them useful in various fields. CeO_2 NPs are used in the glass industry as polishing agents (Janoš et al. 2016). They are also used in automotives as catalysts in diesel fuels (Trovarelli 1996). The first study of CeO_2 NPs was described in 1927

when the CeO_2 NPs were used as catalysts for the oxidation of methane by air (Yant et al. 1927). CeO_2 NPs were used as ceramic electrolytes in fuel cells (Dale et al. 2017; Stambouli, Traversa 2002). Potentially, CeO_2 NPs could be used as environmental chemi-sensors (Khan et al. 2011) for detecting organic pollutants such as ethanol or dyes. One of the latest uses is in biomedicine (Casals et al. 2020; Singh et al. 2020). Historically, CeO_2 has been studied from as early as 1961 for its anti-inflammatory potential (Jancso 1961). However, the notable increase in CeO_2 research began in combination with nanotechnologies – CeO_2 NPs. NPs like CeO_2 can have potential as great anti-inflammatory agents and antioxidants. Evidence of the enormous potential of CeO_2 NPs is the number of medical areas where we can apply them. One of the applications in ophthalmology is the prevention of retinal disorders which can otherwise lead to blindness (Chen et al. 2008). Size-dependent protective effect of CeO_2 NPs on nerve cells has potential use in neurology (Schubert et al. 2006). CeO_2 NPs were used to protect human cells from radiation in oncology (Tarnuzzer et al. 2005). Another use in hepatology is as potential help in the treatment of hepatocellular carcinoma (Fernández-Varo et al. 2020). Due to the anti-inflammatory effect, CeO_2 NPs are one of the potential new ways to treat chronic inflammation (Hirst et al. 2009).

2.8 Toxicity of CeO_2

CeO_2 NPs are the most widespread form of materials containing cerium. They have been applied in different fields, as described in part 2.7. According to the review by Dahle et al. (2015) there are many ways that CeO_2 translocates to the environment from different sources. CeO_2 NPs could potentially escape from automobile emissions and then spread by wind, polluting the air and the soil. In general, different industries could produce waste containing NPs. This waste could come from chemical synthesis, ceramics, electronics, polishing agents, UV protecting agents, and pharmaceuticals. Another way is as waste from wastewater treatment (WWT) and landfill leaches, resulting in water contamination. Needless to say, these WWTs are relatively reliable and comprise most of NPs (about 95 %) (Dahle et al. 2015). The main threat is from inland contamination, such as landfills containing the waste above mentioned. Microorganisms are in general affected by contaminants such as NPs (Kumar et al. 2018). Some studies have investigated the toxicity of CeO_2 NPs on microorganisms. The following examples are some of them.

In general, perhaps the most discussed issue is the issue of ROS formation and its possible link to the toxicity of CeO_2 NPs. However, in their article on CeO_2 NPs toxicity to freshwater algae, Angel et al. (2015) mentioned the possibility of a different origin of toxicity. The ROS formation was investigated on green freshwater algae (*Pseudokirchneriella subcapitata*) exposed to NPs (10–34 nm and 1 μm). UV light was filtered in this experiment to examine

whether it is the main factor causing the ROS. The results showed that the algal growth rate was comparable between UV-filtered light and NP exposure. The results suggested that ROS was not a toxic mechanism. The presence of dissolved organic carbon (DOC) was mentioned in the study as an influencing factor. The DOC was the organic matter that could pass through a filter that removed material between 0.70 and 0.22 mm in size (Zhuiykov 2014). For studies related to pollution, the DOC is an important parameter in characterizing aqueous samples. For example, the DOC enveloped NPs and thus reduced their toxicity. The authors also reported the more significant toxicity of smaller NPs (Angel et al. 2015).

CeO_2 NPs (1 mg/L) were exposed to a bacterial community in activated sludge for 210 days. The results showed that CeO_2 NPs caused inhibition of nitrite oxidoreductase and nitrate reductase enzymes as well as significantly reduced bacterial diversity, especially dominant denitrifying bacteria, *Flexibacter* and *Acinetobacter* (Wang et al. 2018). In a similar study, CeO_2 NPs (20 nm) showed that bacterial composition was only comparable to 41% after 14 days of adding CeO_2 compared to the control (without CeO_2) (Qiu et al. 2015). In freshwater, CeO_2 NPs 1 mg/L (90 nm) showed positive impacts on bacterial communities at 1 mg/L after 4 weeks (Bour et al. 2016). As already mentioned, the work results on the action of CeO_2 NPs are sometimes contradictory. Lawrence et al. (2020) researched the river biofilm microbial community treated with CeO_2 NPs (25 nm) and concentrations of 1 and 10 µg/L for 8 weeks. The study concluded that lower concentrations do not have a toxic effect on aquatic life. Low concentrations have slightly altered community compositions while positively affected at long-term exposure.

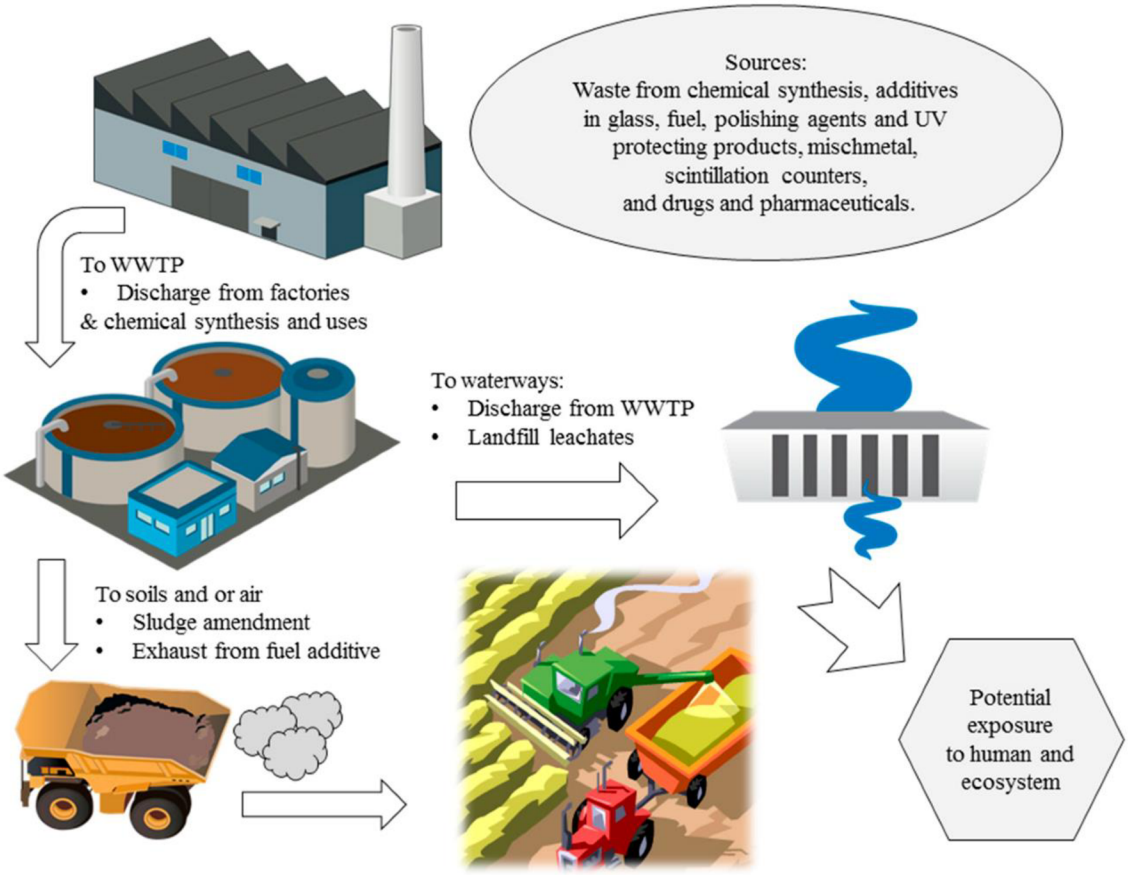

Figure 2.4: Different CeO_2 NPs use and potential contamination sources (Dahle et al. 2015).

2.9 River microorganisms

By microorganisms, we refer to all viruses, bacteria and some of the amoeba, fungi, algae, and protozoa. These microorganisms form communities that are an integral part of the ecosystem. Some microorganisms can survive in various areas, even under extreme conditions (Roane et al. 2009) and play a significant role in aquatic and soil environments. The composition and condition of microbial communities are directly linked to the quality and health of the soil. Poor soil condition can affect its fertility. The state of soil depends on many factors such as temperature, pH, nutrition, humidity, etc. It may also be affected by the consequences of human activity – such as pollution by toxic substances. This can lead to a reduction in the diversity of microbial communities. A single species can directly benefit another and upset the balance of the whole system. In other cases, the disadvantaged species (or organism) may disappear along with its benefits to the environment.

Microorganisms are similarly important for the aquatic environment in freshwater (rivers, lakes, wetlands, etc.) and marine waters (oceans). The importance of the aquatic environment stems from the fact that water makes up 70% of the earth surface. This thesis is focused on freshwater bacteria and algae.

Bacteria are typical representative microorganisms in any environment. They are single-celled organisms (Pepper et al. 2004) with a simple structure, but are an extremely diverse group. They have an average size of μm, a flexible metabolism and adapt quickly (Roane et al. 2009). Additionally, algae are also an important part of river or lake ecosystems. Algae are unicellular or multicellular. Some algal groups belong to macroscopic organisms (Pepper et al. 2004). Basically, they are eukaryotic organisms containing chlorophyll in chloroplasts, which allows them to obtain energy from light and produce glucose and oxygen. This is a key ability that allows algae to thrive and allows them form one of the foundations of the food chain (Roane et al. 2009). Thus, algae undoubtedly play a crucial role in a healthy ecosystem.

3 Materials and Methods

3.1 CeO_2 nanoparticles

Three types of NPs were used: naked CeO_2 (1 g/L) and collyrium CeO_2 (2 g/L) with 4 nm and naked 25 nm. The CeO_2 NPs 4 nm were obtained from Vall d'Hebron–Institut de Recerca, VHIR, Spain. Collyrium is the solution used as eye drops. The exact type and amount of collyrium added to NPs is confidential. A combination of CeO_2 and collyrium is supposed to treat age-related macular degeneration diseases. The CeO_2 NPs 25 nm were purchased from Sigma-Aldrich, Czech Republic. Before the exposure, a suspension (2 g/L) was prepared in 2.2 mM sodium citrate (SC) using a vortex.

For the experiment, concentrations of 0.001 g/L, 0.01 g/L were chosen for the naked CeO_2 and collyrium CeO_2, while CeO_2 25 nm included 0.05 g/L. The reason was that there was not enough naked CeO_2 and collyrium CeO_2 4 nm for the higher concentration (0.05 g/L).

3.2 Freshwater sampling

Before starting the experiments, I was concerned about the concentration of microorganisms in natural waters. Therefore, four waters from different locations were tested to choose the best location having enough natural microorganisms based on their DNA biomass: Jizera (50°31'28.859"N, 14°57'49.295"E), Ještěd (50°44'42.8"N 15°00'36.0"E), Lužická Nisa (50°45'59.846"N, 15°3'8.993"E) and Harcov reservoir (50°46'12.8"N 15°04'32.1"E) with pH of 6.6, 7.3, 7.6 and 8.3, respectively. The water was collected and filtered with 20 µm filters to remove unwanted materials and larger organisms using a vacuum filtration apparatus (Büchner funnel, Germany). Then the water was filtered with 0.22 µm filters to collect microbial biomass for DNA extraction and quantification.

3.3 Exposure of freshwater microorganisms to CeO_2 NPs

For this experiment, water from Lužická Nisa was chosen because the extracted DNA concentration was sufficiently high ($2.01 \cdot 10^{-3}$ g/L).

Before starting the experiment, Erlenmeyer flasks were prepared and sterilized using an autoclave. First, 50 mL of the filtered freshwater was added to each sterilized flask. After that, NP suspensions were pipetted into the flasks to obtain the desired concentrations, as presented in Table 3.1 below. The whole preparation process for the experiment was performed in a microbiological laminar flow hood to prevent contamination. Each sample was prepared in duplicate to achieve higher accuracy and eliminate mistakes.

Samples were taken after 0, 5, 14 and 28 days, when the experiment was stopped. At 0 days, only filtrated freshwater without NPs (control) was taken. 10 samples were taken at each sampling point.

All the flasks were kept on window ledges in the laboratory. The samples were exposed to natural daylight through windows. The average light was 14 hours. The flasks were mixed daily by hand shaking and the temperature recorded with a thermometer every two days.

Table 3.1: Sample labels and CeO_2 NP concentrations in this study.

Sample number	Name and size	Concentration CeO_2 [g/L]
1	Control	0
2	naked CeO_2 4nm	0.001
3	naked CeO_2 4nm	0.01
4	collyrium CeO_2 4nm	0.001
5	collyrium CeO_2 4nm	0.01
6	collyrium CeO_2 4nm	0.05
7	naked CeO_2 25nm	0.001
8	naked CeO_2 25nm	0.01
9	naked CeO_2 25nm	0.05
10	collyrium based 0.5%	0

The experimental design is summarized in Fig. 3.1.

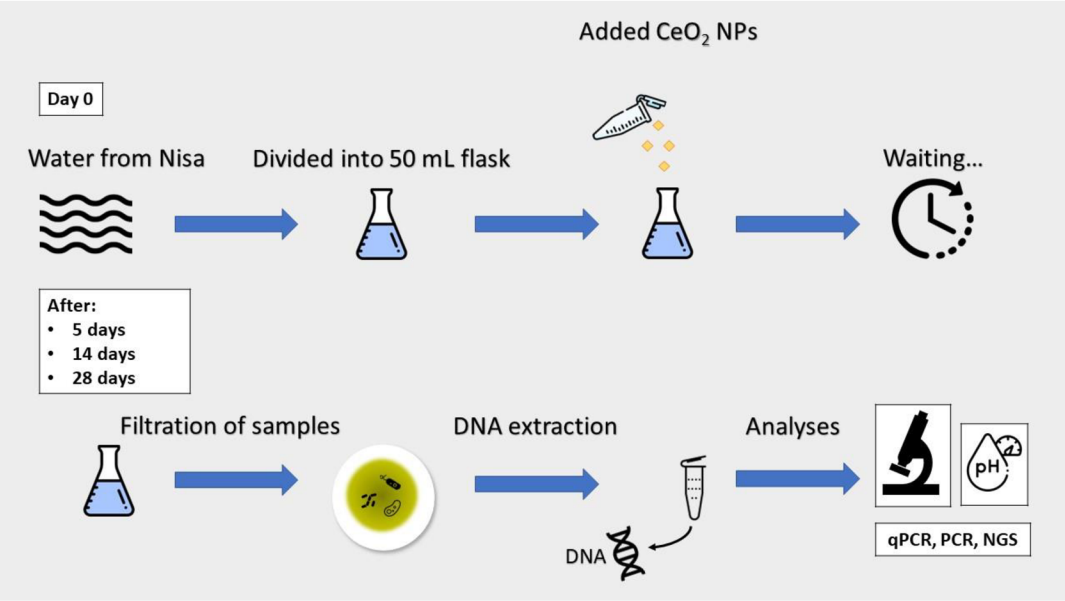

Figure 3.1: Workflow of the first experiment (beginning at the upper left corner): CeO_2 NPs exposed to Lužická Nisa freshwater with pH of 7.6.

3.3 DNA extraction

At each sampling point, the samples were shaken very well to prevent sediment forming at the bottom of the flasks. Each sample was then passed through 0.22 µm filters. The filters were collected and microbial DNA extracted.

A FastDNA™ SPIN Kit was used in the DNA extraction. The DNA was extracted according to the optimized kit´s manual. The workflow of the whole process is described in Figure 3.1. The difference between a typical workflow and this experiment was the sample type. I used the biomass of microorganisms on the filters instead of soil (step 1, Fig. 3.2). The extracted DNA samples (step 8, Fig. 3.2) were placed at 4°C in a fridge before further analyses were performed, as the following description.

Figure 3.2: FastDNA™ SPIN Kit for Soil Typical Work Flow (from Instruction Manual of FastDNA™ SPIN Kit for Soil kit).

The extracted DNA concentrations were measured by a Qubit 2.0 fluorometer (Life Technologies, USA). The following process is shown in Figure 3.3. Firstly, a standard curve of double-stranded DNA (dsDNA) was prepared with two points, Standard #1 and #2. Then reactions were prepared for the extracted DNA samples. Each reaction consisted of 190 µL of broad range (BR) buffer and Qubit™ Reagent, and 10 µL of an extracted DNA sample (Fig. 3.3). Basically, the fluorescent reagent is bound to the DNA. According to the DNA standard

curve, the fluorescent intensity is detected and converted to DNA concentration. The broad range standard detected the DNA concentrations in a range of 0.0002 to 2 g/L. The DNA samples had to be diluted if the DNA concentration was out of that range.

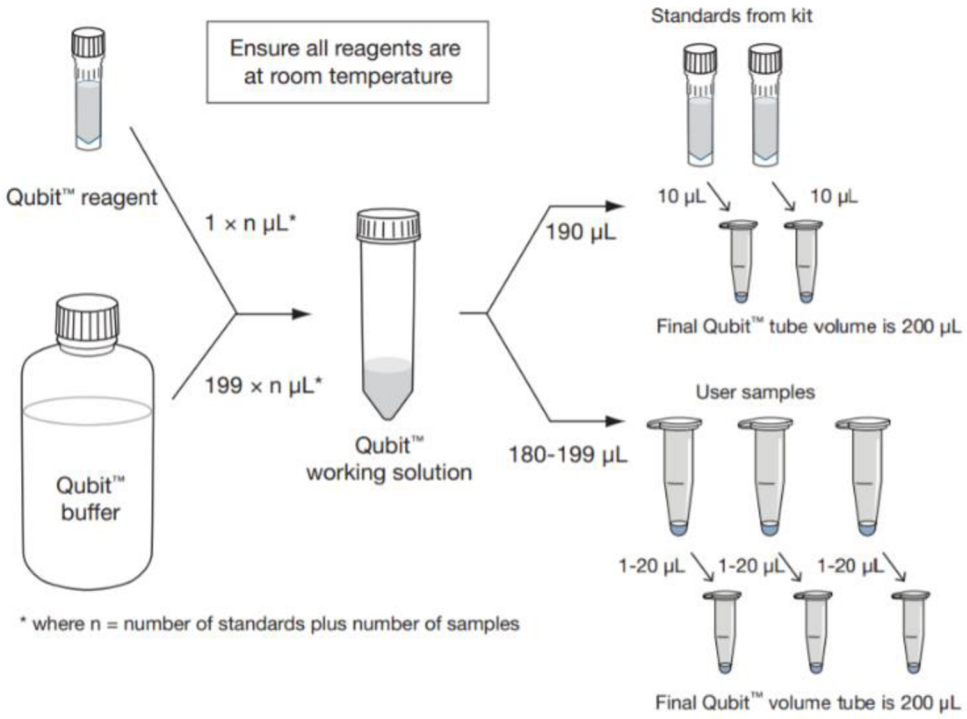

Figure 3.3: Typical Qubit samples and standards preparation workflow (from Quick Reference Qubit Assays).

3.4 Quantitative polymerase chain reaction (qPCR)

All the DNA samples were diluted to achieve a concentration of $0.2 \cdot 10^{-3}$ g/L prior qPCR measurements. A mixture was prepared for qPCR reaction. Each reaction of 10 µL comprised: 1) for bacteria: 5 µL of SybrM, 3.6 µL of qPCR water, 0.4 µL of 16S primer and 1 µL of an extracted DNA sample as a template; 2) for algae: 5 µL of SybrM, 3.2 µL of qPCR water, 0.8 µL of 18S primer and 1 µL of an extracted DNA sample. Non-template controls (NTC) were included for qPCR controls.

A qPCR experiment was performed in a 96 well plate. The plate was placed into Light Cycler 480 (Roche, Basel, Switzerland). The software version was 1.5.1.62. A qPCR with 40 cycles was used. Each cycle of the qPCR was set up with following steps: initial denaturation at 95 °C, 10 seconds for 5 minutes; annealing at 60 °C for 15 seconds, and the extension at 72 °C for 20 seconds. The melting curve was set at 98 °C and the cooling process was at 40 °C for 10 seconds.

Data from the instrument was collected and exported to an Excel file for further analyses. The qPCR generated the threshold cycle (Ct) of each sample. Ct values were averaged in duplicate in each sample and then the averaged value was compared to the control (without NPs) at each time point.

3.5 PCR amplification and NGS

The preparation steps were only observed because only the laboratory technician is authorized to perform this process. The protocol is described in the following paragraph.

All extracted DNA samples for bacterial community analysis were duplicated, with two consecutive PCR performed per sample to amplify DNA from the V4 region (normal and barcode fusion primers used). In silico analysis of primers was performed in order to cover as much diversity as possible while keeping the amplicon size below 400 bp. Amplification of the V4 region of the eubacterial 16S rRNA gene was performed with primers 515F (5′-TGCCAGCMGCNGCGG-3′)47 and barcode 802R (5′-TACNVGGGTATCTAATCC-3′) (Claesson et al. 2010). MOCK community (collection of 6 bacterial genomes prepared in-house) was subsequently sequenced to verify data evaluation. The PCR cycle conditions were as follows: the first PCR 95 °C for 3 min; 10 cycles at 98 °C for 20 s, 50 °C for 15 s, and 72 °C for 45 s, with a final extension at 72 °C for 1 min; the second PCR 95 °C for 3 min; 35 cycles at 98 °C for 20 s, 50 °C for 15 s, and 72 °C for 45 s, with a final extension at 72 °C for 1 min. The concentration of purified PCR products was measured with a Qubit assay. Barcode-tagged amplicons from different samples were then mixed in equimolar concentration. Sequencing of bacterial amplicons was performed on the Ion Torrent platform (Life Technologies, USA).

3.6 Data analysis

This work was performed by the bioinformatician in the Applied Biology department. I learned to understand and interpret the data.

The raw reads were processed by Mothur software. Low-quality reads were removed and sequences were assigned to each sample. Chimeric sequences were identified using UCHIME50 and subsequently removed. Sequences exceeding 400 bases were trimmed, and sequences shorter than 180 bases removed. Sequences were classified against Silva database v. 123 with a bootstrap value set at 80%. A cut-off value of 97% was used for clustering sequences into operational taxonomic units (OTUs). Sequence data were normalized to groups with the least sequences by randomly selecting a selected number of sequences from each sample. Cluster analysis by Bray–Curtis dissimilarity was undertaken using Vegan in the R statistical package (Oksanen et al. 2020).

3.7 Bacteria under epifluorescence

5 µL of each sample was prepared and mixed with 5 µL of a Live/Dead fluorescent staining (Life Technologies, USA) and inoculated for 15 minutes in the dark. Then microbial images were taken and observed using an Axio Imager epifluorescence microscope (Zeiss, Germany), with excitation set at 470 nm and emission at 490–700 nm.

3.8 Characterization of CeO_2 NPs in different pH freshwater

In this experiment, I studied the behavior of CeO_2 NPs in three types of water with different pH, including Jizera, Ještěd and Harcov reservoirs with pH of 6.6, 7.6 and 8.3, respectively (Figure 3.4).

First, water was collected from different sites and filtered through 0.22 µm pore size to remove all microorganisms. Vials were prepared and autoclaved. Then, 10 mL of filtered water was added to each vial and CeO_2 NPs added to obtain concentration of 0.01 g/L. Similar conditions as in the above experiment were experimented with. Samples were taken after 0, 5 and 30 days. The potential of hydrogen (pH) was measured using a standard multimeter (WTW, Germany). CeO_2 sizes were detected using a DLS (Malvern Instruments, UK) with a 633 nm laser source and a detection angle of 173°. The same instrument measured electrophoretic mobility, and subsequently transformed to zeta potential (ZP) using Smoluchowski's approximation. Each sample was measured in triplicate at 30 s intervals. The workflow overview is shown in Fig. 3.4.

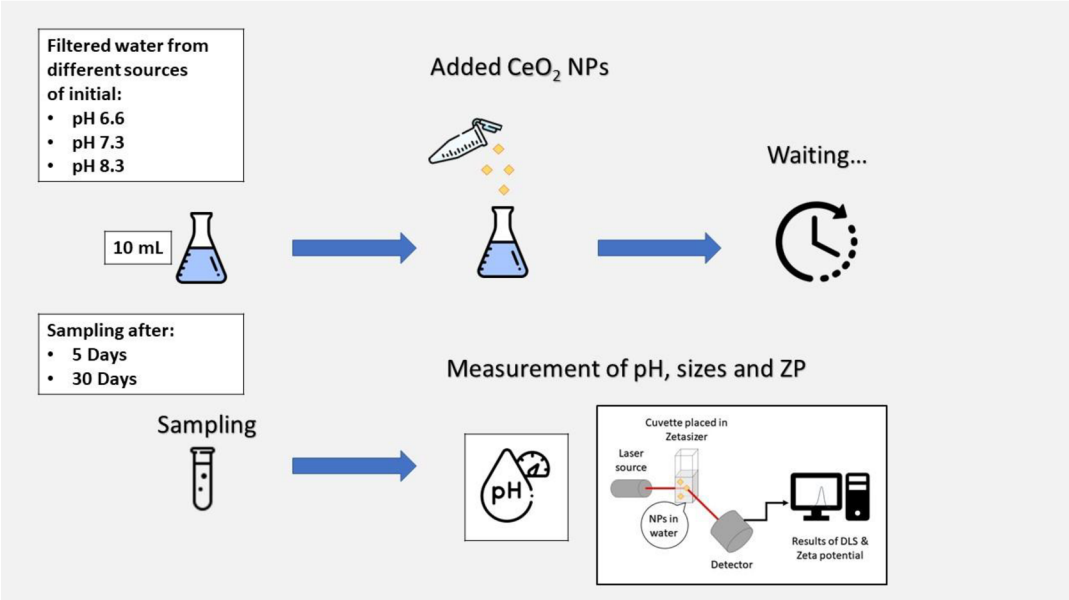

Figure 3.4: Workflow of the second experiment (beginning at the upper left corner): behaviors of CeO_2 NPs in three pH water types (pH, 6.6, 7.6 and 8.3).

4 Results and Discussion

The study was performed in order to deepen our knowledge about the effect of CeO_2 NPs on river algae and bacteria. The bacterial community was studied using next-generation sequencing (NGS). The particles used were of three sizes and added to the samples at different concentrations (Table 3.1). NGS analysis was performed only with particles of the size 4 nm. The reason for this was the assumption of more interesting effects of smaller NPs (Angel et al. 2015; Yokel et al. 2014), a deeper interest in understanding the effect of collyrium and the available budget. The second experiment focused on particles and their behavior in waters of different pH.

4.1 pH in the microbial exposure experiment

The pH of all samples was measured at each sampling point and the pH values are shown in Table 4.1. After 5 days, the pH values in samples of 4 nm CeO_2 NPs were comparable to the control, while they were slightly increased in the case of 25 nm CeO_2. In the presence of collyrium, the pH values were similar to the collyrium control.

All samples showed a similar increase in pH after 14 days. All naked samples of both sizes and collyrium samples became more alkaline, including the control (water without NPs). Naked CeO_2 4 nm 0.01 g/L (pH 8.76) and CeO_2 collyrium 4 nm 0.05 g/L (pH 8.4) were the only samples with pH below 9. The sample which contained collyrium (collyrium CeO_2 4 nm; 0.01 g / L) showed the highest pH value of 9.76.

Interestingly, the pH values were reduced to neural values after 28 days, except for the naked CeO_2 4 nm NPs and collyrium CeO_2 4 nm 0.05 g/L (pH around 8). All flasks with samples but one (collyrium 4 nm 0.05 g/L) showed visible green color. This was probably because algae grew better in those samples compared to others. However, the collyrium 4 nm 0.05 g/L) changed to white-yellow color. The increase and consequent decrease of pH values might correspond to the carbon dioxide (CO_2), taken up by algae (Nyholm et al. 1989). The experiment was performed with sealed flasks which were shaken daily by hand. It could be that between the gas and liquid phase there was no aeration and limited transport of CO_2. Algae could also derive CO_2 from bicarbonate (HCO_3^-) and produce OH^- ions (Nyholm et al. 1989). The pH was increased until the CO_2 sources were exhausted, and algae started being affected. This enervation of nutrients finally led to a decrease of algal growth. The pH returned to the beginning values by reabsorption of CO_2 and OH^- ions. The inorganic nitrogen uptake could have affected the slight difference in final and original pH. The bacterial communities might also effect pH changes. The results are further discussed below (Section 4.3).

Table 4.1: pH measurement of samples after 5, 14 and 28 days. Each sample was performed in duplicate.

	5 Days	14 Days	28 Days
Control (filtered water)	7.485	9.245	7.515
naked 4 nm 0.001 g/L	7.5	9.1	8.045
naked 4 nm 0.01 g/L	7.505	8.755	8.095
collyrium 4 nm 0.001 g/L	7.78	9.06	7.585
collyrium 4 nm 0.01 g/L	7.83	9.76	7.465
collyrium 4 nm 0.05 g/L	8.02	8.395	8.1
naked 25 nm 0.001 g/L	7.96	9.135	7.595
naked 25 nm 0.01 g/L	7.97	9.4	6.765
naked 25 nm 0.05 g/L	8.015	9.395	7.21
collyrium based 0.5% (collyrium control)	8.025	9.415	7.945

4.2 DNA concentration

The DNA concentration was measured using Qubit right after the DNA extraction, and the results are shown in Fig. 4.1. Generally, DNA concentrations were highly increased after 14 days, while they were comparable after 5 and 28 days.

After 5 days, DNA concentrations were more or less comparable to the control (water without NPs). Collyrium CeO_2 4 nm 0.05 g/L had the highest concentration (10 ng/µL). This could suggest that the collyrium stimulated microbial growth.

After 14 days, all samples had higher DNA concentrations, including the control (6.34 ng/µL), which was predicted from the pH results. The DNA concentrations in samples of naked CeO_2 25 nm 0.001 g/L, naked CeO_2 25 nm 0.05 g/L, collyrium CeO_2 4 nm 0.001 g/L and 0.01 g/L exhibited even higher concentrations than the control. For example, naked CeO_2 25 nm 0.001 g/L had 9.56 ng/µL and 0.05 g/L had 8.48 ng/µL; collyrium CeO_2 4 nm 0.001 g/L had 10.14 ng/µL and 0.01 g/L had 7.64 ng/µL. The DNA sample in the collyrium based 0.5% was slightly (4.64 ng/µL) lower than the control. The lowest DNA concentrations were found in the naked CeO_2 4 nm with 0.01 g/L (1.3 ng/µL) and the collyrium CeO_2 4 nm 0.05 g/L (1.71 ng/µL).

At the last sampling point (28 days), the DNA concentration was increased in all samples compared to the control (2.66 ng/µL). Samples with the highest concentration at this sampling point were collyrium based (6.77 ng/µL), collyrium CeO_2 4 nm 0.001 g/L (5.32 ng/µL),

and 0.01 g/L (6.52 ng/µL). Other samples with naked CeO_2 NPs had only a slightly higher concentration.

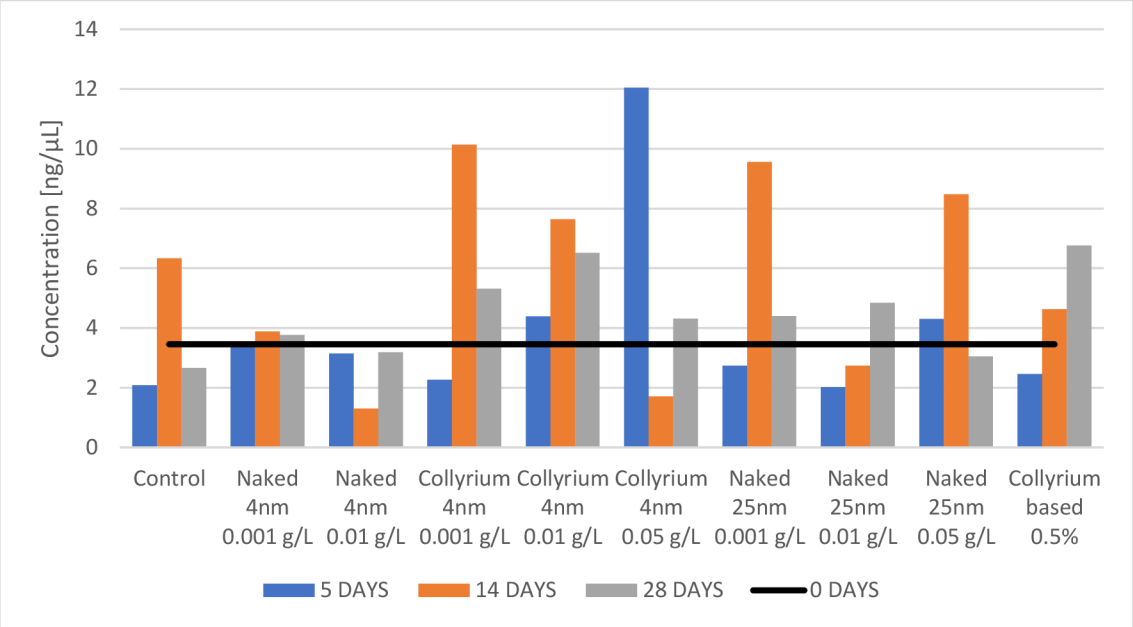

Figure 4.1: DNA concentration after 0, 5, 14 and 28 days measured by Qubit fluorometer. The black line represents the initial concentration (days 0).

4.3 Relative microbial abundance by qPCR

Algal relative abundance

Overall, the algal relative abundance was increased after 5 and 14 days and decreased after 28 days in all samples (Fig. 4.2).

After 5 days, the algal abundance was affected by CeO_2 NPs 4 nm in both naked and collyrium compared to the control. This could suggest that algal growth was affected by CeO_2 NPs sizes in a short time. In the case of naked CeO_2 NPs 25 nm, both concentrations of 0.001 g/L and 0.05 g/L resulted in higher abundance than the control.

After 14 days, the algal relative abundance increased in all samples compared to samples after 5 days. Interestingly, the abundance decreased in all collyrium CeO_2 4 nm samples, except for the collyrium CeO_2 4 nm at the lowest concentration 0.001 mg/L. It had the lowest relative abundance with the value of 12, while the control had the value of 15. In contrast, the relative abundance increased with increasing concentrations of naked CeO_2 25 nm. The relative abundance also increased in the collyrium based sample. This result might indicate that the small size of CeO_2 NPs might have a more negative impact.

After 28 days, the relative abundance was reduced in all CeO_2 types and concentrations as well as the control compared to days 5 and 14, which suggested that the bioavailable nutrients in water were consumed. This led to a higher negative impact on algae in samples with naked CeO_2 NPs, which were already affected by CeO_2. Moreover, the algal growth was also correlated to the pH values from Table 4.1. This presumes that the algae were impacted by the static character of the batch experiment, which is opposite to the dynamic natural conditions in rivers.

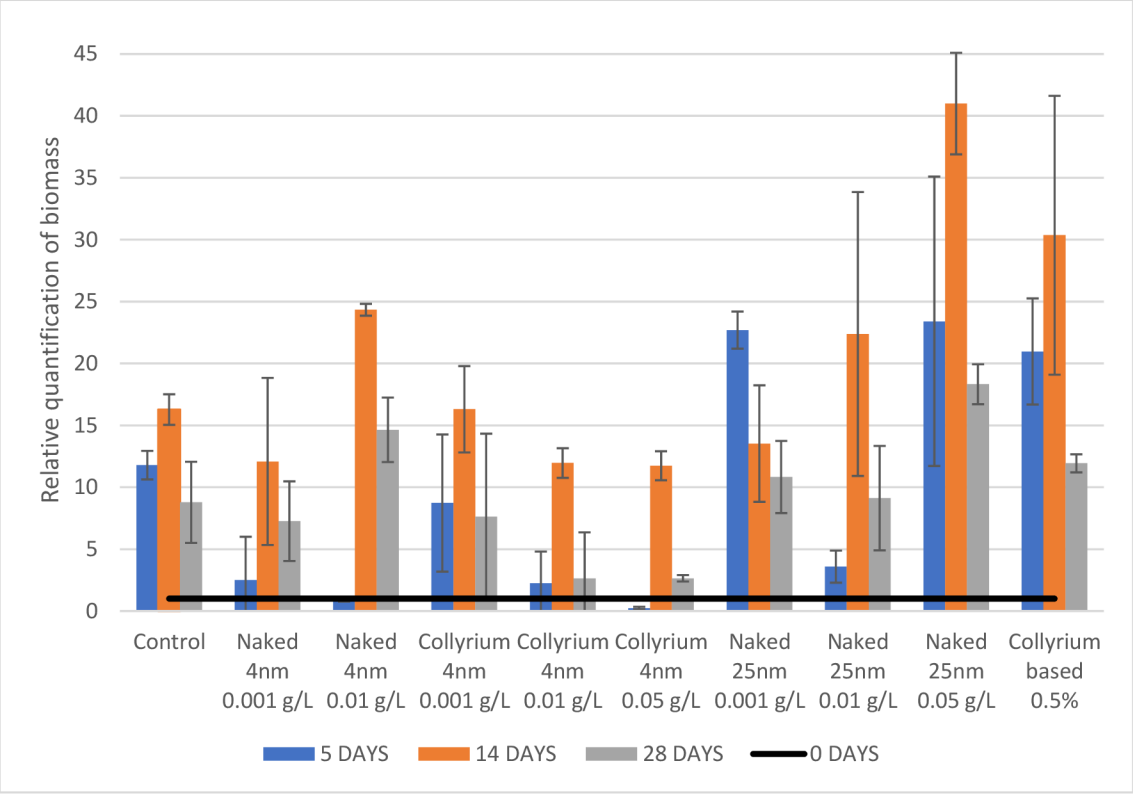

Figure 4.2: Relative quantification of algal biomass. Day 0 is the black line and the relative abundance is 1.

Bacterial relative abundance

Overall, bacterial relative abundance was less fluctuating during 28 days but was generally reduced compared to the control at day 0 (Fig. 4.3).

After 5 days, only two samples, naked CeO_2 4 nm, 0.01 g/L and naked CeO_2 25 nm, 0.01 g/L, showed lower abundance than the control. After 14 days, bacterial relative abundance was comparable or increased in all samples compared to the control. Bacteria continued increasing up to day 28, especially naked CeO_2 4 nm 0.01 g/L.

The bacterial relative abundance showed an opposite trend compared to the algal profile (Fig. 4.2 & 4.3). It could be presumed that algal cells adapted quicker to the static condition

and bloomed at least during the first 14 days. After consuming the nutrients, the algae starved, and their growth was reduced. Dead algal cells might then be used by bacteria as their new nutrient source (Cole 1982; Rhee 1972). This could explain why the bacterial relative abundance could increase after 28 days.

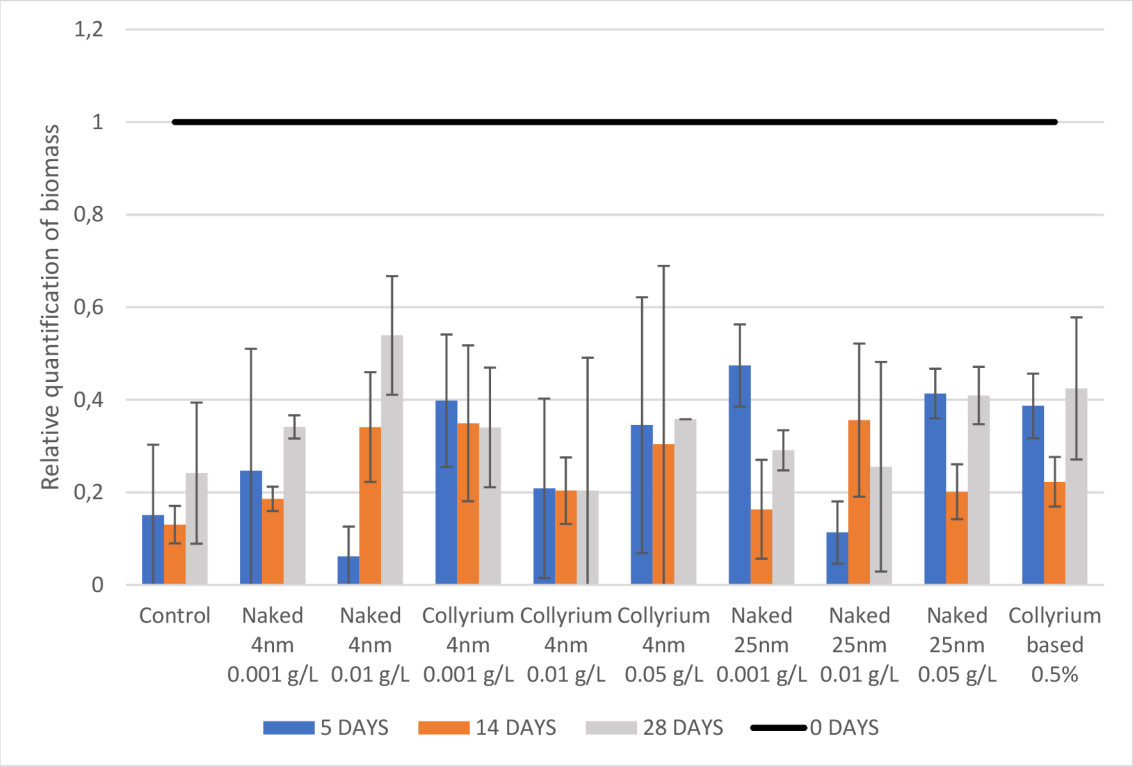

Figure 4.3: Relative quantification of bacterial biomass. Day 0 is the black line and the relative abundance is 1.

4.4 Next-generation sequencing

Next-generation sequencing of bacterial DNA revealed the effect of CeO_2 NPs on bacterial diversity and community structure.

Alpha diversity

Alpha diversity refers to the bacterial diversity within samples (Fig. 4.4). Generally, the diversity was dynamically changed at lower NPs concentrations, while it was continuously reduced at higher concentrations (0.01 and 0.05 g/L) during the experiment. The diversity within the control sample continuously decreased until day 14 and then increased in 28 days. The sample with only collyrium showed a higher diversity after 5 days than the original sample from the river, while it dropped sharply after 14 and 28 days. All samples including collyrium CeO_2 4 nm (0.01 g/L and 0.05 g/L) and naked CeO_2 (0.001 and 0.01 g/L) showed the same pattern in decreasing diversity from days 5 to 28. Notably, the diversity

was similar after 14 and 28 days in naked CeO_2 NPs 0.001 g/L. Thus, it could be concluded that lower CeO_2 NPs concentrations had no or only minor effect on the diversity. At higher concentrations, the diversity tended to decrease. The diversity was more reduced in collyrium with or without CeO_2 NPs 4 nm than in naked CeO_2 samples. It might be that the negative effect was caused by the collyrium or smaller CeO_2 NPs sizes (Brennan et al. 2006; Sapsford et al. 2013; Yeh et al. 2014; Yokel et al. 2014).

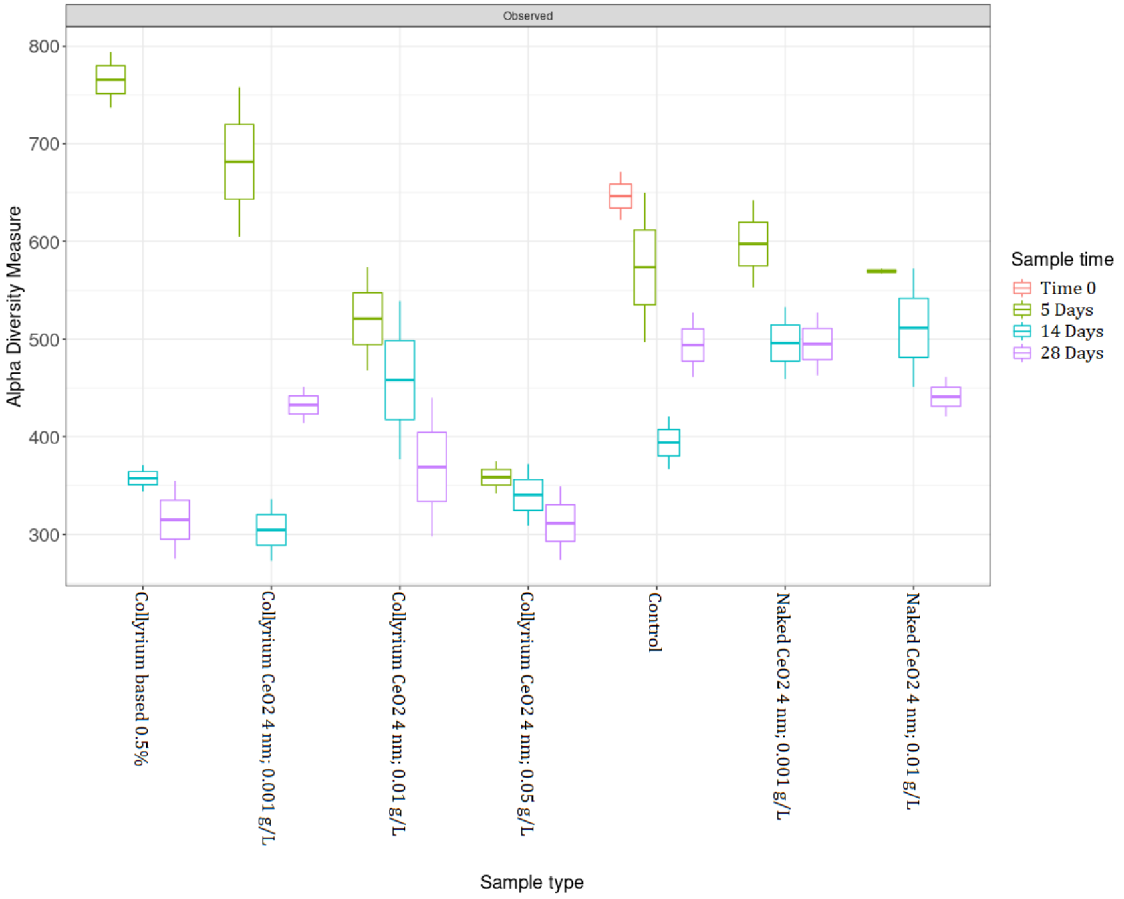

Figure 4.4: Alpha diversity of samples without NPs (Control), with Collyrium, with Collyrium CeO_2 NPs, and naked CeO_2 NPs.

Bray-Curtis dissimilarity

The Bray-Curtis dissimilarity showed the dissimilarity between samples (Fig. 4.5). A value of 1 indicates the maximum dissimilarity, and thus, the minimum microbial diversity was shared in the samples. Most of samples showed different bacterial diversity at other sampling times. However, at the 5 days sampling point, bacterial diversity was the most similar. The group of samples with the highest similarity of microbial abundance was the control (0 days and 5 days), collyrium based (5 days), and collyrium CeO_2 4 nm from 5 days with

the lowest concentration (0.001 g/L). This could indicate a similar development of the microbial communities shared in these samples.

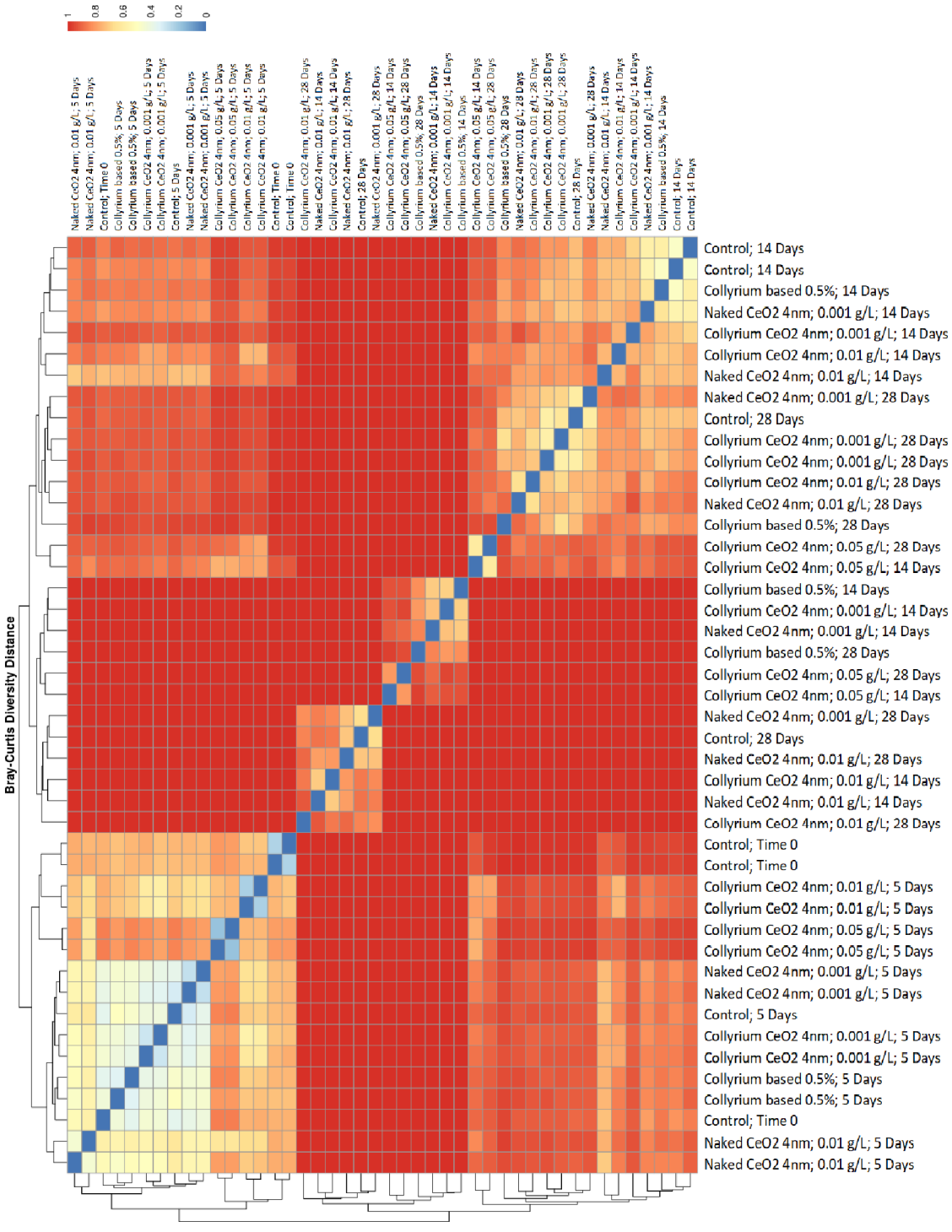

Figure 4.5: Bray-Curtis dissimilarity of samples without NPs (control), with collyrium, with collyrium CeO_2 NPs, and naked CeO_2 NPs.

Composition of bacterial communities

In general, some bacterial taxa proliferated after adding CeO_2 NPs, and while not found in the control samples over the experimental time (Fig. 4.6). There were two predominant bacterial populations, including the genus *Flavobacterium* and the family C*omamonadacea*e, found in the initial sample and exposure samples during the experiment. Genus *Flavobacterium* is regarded as aerobic, gram-negative, and does not form spores (Waśkiewicz et al. 2014). It is a physiologically diverse species and occurs in multiple environments, including freshwater and soil. *Flavobacterium* plays a role in the decomposition and degradation of organic matter. *Comamonadaceae* is also very diverse, large (over 100 species), and comprises mostly environmental bacteria from water and soil (Willems 2014). Representatives can be found in nature as well as in polluted areas. Many of them are saprophytes and they therefore play a significant role in the environment. In addition to the family C*omamonadacea*e and genus *Flavobacterium*, the family *Verrucomicrobiae*, genus *Polynucleobacter* (class Betaproteobacteria), and genus *Sediminibacterium* were present in considerable quantities.

After 5 days, bacterial composition dynamically changed. The genus *Flavobacterium* and family *Comamonadaceae* were slightly reduced and other communities were also present, including *Candidatus Aquirestis* from the *Saprospiraceae* family and genus *Limnobacter*. Interestingly, the class *Verrucomicrobiae* increased in naked CeO_2, collyrium samples and the control, while *Sediminibacterium* was highly presented in collyrium CeO_2 NPs. Genus *Methyloversatilis* showed in all samples containing CeO_2 NPs and increased with increasing CeO_2 concentration. In contrast, the genus *Candidatus Aquirestis* highly decreased at low NPs concentrations and disappeared at higher concentrations of CeO_2. The genus *Limnobacter* in naked CeO_2 0.001 mg/L was comparable to the control and was not found in other samples.

After 14 days, the bacterial composition was changing with no clear pattern. The genus *Candidatus Aquirestis* was found in the control and naked CeO_2. The genus *Limnobacter* recorded a high increase only in naked CeO_2 and collyrium CeO_2 0.001 g/L and one control. The genera *Sediminibacterium* and *Methyloversatilis* continuously increased in collyrium CeO_2, especially at a higher concentration of 0.05 g/L. In a previous study, CeO_2 NPs (20 nm) showed that bacterial composition was only similar to 41% after 14 days of adding CeO_2 compared to the control (without CeO_2) (Qiu et al. 2015).

After 28 days, the composition of the bacterial communities was slightly different. The greatest change was found in the genera *Niveispirillum* and *Cyanobaterium* PCC-6307. The

genus *Niveispirillum* was found in all samples with CeO_2, collyrium based and control. Surprisingly, the genus *Cyanobium* PCC-6307 highly increased in a higher concentration of naked CeO_2 4 nm (0.01 g/L) and a low concentration of collyrium CeO_2 4 nm (0.001 and 0.01 g/L). However, they only appeared in one replicate. The genus *Sediminibacterium* was comparably increased in collyrium CeO_2 after 14 days of samples.

Compared to long-term experiments from previous studies, the impacts of CeO_2 NPs on bacterial communities showed no clear trends. For example, in freshwater, CeO_2 NPs 1 mg/L (90 nm) showed positive effects on bacterial communities after 4 weeks (Bour et al. 2016). CeO_2 NPs (1 mg/L) significantly reduced the genera *Flexibacter* and *Acinetobacter* in activated sludge for 210 days (Wang et al. 2018). At the same time, CeO_2 NPs (25 nm) did not affect bacterial communities at 1 mg/L after 8 weeks of exposure.

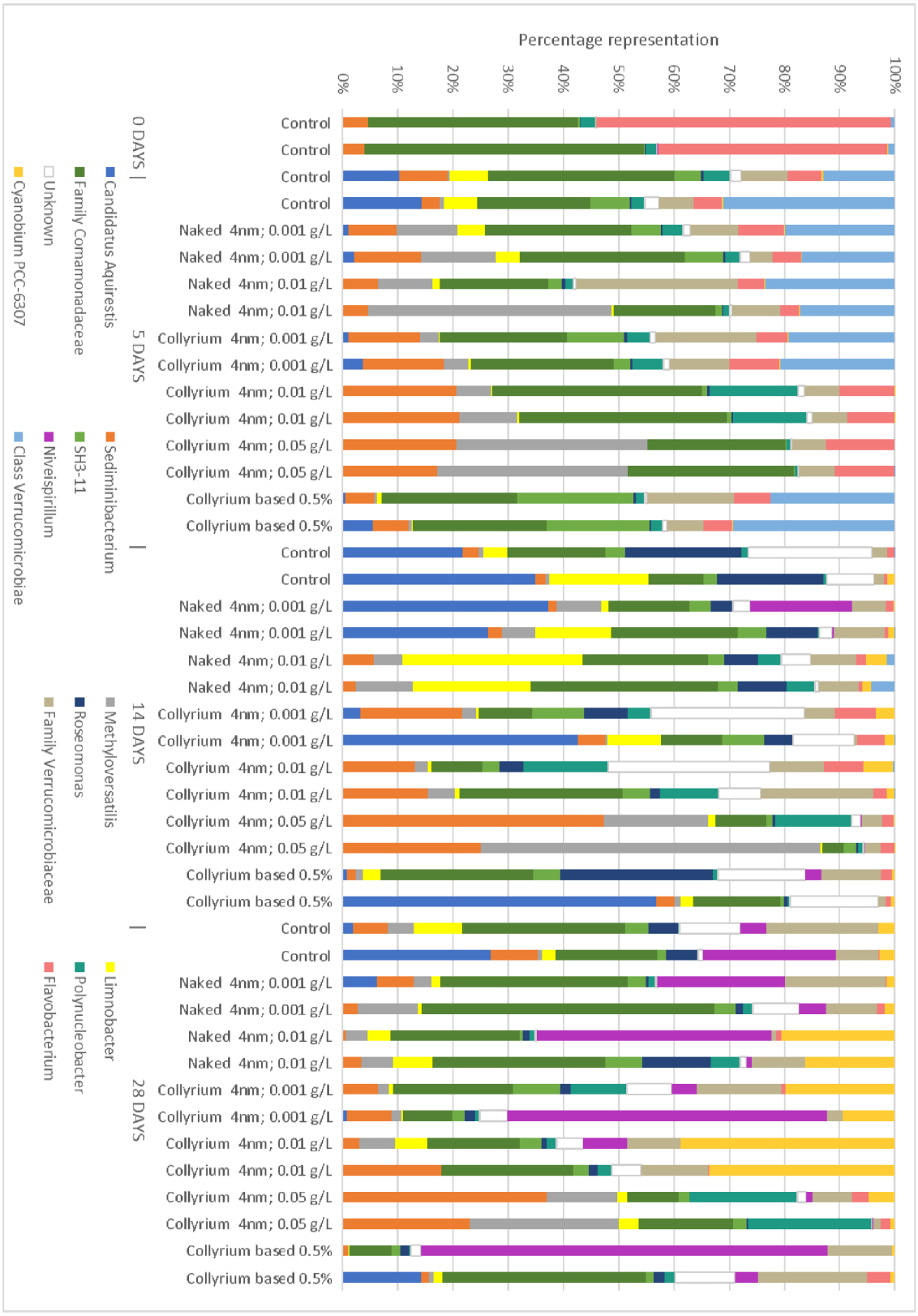

Figure 4.6: Composition of bacterial community from duplicate samples without NPs (control), with collyrium only (collyrium based), with collyrium CeO_2 NPs (collyrium 4 nm), and naked CeO_2 NPs (naked 4 nm).

4.5 Epifluorescence microscopy

Representatives of bacterial cells can be seen in the images obtained by epifluorescence microscopy (Fig. 4.7).

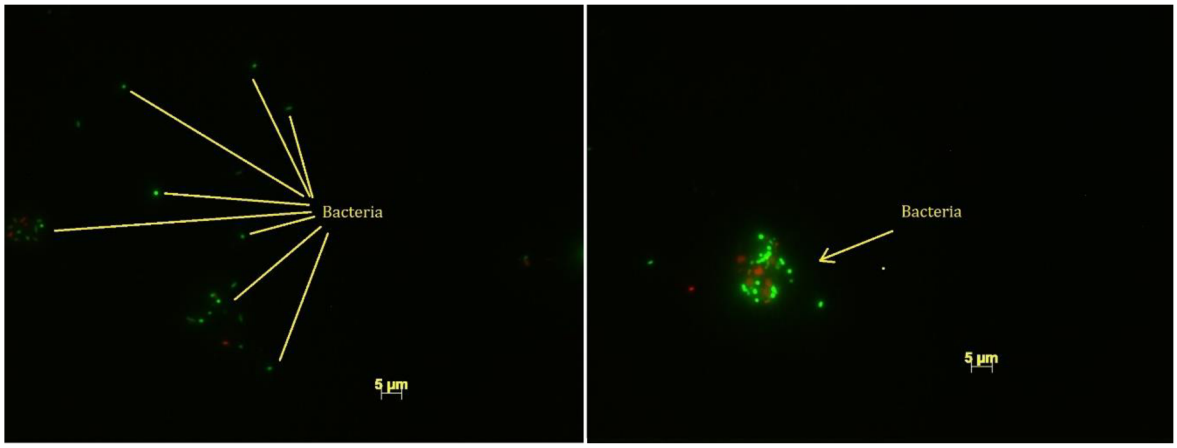

Figure 4.7: Images of living bacteria (green fluorescence) obtained by epifluorescence microscope.

4.6 Scanning electron microscope

SEM images were taken of all CeO_2 NPs (Fig. 4.8). The naked 4 nm NPs could not be identified from the images due to their small size. The collyrium CeO_2 NPs 4 nm are shown in Figure 4.8, part A. It was not possible to distinguish between CeO_2 NPs and collyrium. The collyrium and CeO_2 NPs probably form the web-like structure. The naked CeO_2 NPs 25 nm are shown in Figure 4.8, part B. Here it is possible to distinguish individual particles and the size matches the manufacturer's information.

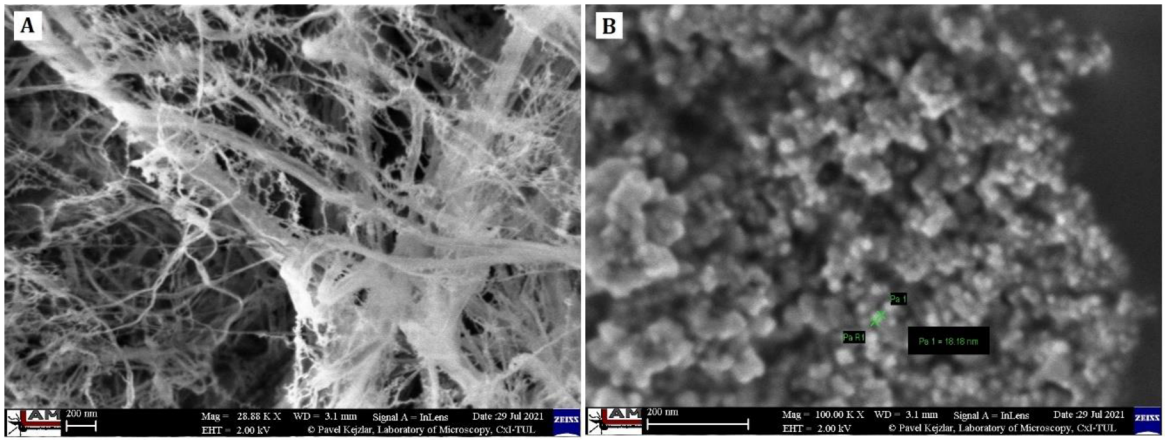

Figure 4.8: Electron microscope images. Figure A shows CeO_2 NPs 4 nm with collyrium. In Figure B are the CeO_2 NPs 25 nm.

4.7 CeO_2 behavior in freshwaters of different pH

The changes in pH were recorded in Table 4.2. Generally, pH values changed to neutral for all samples after 5 days and 30 days compared to the initial samples, and pH values were 6.6 and 7.6. For example, pH of 8.3 was decreased to between 7.2 to 7.7, while pH of 6.6 was increased between pH 7.5 and 8.5. At the last sampling point, the pH was slightly reduced.

Table 4.2 pH values of duplicate samples before DLS measurement. Concentrations of CeO_2 NPs used for all samples were 0.01 g/L.

Source	Particle type	5 days		30 days	
Jizera (pH 6.6)	Naked CeO_2 25 nm	7.93	7.88	7.4	7.74
	Naked CeO_2 4 nm	7.79	7.69	7.13	7.4
	Collyrium 4 nm	7.66	7.59	7.59	7.55
Ještěd (pH 7.6)	Naked CeO_2 25 nm	7.5	7.55	7.06	7.1
	Naked CeO_2 4 nm	7.36	7.45	7.38	7.3
	Collyrium 4 nm	7.4	7.67	7.46	7.6
Harcov reservoir (pH 8.3)	Naked CeO_2 25 nm	7.63	7.55	7.18	7.18
	Naked CeO_2 4 nm	7.56	7.54	7.35	7.39
	Collyrium 4 nm	7.76	7.62	7.67	7.64

Size of CeO_2 NPs in different pH

The average size of CeO_2 NPs generally tended to increase over time. The NPs were distorted by the formation of aggregates. Sizes of naked CeO_2 25 nm were stable at pH 6.6 (180 nm) and 7.6 (500 to 600 nm), while they increased at pH 8.3 (180 to 400 nm) over the experiment.

In contrast, naked CeO_2 4 nm increased in size at lower pH 6.6 (180 to 500 nm) and pH 7.6 (50 to 600 nm) and slightly decreased at higher pH 8.3 (180 to 80 nm).

In the presence of collyrium, CeO_2 NPs 4 nm showed a similar trend to naked CeO_2 4 nm. At an initial pH of 7.6 (closest to a neutral environment), all species of NPs reached a similar hydrodynamic size (slightly above 600 nm) after 30 days, and stayed stable at pH 8.3 of an average of 200 nm. According to the literature, aggregates may show toxicity (Milani et al. 2017; Röhder et al. 2014).

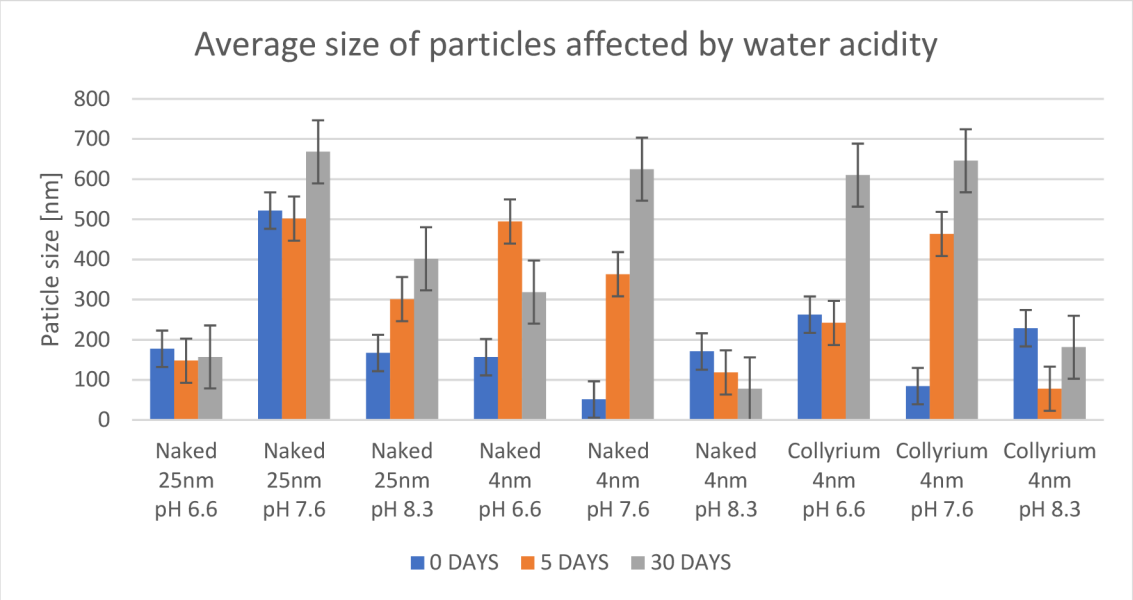

Figure 4.9: Average hydrodynamic particle size in different pH.

Zeta potential

The ZP all showed negative values in a range of -5 and -25 mV and a minor change in different time points (Fig. 4.10) In order for a particle to be considered stable, its ZP should be higher than +30 mV, or lower than -30 mV. Otherwise, NPs tend to aggregate (Malvern 2003). The lower ZP value could mean that NPs might be reactive and impact on microorganisms (Malvern 2003; Milani et al. 2017; Röhder et al. 2014). Connecting with our results, all ZP of CeO_2 NPs were out of the stable range. It could be presumed that CeO_2 NPs tended to aggregate.

The ZP of 25 nm NPs was the most negative at pH 6.6 (-20 mV), less negative at pH 7.6 (-18 mV) and least at pH 8.3 (-15 mV). Both naked and collyrium CeO_2 4 nm NPs had a similar trend at day 0. The ZP of both cases had the same average of -10 at pH 6.6. The ZPs were increased and had an average value of -15 mV at pH 7.6, similar to naked CeO_2 NPs. At pH 8.3, naked CeO_2 had slightly lower ZPs (-10 mV) compared to collyrium CeO_2 NPs (-13 mV). The lowest ZP value was -5 mV, happening in the naked CeO_2 4 nm pH 8.3 sample.

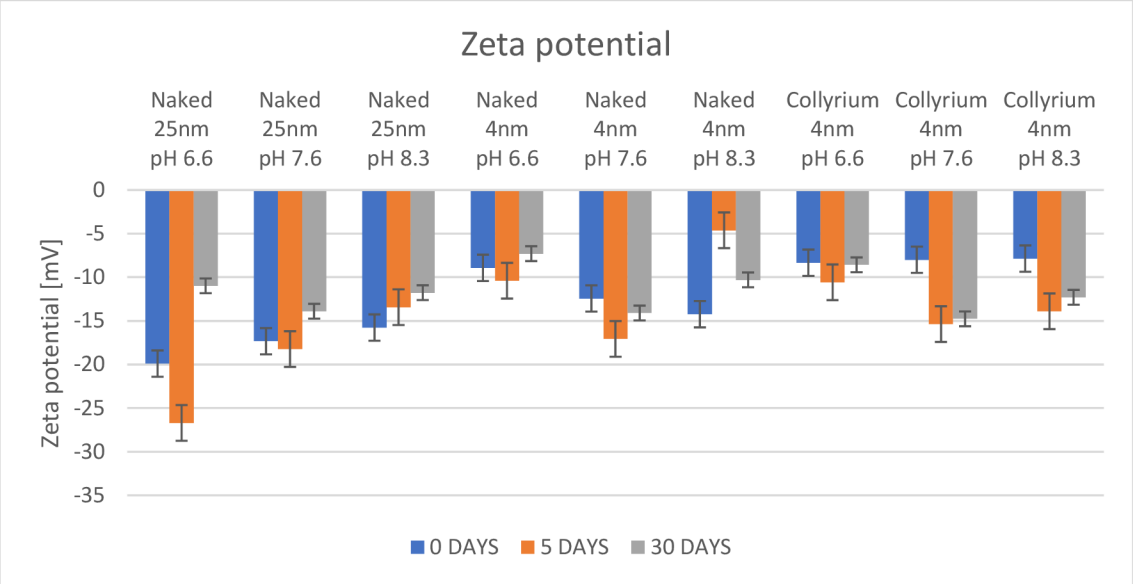

Figure 4.10: Zeta potential of naked CeO_2 NPs (size 25 nm and 4 nm) and NPs with collyrium (size 4 nm).

5 Conclusion

In summary, the thesis explored the influence of CeO_2 NPs on freshwater microorganisms (algae and bacteria) during the 28-day study, whilst a more detailed part of the thesis was devoted to bacteria. In addition, the behavior of CeO_2 NPs in natural water of different pH was investigated.

Based on the qPCR results, the algal relative abundance highly increased after 14 days and was almost unaffected by CeO_2 NPs 25 nm during the experiment. The CeO_2 NPs 4 nm with or without collyrium slightly decreased the algal abundance after 5 and 28 days. The relative bacterial abundance decreased compared to the initial abundance. However, it did not show any clear trend, only slightly increased after 28 days, probably due to extra nutrients gained from decomposed algal cells.

The NGS analysis revealed that lower CeO_2 NPs concentrations had no or negligible effect on the bacterial diversity, while at higher NPs concentrations, the diversity tended to decrease. The bacterial community composition developed differently after adding CeO_2 NPs over 28 days. In the presence of collyrium CeO_2 NPs, *Sediminibacterium* increased and remained high for the whole time, and *Cyanobacterium* PCC-6307 increased after 28 days. In naked CeO_2 4 nm samples, *Verrucomicrobiae* developed after 5 days, *Candidatus Aquiresis* was found after 14 days, and *Limnobacter* was detected after 28 days. *Niveispirillum* showed in one sample of both CeO_2 after 28 days.

All CeO_2 NPs were negatively charged in a range of -5 to -25 mV in all pH 6.6, 7.6 and 8.3. They tended to aggregate during 28 days in all pH, except both sizes of naked CeO_2 were stable in pH 6.6 and collyrium CeO_2 was stable in pH 8.3.

To conclude, CeO_2 NPs changed the composition of the bacterial community. Samples treated with NPs resulted in the different development of communities. However, the biodiversity remained relatively high. In our study, CeO_2 NPs showed more negative impacts on algae than bacteria. However, the impacts were also influenced by the addition of collyrium. Therefore, it is recommended that studies on the toxicity of NPs should include both pristine (naked) and modified NPs as well as additive materials (collyrium). Additionally, the characterizations of NPs should be investigated.

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