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CONTENTS

Aquatic environment

Atmospheric environment

Terrestrial environment

Environmental biology

Environmental health and toxicology

Environmental catalysis and materials

Serial parameter: CN 11-2629/X*1989*m*223*en*P*26*2014-3

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Influence of stabilizers on the antimicrobial properties of silver nanoparticles introduced into natural water

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ABSTRACT

Physical, chemical and biochemical properties of silver nanoparticles (AgNPs) depend to a great extent on their size, shape, size distribution, and stabilizers located on their surface. This study focused on two typical stabilizers, namely citrates (cit), low molecular ions protecting nanoparticles by electrostatic repulsion, and polyvinylpyrrolidone (PVP), a hydrophilic, neutral, high molecular polymer protecting nanoparticles by steric stabilization. Natural bacterioplankton was collected from a eutrophic, downtown lake and exposed to five concentrations (0.1–5 mg/L) of AgNPs-PVP and AgNPs-cit. Responses were monitored after 1, 3, 5 and 7 days of exposure, by evaluating the survival rate of bacteria, their respiratory activity, and the general activity of extracellular esterases. A significantly better (greater) survival rate of bacterioplankton was observed in water with an addition of AgNPs-cit. The inhibition of extracellular esterases was observed only in samples containing AgNPs-PVP. The inhibitory effect increased proportionally to the concentration of AgNPs-PVP applied. Within the studied concentration range, there was no statistically significant inhibition of bacterioplankton respiratory activity by AgNPs-PVP and AgNPs-cit.

Introduction

The last two decades saw a rapid development of nanotechnology. The structure, properties, and methods for obtaining nanoparticles (NPs) have been widely discussed in scientific literature (Kahru and Dubourguier, 2010). Nanoparticles have been successfully applied in electronics, tissue engineering, biomedicine, nanocomposite manufacturing, and everyday objects and substances such as paints, cosmetics, and underwear (Nanowerk Nanomaterial Database Inventory, 2012).

At the same time information on potential risks connected with NPs is 10–20 times less abundant that the information on their obtaining and applying. Recent research indicates that NPs are not as indifferent to human health and natural ecosystems as previously believed. Their large active surface area, biological reactivity, size, shape, durability, and hydrophobicity may facilitate their movement in the air, soil, and water (Colvin, 2003; Lecoanet et al., 2004; Biswas and Wu, 2005; Wiesner et al., 2006; Holbrook et al., 2008). Moreover, NPs can also act as carriers for dangerous contaminants dispersed on their surface, and thus promote their translocation in the environment (Kleiner and Hogan, 2003; Shelley, 2005; Maynard and Aitken, 2007). Although toxicological data on NPs are easily available (for different biological levels, from *in vitro* cell cultures to *in vivo* research in rodents), they cannot be directly related to environmental conditions. Although not yet extensively developed, the research on ecotoxicological properties of NPs seems to be of great importance (Nowack and Bucheli, 2007; Kahru and Dubourguier, 2010).

Silver nanoparticles (AgNPs) are currently one of the most common metal nanoparticles found in consumer

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products. From a practical point of view bactericidal and fungicidal properties of AgNPs, undeniably their most valuable features, guarantee a variety of medical applications: silver-based dressings as well as surgical clothes and medical instruments coated with AgNPs have gained immense popularity (Furno et al., 2004; Li et al., 2006; Duran et al., 2007; Rai et al., 2009; Hamouda, 2012). Owing to their bactericidal properties, materials containing AgNPs are widely applied in everyday products as well – they are added to detergents, food packaging, textiles including socks, underwear, and bed linen (Morones et al., 2005; Maynard and Michelson, 2006). This widespread use inevitably leads to the release of AgNPs to natural water bodies (Benn and Westerhoff, 2008; Hassellv and Kaegi, 2009; Kim et al., 2010); the results of numerous studies indicate that AgNPs have a negative impact on the crustaceans, algae and fish, and can be considered highly toxic because L(E)C50 < 0.1 mg/L (Kahru and Dubourguier, 2010).

Considering both the importance of microorganisms in the aquatic environment and the bactericidal properties of nanosilver, the impact of AgNPs on microorganisms in the natural ecosystem should be carefully examined. This study involved investigating bacterioplankton from a natural water body and assessing several indicators of microbial activity, including respiration and enzymatic activity of microorganisms.

Physical, chemical, and biochemical properties of AgNPs depend crucially on their size and shape (Morones et al., 2005; Pal et al., 2007), as well as on their surface functionalization (Wang et al., 2005). Stabilizers are used to control the process of obtaining AgNPs and then to prevent their aggregation. What happens to AgNPs in the environment (aggregation, sedimentation, oxidation) is directly related to their stability guaranteed by appropriate surface modification (Lok et al., 2007; Kittler et al., 2010). The study is based on two typical stabilizers, i.e. citrates: low-molecular ions protecting nanoparticles by electrostatic repulsion and polyvinylpyrrolidones (PVP), hydrophilic, neutral, high-molecular polymer protecting nanoparticles by steric stabilization. In previous studies of surface-dependent toxicity of AgNPs were used only bacterial strains (El Badawy et al., 2011; Xiu et al., 2012; Sadeghi et al., 2012) or model organisms (Yang et al., 2012; Tejamaya et al., 2012). In this study, we compared the antimicrobial activity of AgNPs introduced into natural water, because the research on influence of AgNPs on environmental microorganisms seems to be not yet extensively developed.

1 Materials and methods

1.1 Materials preparation

Silver nanoparticles were prepared in water solution by a chemical reaction of silver nitrate ($AgNO_3$) with sodium borohydride ($NaBH_4$) in the presence of one of the stabilizers: polyvinylpyrrolidone (AgNPs-PVP) or sodium citrate (AgNPs-cit). Reference solutions of the same composition except for silver nanoparticles were prepared using HNO_3 instead of $AgNO_3$. All solutions were aged for a few days, then pH was set at 7, and the solutions were diluted to obtain the concentration of Ag equal to 100 mg/L.

Typical UV-Vis spectrum of AgNPs obtained by reduction of $AgNO_3$ with $NaBH_4$ possesses one maximum at 390–410 nm and FWHM (full width at half maximum) equal 50–70 nm. TEM enables to determine their shape, size and size distribution, silver nanoparticles obtained in this way and characterized by such UV-Vis spectrum are spherical, their size is in the range 5–20 nm with the maximum frequency equal 12–14 nm (Solomon et al., 2007).

UV-Vis spectrum of our AgNPs with maximum at 408 and 397 nm and FWHM equal 57.4 and 66 nm for AgNPs-PVP and AgNPs-cit respectively, enables to assume that our AgNPs are typical, similar to those obtained and characterized in other articles.

1.2 Natural bacterioplankton

Natural bacterioplankton was collected from a eutrophic, downtown lake Martówka in Toruń, Poland (**Table 1**). Natural lake water (1 L) in glass flasks was mixed with AgNPs-PVP and AgNPs-cit to achieve their final concentrations of 0.1, 0.5, 1.0, 2.0, and 5.0 mg/L. Responses were monitored after 1, 3, 5 and 7 days of exposure at 20°C. Control samples contained natural lake water. Reference samples (water with the solutions of the two stabilizers, i.e., citrates and PVP, the concentration of 5 mg/L) were prepared in order to exclude the influence of the stabilizers on bacterioplankton.

Table 1 Morphometric and trophic characteristics of "Martówka"

Characteristic	Value
Latitude	53°00′N
Longitude	18°34′E
Area	2.8 ha
Maximum depth	2.5 m
Maximum length	640 m
Maximum width	61 m
pH	8.3–8.5
Electrolytic conductivity	668–955 μS/cm
Water transparency	0.8-1.2 m
Total organic carbon	8.5–19.7 mg/dm^3
Chlorophyll *a*	1.44–16.88 μg/dm^3

The data source was from Dembowska, Nicolaus Copernicus University, Department of Hydrobiology, unpublished data.

1.3 Survival of bacteria

Survival of bacteria was assessed by using the fluorescent markers targeting the cell membrane integrity (LIVE/DEAD®BacLight™ Bacterial Viability Kit). Stained bacteria were counted under the epifluorescent Jenalumar microscope fitted with a set of inducing filters 2 × KP490 + B229, lens "Planachromat fl" with a 100× magnification, 1.30 aperture, and an eyepiece with a 10× magnification.

1.4 Activity of esterases

Activity of esterases was assessed by measuring the rate of fluorescein release from fluorescein diacetate (FDA) (Adam and Duncan, 2001). The amount of the released fluorescein was measured with a Hitachi f-2500 spectrofluorimeter using an excitation wavelength of 480 nm and an emission wavelength of 505 nm.

1.5 Respiration activity of bacteria

Respiration activity of bacteria was assessed using an OxiTop Control 12 system. Biochemical oxygen demand was determined according to the instruction manual (WTW, 1998). Natural lake water exposed to AgNPs-PVP and AgNPs-cit was incubated for 7 days at 20°C. Respiration was expressed in μg O_2/dm^3 of the sample.

1.6 Stability of silver nanoparticles

The stability of silver nanoparticles in environmental conditions was determined spectroscopically (UNICAM 8620 UV-Vis spectrometer). The concentration equals 5 mg/L was chosen to obtain the best accuracy. Absorbance of the samples was measured at 0.5 hr, 4 hr, 1, 5 and 7 days after mixing the stock solutions with the natural lake water. The samples were exposed to light and air. Additionally, for the comparison, the measurements were performed for the samples obtained by mixing the stock solutions with distilled water and for the samples kept in dark and without air.

2 Results and discussion

2.1 Survival of bacteria

Figure 1 shows that the survival rate of bacteria in the lake water (assessed with LIVE/DEAD method) was significantly lower after introducing AgNPs-PVP than after introducing AgNPs-cit. The number of dead cells increased considerably after only one day of incubation in the presence of AgNPs-PVP within the concentration range of 1–5 mg/L. After seven days of incubation in the presence of 5 mg/L AgNPs-PVP dead cells constituted as much as 65.9% of all bacterial cells. In the presence of AgNPs-cit, the concentration range of 2–5 mg/L, the number of dead cells increased after three days at the earliest. After seven days of incubation, at the maximum concentration of 5 mg/L, dead cells constituted 22.4% of all bacterial cells, which is less than two times more than in the control samples.

Although the mechanism of bactericidal activity of AgNPs is not yet fully understood, it has been presumed that silver nanoparticles may cause morphological and structural changes in bacterial cells (Rai et al., 2009). Moreover, they can affect some proteins and phospholipids in the cell membrane causing its disintegration, which inevitably leads to the cell death (Li et al., 2010). The results of proteomic studies indicate a strong response of bacterial cells to the presence of AgNPs, which suggests the destabilization of the cell membrane (Lok et al., 2006,

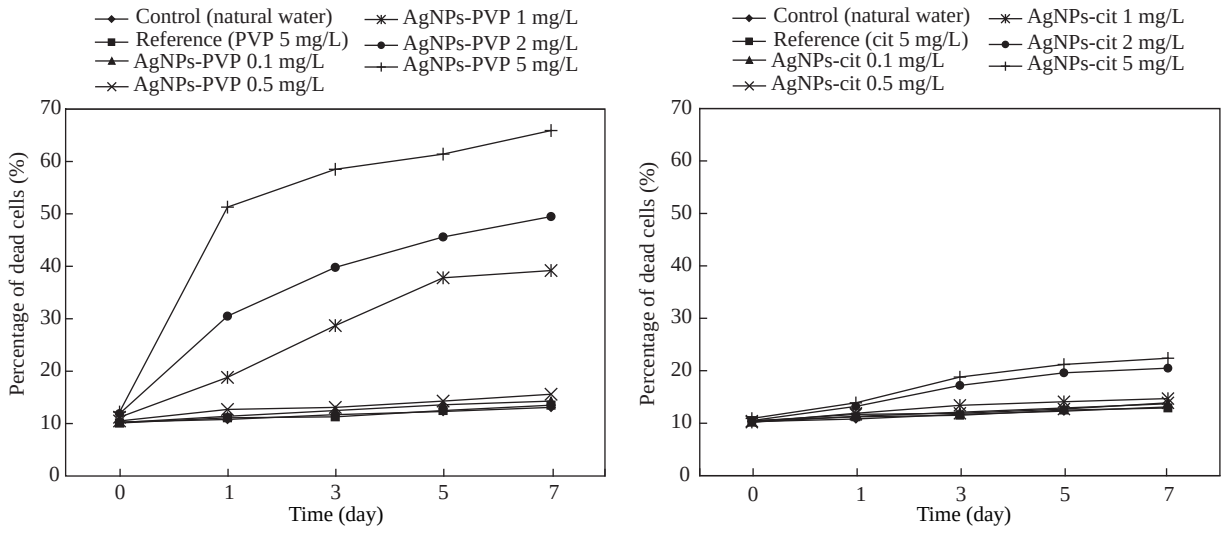

Fig. 1 Influence of silver nanoparticles introduced into natural water on survival of bacteria.

2007). However, Choi and Hu (2008) noted no statistically significant effect of AgNPs at a concentration of 1 mg/L on the survival rate of *E. coli* and autotrophic nitrifying bacteria, assessed with LIVE/DEAD method, which indicates no obvious damage to the cell membrane caused by AgNPs. The results of the present study show that at a concentration of 1 mg/L only AgNPs-PVP caused lethal damage to the cytoplasmic membrane, which indicates that both the concentration and the stabilization of AgNPs influence their force and mechanism of damaging cell structures.

Additional, unintended observation was also made: algal cells present in the lake water displayed far greater sensitivity to the presence of AgNPs, both stabilized by the citrates and the PVP. Throughout the experiment algal cells remained alive only in the control sample and in the reference samples while in samples containing AgNPs the disintegration of cell membranes was observed after only three days of the incubation (**Fig. 2**).

The results obtained by Kahru and Dubourguier (2010)

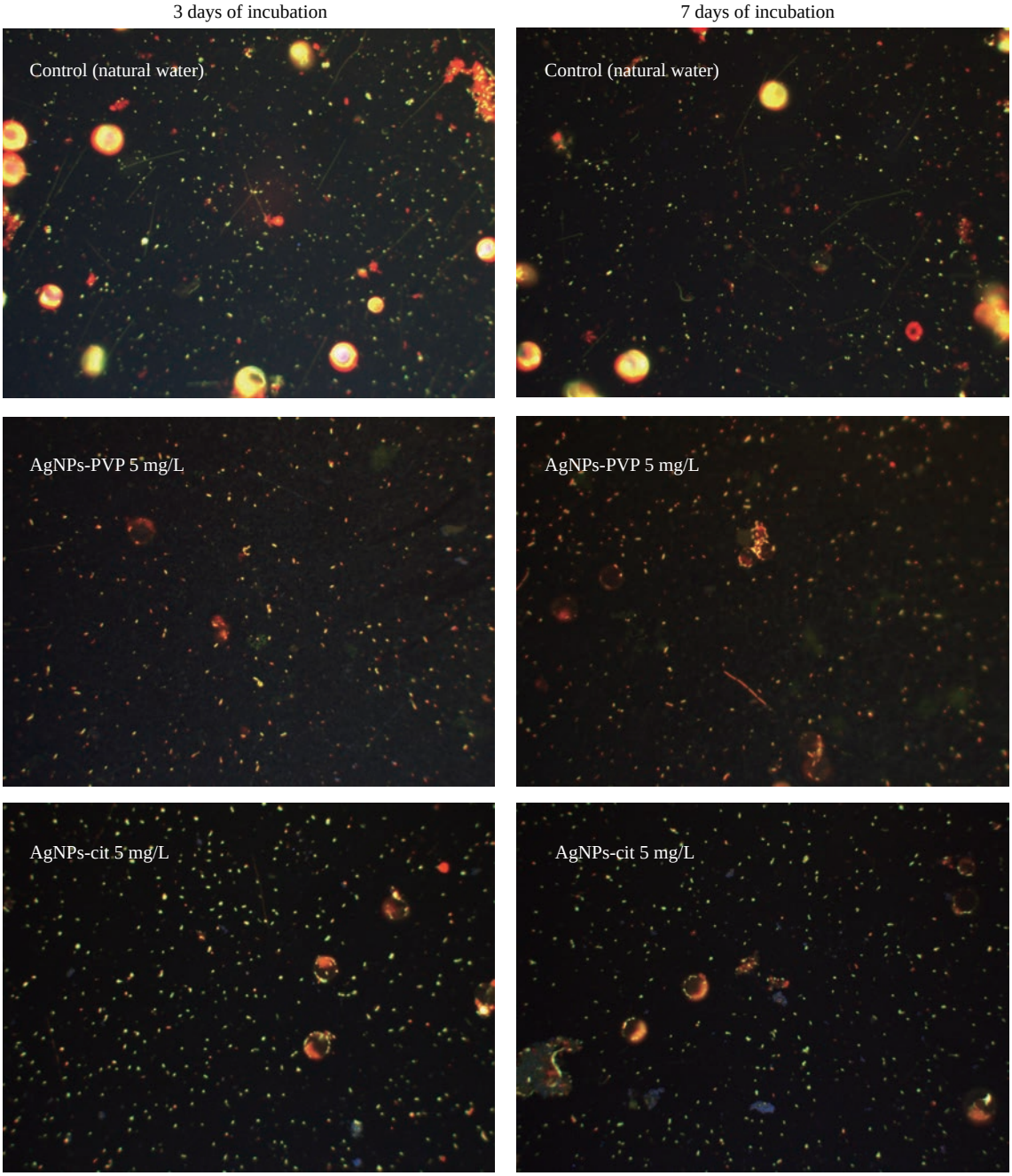

Fig. 2 Survival of bacteria assessed by using the fluorescent markers targeting the cell membrane integrity (LIVE/DEAD®BacLight™ Bacterial Viability Kit). Green: living bacteria, red: death bacteria. The algal cells remained alive only in the control sample; in samples containing AgNPs the disintegration of cell membranes was observed after only three days of the incubation.

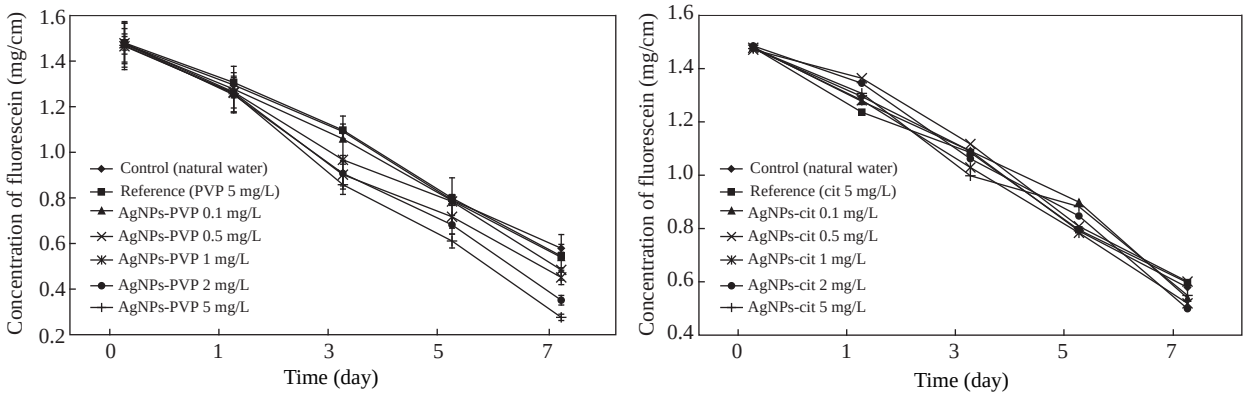

Fig. 3 Influence of silver nanoparticles introduced into natural water on extracellular esterases activity.

confirmed a significantly higher sensitivity of algae (median L(E)C50 0.235 mg Ag/L) than of bacteria (median L(E)C50 7.6 mg Ag/L) to AgNPs. According to Moore (2006) prokaryotic cells may be effectively protected against the introduction of several types of NPs as they are unable to transport colloidal particles through the cell wall (Moore, 2006). In addition, according to Navarro et al. (2008), Ag^+, much more toxic for algae than AgNPs, are created as a result of oxidation when AgNPs interacts with algal cells.

2.2 Activity of esterases

As shown in **Fig. 3**, a significant decrease in the activity of esterases was observed in all water samples collected from a natural water body. The analysis of variance indicates that the type of AgNPs stabiliser was always a statistically significant factor affecting esterase activity ($p = 0.045$). Silver nanoparticles introduced to water inhibited the activity of esterases only when stabilized with PVP. Moreover, this inhibitory effect increased proportionally to the increase in the concentration of AgNPs-PVP (correlation coefficient $r = –0.61$). The differences were easily noticed from the third day of the incubation. In the water samples containing AgNPs-cit the decrease in the activity of esterases was comparable to that observed in the control and references samples.

A number of bacterial proteins (including enzymatic proteins) which bind specifically to AgNPs have been identified (Li et al., 2010; Wigginton et al., 2010). According to Wigginton et al. (2010) strong bonds between AgNPs and the enzyme (tryptophanase) decrease the enzymatic activity, which confirms the thesis that direct interactions between AgNPs and enzymes may lead to certain metabolic irregularities. Although all forms of Ag display certain toxicity, numerous observations suggest that AgNPs are more toxic to bacteria than Ag^+ (Choi and Hu, 2008; Fabrega et al., 2009), which may result from the fact that the binding of AgNPs to the surface of proteins modifies the structure of active sites in enzymes. The results of this study confirm that only AgNPs-PVP, more stable in the environment, decrease the enzymatic activity of esterases.

2.3 Respiratory activity of bacteria

The assessment of bacterial respiratory activity is yet another key parameter for evaluating metabolic processes in the ecosystem; lowered respiratory activity undeniably indicates abnormalities within the ecosystem. **Figure 4** shows that no inhibition of bacterioplankton respiratory activity was observed in water samples after the introduction of AgNPs. In addition, no statistically significant differences in bacterioplankton respiratory activity were noted in water samples containing different concentrations of AgNPs-PVP and AgNPs-cit.

The results obtained by Li et al. (2010) indicate that the dehydrogenase activity of the respiratory chains of *E. coli* may be inhibited by AgNPs. This inhibition, proportional to the concentration of AgNPs, impairs the respiration and growth of bacterial cells, most commonly leading to their death (Rai et al., 2009). However, Shahrokh and Emtiazi (2009) observed no clear inhibitory effect of AgNPs on the dehydrogenases of the respiratory chains of soil bacteria. Moreover, in the research conducted by Colman et al.

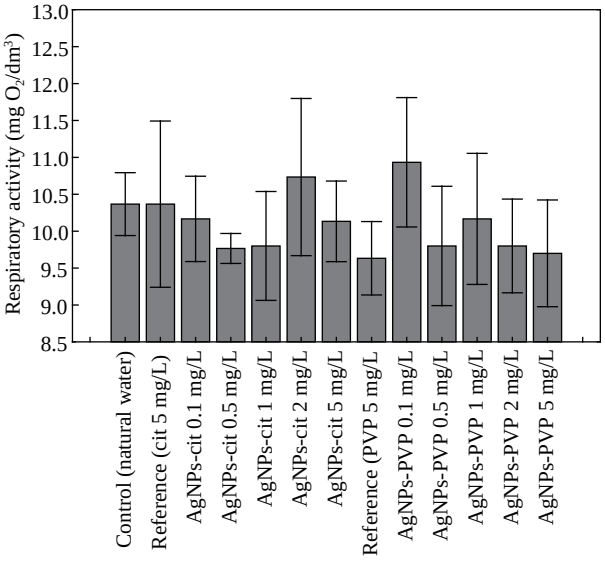

Fig. 4 Respiration activity of bacteria in the presence of AgNPs, oxygen consumption after 7 days. Data are expressed as average ± standard error.

(2009) even the concentration of AgNPs 250 mg/L had no inhibitory effect on the respiratory activity of bacteria inhabiting wet soils. This indicates that environmental conditions such as temperature, salinity, and the presence of organic matter can change the properties of AgNPs (Kittler et al., 2010). The toxicity of AgNPs in natural water bodies is controlled to some extent by their interactions with organic matter (Wigginton et al., 2010). The affinity of AgNPs to certain bacterial proteins may limit the mobility of NPs in the environment and reduce the bactericidal effect of AgNPs on bacteria populating natural water bodies.

2.4 Differences in the bactericidal effect of AgNPs-cit and AgNPs-PVP

The results of this study indicate that AgNPs-PVP has significantly stronger bactericidal effect than AgNPs-cit (**Fig. 5**). As with other nanomaterials, toxicity mechanisms of AgNPs depend on their interactions with biomolecules and may be controlled by the surface of nanoparticles (Lundqvist et al., 2008). All factors affecting the reactivity of the surface of nanoparticles such as their size (Lok et al., 2007; Choi and Hu, 2008) and shape (Pal et al., 2007), as well as stabilizers may also affect their toxicity. PVP, the most commonly used stabilizer (Jiang et al., 2005; Chen et al., 2009; Crespy and Landfester, 2009; Kittler et al., 2010), has a polyvinyl skeleton containing polar groups, in which N and O atoms show a strong affinity with AgNPs. As a result, PVP particles cover the surface of AgNPs preventing them from aggregation (Chen et al., 2009).

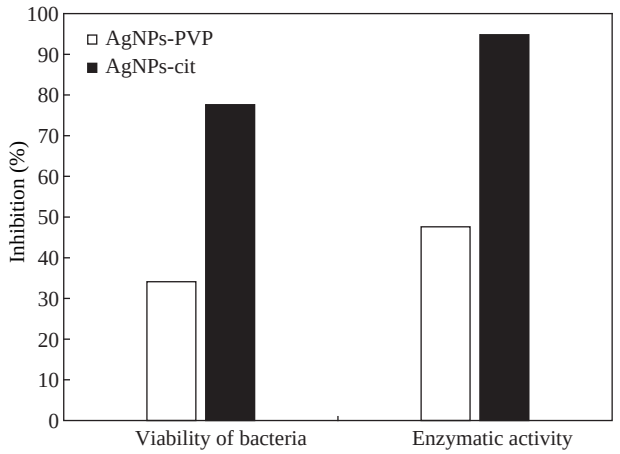

Fig. 5 Inhibition (relative to control) of bacteria viability and extracellular esterases activity in the presence of 5 mg/L AgNPs.

Our experiments performed to determine the stability of AgNPs-PVP in environmental conditions of lake (electrolytes, oxygen, organic matter, pH, light) prove the high quality of PVP as a caping agent. The solutions of AgNPs-PVP retain their characteristic yellow colour for many days. The concentration of AgNPs-PVP measured spectrophotometrically decreased by 9.8% of the initial value after 7 days. The decrease in the concentration of AgNPs-PVP kept in dark without air was even less, equal 1.2% (**Fig. 6a**). It means that silver nanoparticles stabilized by PVP do not aggregate in the natural lake water, however they slowly oxidize due to the presence of oxygen and chloride ions. Silver ions and colloidal silver chloride formed in this process are substantially less active than AgNPs (Lok et al., 2006; Choi and Hu, 2008; Fabrega et al., 2009; Navarro et al., 2008).

Silver nanoparticles stabilized by citrates behave quite differently. They lose their characteristic yellow colour and turn violet in seconds after introducing into the lake water, then become gray. The solution becomes colourless and a gray deposit is visible on the bottom after few days. The loss of concentration of silver measured spectrophotometrically was equal 12%, 50% and 91% after 4 hr, 1 day and 5 days, respectively (**Fig. 6b**). It means that AgNPs-cit introduced into the lake water form aggregates and then precipitate. Aggregation of silver nanoparticles stabilized by citrates occurs due to the presence of electrolytes, especially multivalent cations. All electrolytes decrease electrostatic repulsion of charged nanoparticles decreasing their stability, but divalent calcium and magnesium cations located between negatively charged silver nanoparticles act additionally as linking agent.

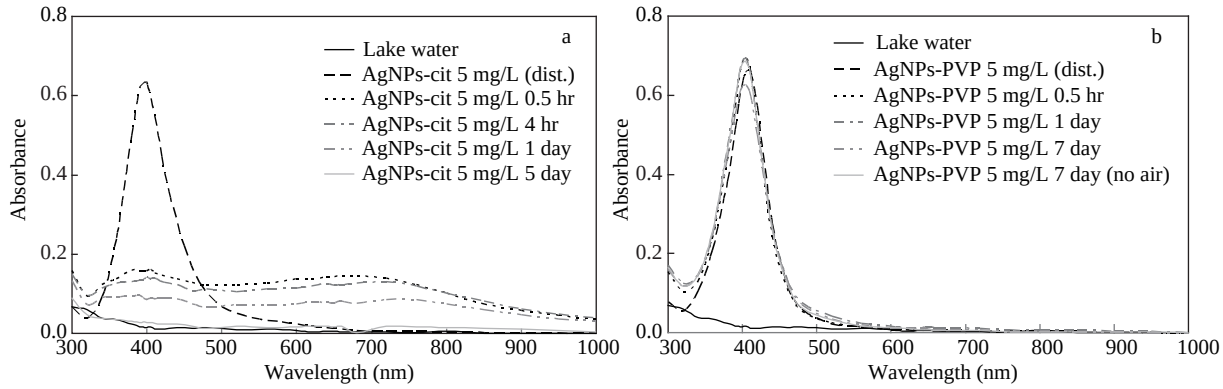

Fig. 6 Time evolution of UV-Vis spectrum of AgNPs in lake water AgNPs-cit (compared to AgNPs-cit in distilled water) (a), and AgNPs-PVP (compared to AgNPs-PVP kept without air and AgNPs-PVP in distilled water) (b).

3 Conclusions

The bactericidal effect of AgNPs on bacterioplankton depends not only on the size and shape of nanoparticles but also on the functionalization of their surface. Stabilizers which make stronger bonds with AgNPs stabilize and strengthen their bactericidal effect, which explains why AgNPs-PVP decreased bacterial survival rate and inhibited the activity of hydrolytic enzymes. Not all metabolic parameters of microorganisms are equally inhibited by AgNPs; respiratory activity of bacterioplankton was never affected, which may be connected with the interactions between AgNPs and the elements of the natural environment (e.g. organic matter). Due to the widespread use of AgNPs, the interactions between these nanoparticles and all elements of the natural environment (both animate and inanimate) should be carefully investigated.

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