DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

# Tolerance and co-tolerance of microbial communities on leaf litter to silver nanoparticles and antibiotics

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob a orientação científica da Professora Doutora Cristina Maria M. Monteiro Leal Canhoto (Universidade de Coimbra) e do Professor Doutor Mark Gessner (Technische Universität Berlin)

**Marina Kuburić**, 2014

## ACKNOWLEDGMENTS

Realization of this work would not be possible without unconditional help and support of many people, that I was lucky to meet during these two years of EMAE program.

First, I would like to express my deepest gratitude to Professor Cristina Canhoto, for her guidance and support during this project. She had the patience to answer to all my doubts and questions, she was always there to support me, when I needed it the most. Professor Canhoto discovered to me how amazing stream ecology is and how cool microbes can be.

I am thankful to Professor Mark Gessner, for giving me an amazing opportunity to work in IGB Neuglobsow, and for all his advices and support during my work.

To Doctor Ahmed Tlili, for all the patience to answer to million of my questions and to teach me all the techniques that I wasn't familiar with, for his ideas and for performing some of analysis that I couldn't, due to administrational issues.

I want to thank to Ana Gonçalvez and Ana Lírio, for all their help and for sharing their knowledge with me.

To Daniela Batista, for performing some of the analysis, that I couldn't, due to administrational issues.

I would like to thank to all those smiley faces from IMAR and IGB Neuglobsow, for making bad days more beautiful.

To EMAE consortium and European Commission, for making my dreams come true.

I would like to thank to my parents and sister, for all the love and encouragement, for all the sacrifices for me and my education.

I want to thank to Boris, for having faith in me, when I was loosing it, for all the patience for my mood swings and all the love.

Great thanks to my EMAErs, those amazing nineteen people, who became my friends and my family, for making my life more beautiful and my heart full.

## ABSTRACT

**Keywords:** silver nanoparticles, antibiotics, co-tolerance, bacteria, aquatic hyphomycetes

Antibiotics and silver nanoparticles present emerging toxicants, more and more present in freshwater, due to their increase use in medicine and for other purposes. The aim of this work was to evaluate the effects of long-term exposure to silver nanoparticles (AgNP), silver nitrate ($AgNO_3$) and a mixture of five antibiotics (AB) on the pollution-induced community tolerance (PICT) of microbes colonizing leaf litter in streams. Furthermore, I aimed to assess the specificity of PICT by evaluating the occurrence of co-tolerance between AgNP, $AgNO_3$ and AB. Tolerance of pre-exposed and control communities to the toxicants were assessed in short-term bioassays by determining bacterial production measured as $^{14}C$-leucine incorporation into protein and by estimating fungal sporulation rate determined by microscopic counts of spores induced during 12 hour incubations of decomposing leaves in distilled water. In addition, I estimated fungal diversity based on microscopic identification of spores produced by both control and pre-exposed communities.The bacterial communities pre-exposed to silver nanoparticles, silver nitrate and antibiotics increased their tolerance to these toxicants, and co-tolerance between the toxicants also occurred. In contrast to bacteria, fungal communities on decomposing leaf litter showed no tolerance or co-tolerance to any of the toxicants assessed. Our study confirms possibility of enhanced antibiotic resistance of bacteria to antimicrobial agents, potentially presenting a serious threat to both human and environmental health.

# RESUMO

Os compostos farmacêuticos constituem atualmente uma das principais ameaças aos sistemas de água doce. Motivo especial de preocupação é a ocorrência de agentes antimicrobianos, que podem determinar um aumento da tolerância da comunidade microbiana em relação a eles. O objetivo do nosso estudo foi, portanto, avaliar as consequências de uma exposição a longo prazo a nanopartículas de prata (AgNP), $AgNO_3$ (como fonte de $Ag^+$) e a uma mistura de 5 antibióticos (AB) na tolerância à poluição induzida na comunidade de decompositores microbianos (PICT) associados à folhada em águas doces. Além disso, pretendeu-se avaliar a especificidade de PICT avaliando a co - tolerância entre estas substâncias tóxicas. Após uma exposição longa aos tóxicos, foram realizados bioensaios de curta duração. A tolerância das comunidades controlo e pré-expostas aos tóxicos foi avaliada através da produção bacteriana por incorporação de 14C -Leucina etaxas de esporulação de fungos determinada por microscopia. Adicionalmente, foi também avaliada a diversidade fúngica nas comunidades controlo e pré-expostas. Os resultados mostraram que a pré-exposição da comunidade bacteriana a AgNP, $AgNO_3$ ou AB aumenta a tolerância a estes agentes tóxicos, masco-tolerância entre estes tóxicos também ocorre. No entanto, não observamos qualquer tolerância em relação a essas substâncias tóxicas, nem co- tolerância na comunidade de fungos presente na folhada. Isto pode ser devido ao facto de que, em geral, as comunidades fúngicas pré-expostas não alterama sua diversidade, em comparação com o controlo.

# INDEX

**CHAPTER I –** ***General introduction*** ........ 9

Emerging substances in streams ........ 11

Low order streams ........ 11

Assessment tool of streams functional integrity ........ 12

Main objectives ........ 12

**CHAPTER II –** ***Co-tolerance of microbial communities on leaf litter to silver nanoparticles and antibiotics*** ........ 13

2.1. Introduction ........ 15

2.2. Materials and methods ........ 16

2.3. Results ........ 20

2.4. Discussion ........ 27

**CHAPTER III –** ***Final remarks*** ........ 31

**CHAPTER IV –** ***References*** ........ 35

# CHAPTER I

## *General introduction*

## Emerging substances in surface waters

In the last twenty years, many studies have revealed presence of drugs and personal care products in the surface and drinking water. These substances, present in small concentrations in the water are known as emerging toxicants. According to the U.S. Geological Survey's Toxic Substances Hydrology Program, the most common compounds found in rivers are steroids, antibiotics, nonprescription drugs such as ibuprofen and caffeine, but also insect repellents. Special concern present antibiotics, as it is known that exposure to antibiotics can lead to resistant strains of bacteria, reducing the effectiveness of current classes of drugs (e.g. Young *et al.*, 2013; Watkinson *et al.*, 2007). Martinez (2008) showed that more than 90 % of bacterial strains originated in seawater are resistant to more than one antibiotic, and 20 % are resistant at least to five. Also, these compounds can change community structure in streams, by affecting present microbial community (Proia *et al.*, 2013). As there appears to be great potential of complementarity between microbial decomposers, which improves overall efficiency to degrade different litter types and consequently increase litter decomposition rate (Gessner *et al.*, 2010), change in a microbial community structure would potentially impact the decomposition process. Changes in microbial community structure would also impact macroinvertebrate functional group that feed on detritus and most likely entire stream community and multiple trophic levels (Wallace *et al.*, 1997).

Relatively new emerging substances in surface water, that are causing special concern, are nanoparticles. Mostly used nanoparticles are nano silver, nano $TiO_2$ and carbon nanotubes (Mueller & Nowack, 2008). Since ancient times, silver has been known for its antimicrobial activity. This resulted in widespread use of this metal. Lately, silver is mainly used in its nano form, which antimicrobial activity was confirmed (Kim *et al.*, 2007). Silver nanoparticles are mostly used in medicine to treat wounds (Woo *et al.*, 2009), to coat central venous catheters (Kaflon *et al.*, 2007), urinary catheters (Seymour, 2006) etc. They have also been used a lot not only in medicine and pharmacy, but also in photography, production of jewelry and industry (Mueller & Nowack, 2008), which all increased occurrence of these particles in the environment, specially in surface water, such as streams. It has been shown that presence of heavy metals induce tolerance to antibiotics and vice versa (e.g. Stepanauskas *et al.*, 2005), but it is still not well studied if silver nanoparticles and antibiotics can promote tolerance to one another.

## Low order streams

Low order streams represent more than 75% of total length of river network in a catchment basin (Benda *et al.*, 2005; Clarke *et al.*, 2008). They are located at the head of the river continuum (Vannote *et al.*, 1980). These streams are shadowed, with characteristic low primary productivity, a high edge to surface area ratio and a local microclimate. Temperate forested streams are lined up by trees, which treetops limit light penetration and primary production. Leaves, bark and wood, mainly from the riparian areas are, though, the main energy source for the heterotrophic food webs. Because of this, and although the riparian area presents just a small part of the watershed area, these ecotones are very important for the metabolism of low order streams.

Leaf litter decomposition is an integrative process, that links the riparian area with the microbial and invertebrates activities. Litter decomposition is important for cycling of organic carbon in stream ecosystems, as this material often presents the major energy source for aquatic food webs ( Webster *et al.*, 1999; Wallace *et al.*, 1997).

The process of leaf decomposition in streams occurs in three more or less defined phases - leaching, conditioning and physical and biological fragmentation (Gessner *et al.*, 1999; Webster & Benfield, 1986). Although in general, this process consists of sequential phases, some overlapping usually occurs. Immediately after the immersion of leaves in water, leaching of organic and inorganic soluble substances starts and lasts from two to seven days. 25 % or more of the initial dry mass of fresh leaves is lost during the first 24 hours of leaching. The leaching rate depends on leaf species, leaf structure and chemistry (McArthur & Richardson, 2002; Webster & Benfield, 1986), and abiotic factors such as nutrients in the stream water (Gulis *et al.*, 2006), temperature (Robinson & Jolidon, 2005) and current velocity (Ferreira *et al.*, 2006).

As leaching progresses, microbial colonization takes place. Microbial activity leads to increased leaf litter nutritional value and production of fine particular organic matter. This activity softens leaves tissues and increases its palatability (Gessner *et al.*, 1999). Microbial colonization largely occurs during the first two weeks of decomposition (Gessner *et al.*, 1993). Microbial decomposition of leaf is dominated by a group of aquatic fungi, mainly aquatic hyphomycetes, but also bacteria (Hieber & Gessner, 2002; Bärlocher, 2005). The leaves are fully conditioned when fungal biomass and activity reaches its peak.

The final phase in leaf decomposition is fragmentation, which is caused by physical forces and/or biological activity, such as feeding of invertebrates. Fragmentation depends on abiotic factors such as discharge, turbulence, floods, type of substratum and biotic factors, such as leaf type, conditioning level, shredder species present in the stream (Molinero *et al.*, 1996; Abelho, 2001; Gonçalves *et al.*, 2007; Allan, 1995; Graça & Canhoto, 2006).

## Assessment tool of streams functional integrity

Streams health traditionally have been evaluated with tools such as physicochemical characteristics and community structure (Barbour *et al.*, 1999; Boulton, 1999). However, streams are also characterized by functional parameters, which together with structural ones would provide us complete information on ecosystem health. Litter decomposition, as a technique, is inexpensive, well studied and an easy to measure functional parameter for streams health assessment. As said before, this ecosystem level process is complex multitude of processes and involves different organisms. This characteristic makes litter decomposition sensitive to physical and chemical stressors (Gonçalves *et al.*, 2013; Niyogi *et al.*, 2001) and by that, sensitive to many anthropogenic impacts. Therefore, decomposition is proposed as assessment tool of the stream functional integrity (Gessner & Chauvet, 2002). This tool can be used to assess impacts of emerging contaminants on streams ecosystems and its processes (Bundschuh *et al.*, 2008).

## Main objectives

In this work, we assessed the effects of silver, silver nanoparticles and antibiotics on leaf litter decomposition and associated microbial activity. We examine if the long-term exposure of microbial decomposers to silver, silver nanoparticles or a mixture of antibiotics will induce tolerance of the bacterial and fungal communities, but also if the exposure to one toxicant will induce tolerance to other, to which the community wasn't exposed.

# CHAPTER II

## *Tolerance and co-tolerance of microbial communities on leaf litter to silver nanoparticles and antibiotics*

## 2.1. Introduction

Freshwaters are among the most threatened ecosystems in the world (Malmquist & Rundle, 2002). According to Dudgeon *et al.* (2006), the main threats to freshwaters are their overexploitation, presence of invasive species, flow modification, habitat degradation and water pollution. Of special concern is water pollution, particularly presence of xenobiotics. Xenobiotics are chemicals, such as pharmaceuticals, industrial chemicals, pesticides or other toxins produced by molds, plants or animals, that are found in ecosystems, where they are not normally present. Of particular concern are pharmaceuticals in freshwaters.

In the last century, development of the pharmaceutical industry has led to an increased use of antibiotics, for both human and veterinary purposes. It has been shown that antibiotics are not totally removed by wastewater treatment plants, leading to their release into surface waters (Hirsch *et al.*, 1999; McArdell *et al.*, 2003). Presence of antibiotics or their metabolites in freshwaters might present an important environmental issue due to development of bacterial tolerance to these substances (Young *et al.*, 2013).

Another xenobiotic, whose large-scale utilization has increased its occurrence in the environment, especially in aquatic ecosystems, are silver nanoparticles (AgNP). AgNP may present a threat to aquatic ecosystems as silver is a very effective antibacterial agent (Kim *et al.*, 2007). It causes destabilization of the outer bacterial membrane in gram-negative bacteria (Lok *et al.*, 2006), pits in the bacterial cell wall in gram-positive bacteria (Sondi & Salopek-Sondi, 2004), inhibition of growth due to the generation of reactive oxygen species (Choi *et al.*, 2008), and inhibition of bacterial respiration (Choi & Hu, 2008). AgNP can provoke membrane alterations, increase membranes porosity and change the capacity of cells to regulate transport through the membrane (Pal *et al.*, 2007; Sondi & Salopek-Sondi, 2004). Silver has a wide range of targets in microbes, and since many different processes are affected, resistance to silver is not wide-spread and has not been of major concern in the past.

Some studies have raised the question of whether increased bacterial tolerance to antibiotics in the presence of metal contamination and vice versa. Gupta and co-workers (1999) showed the existence of plasmids that carried tolerance genes to both antibiotics and metals. If similar tolerances exist for AgNP and antibiotics, presence of both toxicants in the environment will increase the tolerance of microbial communities to them and present a threat to both environmental and human health. However, most of toxicity studies of AgNP were performed with single species (e.g. Yoon *et al.*, 2007, Sudheer Khan *et al.*, 2011). However, effects of toxicants on communities and to some extent on ecosystems are not necessarily predictable from single species, for example because of species interactions (McClellan *et al.*, 2008). Being composed of many species, with varying sensitivites to contaminants, communities would reflect to some extent the complexity and variability occurring in the environment (Clements & Rohr, 2009). Communities useful to assess impacts of toxicants in aquatic ecosystems are microbial communities associated with decomposing leaf litter. This community is composed of fungi, mainly aquatic hyphomycetes, and bacteria. Bacteria and fungi associated with decomposing leaf litter may interact synergistically or antagonistically during decomposition. Gulis and Suberkropp (2003) found that microbial biomass and production is always dominated by fungi. The bacterial contribution was increased only in treatments where fungi were excluded, suggesting competition between bacteria and fungi. They also found that bacteria may benefit from fungal activity during decomposition, indicating that interactions between bacteria and fungi can be synergistic in facilitating leaf decomposition but antagonistic in resources acquisitions.

Long-term exposure of communities to xenobiotics leads to structural and functional community changes of the community toward the most resistant species and strains and an elimination of the most sensitive ones. This process is the basis of the pollution induced community tolerance (PICT) concept (Black *et al.*, 1988). PICT aims to compare sensitivities to a toxicant of two communities. One community is exposed to the toxicant for an extended period, whereas an other, originally identical community, which is called reference community, is not. Tolerance of both communities is subsequently tested in short-term bioassays. Based on the results of these bioassays, community tolerance is expressed as an effective concentration (ECX). PICT provides a causal link between exposure to a toxicant and a measurable effect. It can give information about previous exposure to a toxicant and can be used as a tool for ecological risk assessment.

Applicability of PICT as an ecotoxicological tool depends on PICT specificity. However, this specificity is not absolute and co-tolerance may occur. Co-tolerance may happen when a community that had been exposed to one toxicant, but not another, becomes tolerant to both of them. Co-tolerance mostly occurs when the two toxicants have similar modes of action or when they induce similar detoxification mechanisms (Blanck *et al.*, 1988; Soldo & Behra, 2000). Co-tolerance has been observed between metals, such as copper and zinc or copper and silver, in periphyton community (Soldo & Behra, 2000; Tlili *et al.*, 2011).

This study adressed the following questions: (1) Does exposure of a microbial community to $AgNO_3$, AgNP and AB change the structure of microbial communities on leaf litter? (2) Does exposure of a microbial community to $AgNO_3$, AgNP and AB induce tolerance of bacterial and fungal community to those toxicants? and (3) Does exposure of a microbial community to $AgNO_3$, AgNP and AB induce co-tolerance of to these toxicants?

## 2.2. Materials and methods

### 2.2.1. Chemicals

Clarythromycin (CAS 81103-11-9, purity> 98 %), roxythromycin (CAS 80214-83-1, purity> 90 %), erythromycin (CAS 114-07-8, purity> 95 %), sulfamethoxazole (CAS 723-46-6, purity> 98 %) and trimethoprim (CAS 738-70-5, purity> 98.5 %) were purchased from Sigma-Aldrich, Germany. Erythromycin - H20, the metabolite of erythromycin most commonly found in wastewater (Hirsch *et al.*, 1999), was made from erythromycin before the experiment, by adding 3M H2SO4 until the pH reached the value 3 and stirring for 4 hours (McArdell *et al.*, 2003).

Citrated coated AgNP were purchased from NanoSys GmbH (Wolfhalden, Switzerland) with a nominal concentration of 9.27 mM as total silver, size 25 ± 13nm; zeta potential -36.6 ± 3.2 mV in nanopure water (Navarro *et al.* 2008).

$AgNO_3$ was purchased from Sigma-Aldrich, Germany.

### 2.2.2. Experimental design

Coarse, nylon mesh bags (50x50 cm, 1 cm mesh size) were filled with similar amounts of various leaves and placed in a pristine stream (Northern Germany; 51°41’31”N, 10°21’31”E) for 13 days, in

September 2013. Afterward, the leaves were retrieved and transported to the laboratory in cooling boxes, where they were rinsed with deionized water and incubated for 48 hours. The medium with the spores and bacteria was then used as the inoculum in the experiment.

Poplar leaves (*Populus gr. nigra L.*) were sinked in deionized water and 2080 12-mm diameter discs were cut. Then discs were dried at 40 °C for 48 hours and weighted in groups of 130. Each group of discs was placed in one microcosm, containing 250 ml of 'Volvic' water. All microcosms were agitated for 24 hours, to induce the leaching of soluble matter. After this period, the water was replaced by 400 ml of 'Volvic' water and 40 ml of inoculum. The incubation was carried out for 5 days, at 16 °C, under a 12h light: $AgNO_3$ 12 h dark photoperiod, to allow leaf inoculation with fungi and bacteria.

After the inoculation phase, the medium from all microcosms was renewed. Four microcosms were contaminated with 200 µg/l of AgNP and four others with 20 µg/l of $AgNO_3$, as source of $Ag^+$ (corresponding to 10 % of dissolved silver ions in the AgNP solution). Four other microcosms were contaminated with 200 µg/l of the antibiotic mixture (five antibiotics in equal concentrations). Antibiotics (i.e. sulfamethoxazole, erythromycin-$H_20$, roxithromycin, clarythromycin and trimethoprim) were chosen, because they are often present in wastewater effluents (McArdell *et al.*, 2003; Zhou *et al.*, 2013). Roxythromycin and clarythromycin were hardly soluble in the water, so we had to add solvent DMSO, to a final concentration of 0.03 %. Finally, four microcosms were used as control, without addition of any toxicant.

The long-term exposure lasted 25 days, at 16 °C, under a 12 h light: 12 h dark photoperiod. The media were renewed every 5 days. Water samples were collected from the microcosms just before water replacement for chemical analysis. For the analysis of total silver, 10 ml of water samples were taken from AgNP and $AgNO_3$ treatments, acidified with 100 µl of 65 % suprapure $HNO_3$ and stored at 4 °C until their analysis. For the analysis of dissolved silver, 4 ml water samples were taken from AgNP treatment, filtrated with Ultracel 3k Centrifugal Filter Devices (Amicon Millipore) with a molecular cutoff of 3kDa (pore size < 2 nm) (Navarro *et al.*, 2008). After centrifugation at 4700/ min for 30 min and at 16 °C, 40 µl of 65 % $HNO_3$ was added and the samples were kept at 4 °C until their analysis. For the analysis of antibiotics, 20 ml water samples were taken from AB treatment and frozen at -20°C till their analysis.

### 2.2.3. Water analysis

Temperature, oxygen concentration and pH were measured daily, for each treatment.

At the end of the experiment, water samples were collected for the nutrient analysis. Dissolved organic carbon (DOC) concentrations were determined with an organic carbon analyzer (Shimadzu, TOC-V CPH, Germany) as non-purgeable carbon after acidification. Soluble reactive phosphorus (SRP) was determined photometrically by applying the molybdenum-blue method. Total phosphorus (TP) was determined after digestion in potassium peroxidisulfate for 30 minutes at 134 °C in a steam autoclave. Phosphorus was measured photometrically (FIA compact analyser MLE, Germany). Nitrogen was oxidized with Oxisolv (Merck, Germany) at 120 °C for 45 min in an autoclave and measured with a Foss FIAstar 5010 analyzer (Rellingen, Germany).

Total silver concentration in AgNP suspension was measured by ICP-MS (Element 2 High Resolution Sector Field ICP-MS; Thermo Finnigan). From 10 ml water sample, 1 ml was taken and digested with 4 ml of 65 % $HNO_3$ for 90 minutes in a high-performance microwave digestion units mls 1200 mega (mls GmbH, maximal temperature 195 °C). Before ICP-MS measurement, the sample was taken out from the microwave oven and diluted with ultra pure water until total volume of 25 ml.

The measurement of dissolved silver was performed as for the total silver.

The concentration of antibiotics was determined with the high performance liquid chromatography (HPLC).

### 2.2.4. Silver in the leaf discs

Three random discs were taken from each replicate, from AgNP and $AgNO_3$ treatment and freeze-dried. Dry leaf discs were weighted before their digestion with 4 ml of concentrated supra pure $HNO_3$ in a high performance microwave and 25 ml ultra-pure water was added to dilute the content. Afterwards, the samples were analyzed following the same procedure as for total and dissolved silver concentration. The results were expressed as μg/g DM.

### 2.2.5. AgNP characterization

AgNP were characterized in the exposure medium before (t5) and after (t0) every water renewal and in 'Volvic' water. Z-average size and the size distribution of AgNP was measured by Dynamic Light Scattering (DLS), using a Zetasizer (Nano ZS, Malvern Instruments), with a red laser (633 nm). Zeta potential reflecting the surface charge of AgNP was measured by electrophoretic mobility using a Zetasizer.

### 2.2.6. Biological analysis

*Decomposition rate*

Leaf discs of each replicate were dried at 40 °C to a constant mass (72 ± 24 h) and weighed to the nearest 0.001 g. Decomposition rate (k) was calculated based on the formula: $Wt = W0\ e^{-kt}$, where Wt is the leaf dry mass remaining at time t, W0 is initial dry mass of the leaves and k is the slope of the plot of the natural logarithm, of leaf mass versus time, in units $day^{-1}$

*Bacterial abundance*

Bacterial abundance was determined by flow cytometry (according to Frossard *et al.*, 2012, with a few modification). Bacterial cells were detached with a sonifier during 60 seconds. After homogenization, a 1 ml aliquot of the cell suspension was placed stained with SYBRGreen I (Promega, Switzerland) in anhydrous dimethylsulfoxide and incubated in the dark for 15 minutes. The samples were diluted ten times with filtered (0.22 mm Millex-GP, Millipore, Switzerland) 'Volvic' water. The cell concentration didn't exceed $10^6$/ml. Samples were then analyzed with a CyFlow space Flow Cytometer System (Partec, Germany) equipped with a 200mW solid state laser (488 nm). Green and red fluorescence were measured at 520 nm and 630 nm. The flow cytometer was set: gain FL1.495, gain FL3.50, speed 4. Counts were recorded as logarithmic signals. Data were processed with Flowmax software (Partec, Germany).

*Fungal biomass*

Ergosterol content was determined as a proxy of fungal biomass (Gessner & Chauvet, 1993). Four random discs were chosen from each microcosm and freeze-dried. They were then weighted and heated at 80 °C for 30 minutes in a solution of 0.8 % KOH-methanol. Afterwards, extracts were purified by solid phase extraction using SPE-catridges (SPE; Waters Sep-Pak®, Vac RC tC18, 500 mg) and eluted in isopropanol. The extraction efficiency was monitored by using an external ergosterol standard.

Radiolabelled ergosterol in the extract was quantified by high-performance liquid chromatography (Kontron HPLC-System Series 400, Kontron Instruments, Germany) at 282 nm. The ergosterol was converted in fungal biomass using a conversion factor of 5.5 µg ergosterol/mg fungal dry mass (Gessner & Chauvet, 1993). The results were expressed as mg fungal biomass/g dry mass.

*Bacterial productivity*

Bacterial productivity was measured according to Buesing and Gessner (2003). Three discs and 2.9 ml of medium from each bioassay, were transferred in a 20 ml vial, and 100 µl leucine with final concentration of 50 µM, which was obtained by mixing 35.5 µl 14C-leucine (4.5 µM) and 61.5 µl 2,4 M non-radioactive leucine, was added. For control, in one sample from each treatment, first 300 µl 50% TCA was added and later leucine. All samples were incubated for 30 minutes, at room temperature and while shaking. After this period, the leucine incorporation was stopped by adding 300 µl 50% TCA.

Samples were sonicated for one minute at 5-6 watts in order to separate bacteria from the leaves. Three milliliters of samples were filtered through 0.2 µm polycarbonate filter and the plant litter residual at the filter was washed two times with 5 % TCA and once with 40 mM leucine, 80 % ethanol and bidestiled water. Every washing step was followed by the filtration through the same polycarbonate filter.

After these steps, the plant material and the filter were transferred to 2ml Eppendorf tubes, in which we added 500 µl of 0.3 % SDS, 75 mM EDTA and 1.5 M NaOH. Samples were heated for 60 minutes at 90 ºC, to dissolve proteins, and cooled down at room temperature. The tubes were centrifuged for 10 minutes at 13 000 x g, and after that 250 µl aliquot was pipetted into a scintillation vial. Five milliliters of Ultima Gold XR scintillation cocktail was added, and radioactivity was determined using a Liquid Scintillation Analyzer Tri-Carb 2810 TR (PerkinElmer GmbH, Germany).

*Fungal sporulation*

From each bioassay, one disc and 10.1 ml of medium were used to analyse fungal sporulation. Into this, 500 µl of formaldehyde (to fix the spores) and 30 µl of Triton X 100 (to avoid conidial adherence to the flask) were added. These samples were filtered (Millipore SMWP, 5 mm pore size) and retained spores were stained with 0.05 % cotton blue in lactic acid (60 %). Conidia of aquatic hyphomycetes were identified and counted under light microscope at 200 x magnification. Sporulation rates were expressed as number of conidia released/g DM /day.

*Tolerance and co-tolerance to AgNP, $AgNO_3$ and AB*

In order to measure tolerance to AgNP, $AgNO_3$ and AB of bacterial and fungal communities, short term bioassays with the long-term pre-exposed and control communities were performed. All the communities were exposed separately for 12 hours to eight increasing concentrations of AgNP, $AgNO_3$ and AB. Final concentrations ranged from 0 to 1830 µg/l for AgNP and $AgNO_3$ and from 0 to 20000 µg/l for AB. After exposure, sporulation and bacterial production were used as functional endpoints (see previous sections). Since antibiotics were dissolved with DMSO, controls with DMSO were also included, to exclude any solvent effect.

## 2.2.7. Statistical analysis

$EC_{50}$ values were calculated with models with log(c) transformation and three parameters, simple or normalized response, depending which gave the best fit. The tolerance (R>1) or sensitivity (R<1) ratio were calculated by dividing $EC_{50}$ for the pre-exposed fungal or bacterial community with the $EC_{50}$ for the control fungal or bacterial community.

Shannon indices was calculated using formula $H' = - \Sigma p_i \ln p_i$, where p*i* is the proportion of individuals found in species i. Species richness presents the number of species found in a treatment, while Sørensen similarity index is calculated using the formula QS=2C/(A+B), where A is the number of species in one treatment, B is the number of species in other treatment and C is the number of species common to both treatments.

Shannon indices and species richness values for control and pre-exposed communities were tested by factorial one-way ANOVA, and if the effect was significant, the ANOVA was followed by a Tukey test. $EC_{50}$ values were compared by the comparison of 95 % confidence interval.

For these calculations and analysis, Graphpad Prism 6.04 version (GraphPad software Inc., CA, USA) was used.

## 2.3. Results

### 2.3.1.Water and leaf discs analysis

Values of pH for control, AgNP, $AgNO_3$ and AB treatment were not significantly different between the different treatments (1-way ANOVA, $p \geq 0.05$), same as oxygen concentrations (1-way ANOVA, $p \geq 0.05$).

As it is shown in Table 1, controls were characterized by lower nitrite (Tukey test, $p \leq 0.01$) and nitrate concentration (Tukey test, $p \leq 0.05$) than AgNP treatments and lower DOC concentration than AgNP (Tukey test, $p \leq 0.01$), $AgNO_3$ and AB treatment (Tukey test, $p \leq 0.001$). Controls were also characterized by higher ammonium concentrations than $AgNO_3$ and AgNP treatments (Tukey test, $p \leq 0.0001$) and higher total nitrogen than $AgNO_3$, AgNP and AB treatments (Tukey test, $p \leq 0.01$). AB treatments were characterized by higher ammonium concentration than AgNP and $AgNO_3$ treatments (Tukey test, $p \leq 0.0001$). Mean values for soluble reactive phosphorus and total phosphates were not significantly different for all the treatments (1-way ANOVA, $p \geq 0.05$).

***Table 1.***

pH, oxygen values and nutrient concentration values for treatments at the end of the experiment. Values are mean (± standard deviation).

| | pH | $O_2$ [mg/l] | $NO_2$-N [mg/l] | $NO_3$-N [mg/l] | $NH_4$-N [mg/l] | TN [mg/l] | SRP [mg/l] | TP [mg/l] | DOC [mg/l] |
|---|---|---|---|---|---|---|---|---|---|
| **Control** | 6.73 ± 0.56 | 7.28 ± 0.49 | 0.006 ± 0.001 | 0.026 ± 0.014 | 0.196 ± 0.016 | 2.465 ± 0.795 | 0.006 ± 0.001 | 0.181 ± 0.152 | 11.285 ± 1.126 |
| **AgNP** | 6.79 ± 0.48 | 7.19 ± 0.44 | 0.012 ± 0.002 | 0.305 ± 0.102 | 0.051 ± 0.011 | 1.412 ± 0.145 | 0.005 ± 0.001 | 0.04 ± 0.027 | 32.845 ± 12.965 |
| **$AgNO_3$** | 6.89 ± 0.43 | 7.22 ± 0.60 | 0.009 ± 0.002 | 0.130 ± 0.176 | 0.042 ± 0.012 | 1.436 ± 0.396 | 0.007 ± 0.002 | 0.07 ± 0.014 | 40.77 ± 3.696 |
| **AB** | 6.93 ± 0.41 | 7.30 ± 0.62 | 0.009 ± 0.003 | 0.146 ± 0.099 | 0.185 ± 0.035 | 1.128 ± 0.348 | 0.007 ± 0.002 | 0.107 ± 0.037 | 41.475 ± 1.717 |

*Total and dissolved Ag in the water*

Total $Ag^+$ concentration in $AgNO_3$ treatments, at the end of the experiment, was 12.625 ± 3.854 µg/l, while value for total Ag in AgNP microcosms, at the end of the experiment, was 71.75 ± 31.598 µg/l. The concentration of dissolved Ag in AgNP was below the level of detection.

*Antibiotics*

Values for antibiotics concentrations, measured in each replica of antibiotic treatment at the end of the experiment, are presented in Table 2. Knowing that at every medium renewal, we added 40 µg/l of every antibiotic in AB treatment, it can be seen that during the exposure concentration decreased for clarythromycin (Tukey test, $p \leq 0.01$), erythromycin-$H_2O$ (Tukey test, $p \leq 0.001$), sulfamethoxazole (Tukey test, $p \leq 0.01$) and trimethoprim (Tukey test, $p \leq 0.001$), while did not change significantly for roxythromycin (Tukey test, $p \geq 0.05$).

***Table 2.***
Mean values (± standard deviation) of antibiotic concentration, in the antibiotic treatments, at the end of the experiment. N-acetyl- sulfamethoxazole is a metabolite of sulfamethoxazole

| | Clarythromycin [µg/l] | Erythromycin - $H_2O$ [µg/l] | Roxythromycin [µg/l] | Sulfamethoxazole [µg/l] | N-acetyl-Sulfamethoxaz-ole [µg/l] | Trimethoprim [µg/l] |
|---|---|---|---|---|---|---|
| **Concentration** | 34.5 ± 3 | 27.75 ± 4.031 | 36 ± 4.546 | 34.25 ± 2.63 | 5 ± 0 | 28.5 ± 3.416 |

*Silver in leaf discs*

Mean value for the Ag in the leaves, at the end of the experiment, in $AgNO_3$ microcosms is 127.2 ± 36.878 µg/g DM, while for AgNP microcosms is 180.76 ± 22.93 µg/g DM.

## 2.3.2. AgNP characterization

AgNP in 'Volvic' water has an average size of 76.1 ± 1.17 nm and zeta potential of -15.07 ± 0.7 mV at t0, while after five days average size was 50.15 ± 1.53 nm and zeta potential -16.4 ± 1.3 mV. The size of nano particles in the medium at the t0, but also at t5, didn't change during the experiment (1-way ANOVA, $p \geq 0.05$), as it can be seen in Fig. 1. Figure 2. shows that average zeta potential at t0 and t5 didn't change significantly during the experiment (1-way ANOVA, $p \geq 0.05$).

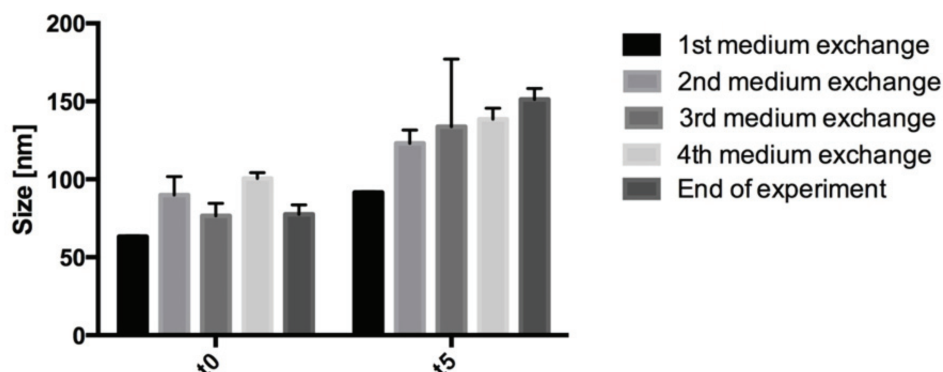


***Fig. 1.*** *Average size (+ standard deviation), of nanoparticles in the medium, at t0 and t5, during the experiment.*

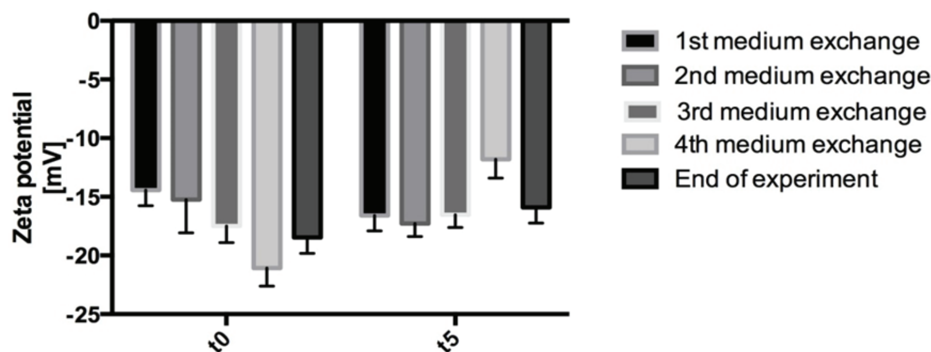


***Fig. 2.*** *Zeta potential (+ standard deviation) of nanoparticles in the medium, at t0 and t5 during the experiment.*

## 2.3.3. Biological analysis

Values for biological analysis are presented in Table 3.

Decomposition rate decreased significantly in all treatments in comparison to the control (1-way ANOVA, p≤0.0001). AB treatments displayed the lowest rate (Tukey test, p≤0.0001), followed by $AgNO_3$ treatment (Tukey test, p≤0.01) and AgNP treatment (Tukey test, p≤0.05).

Bacterial counts was significantly different between the treatments (1-way ANOVA, p≤0.01) and AB treatment displayed the lowest number of bacterial cells/g DM (Tukey test, p≤0.05), (Fig. 3.).

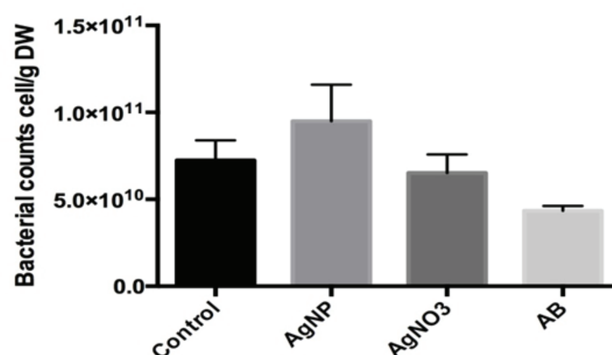


***Fig. 3.*** *Bacterial abundance in different treatments, at the end of the experiment, after 25 days of incubation.*

Bacterial production between the treatments was similar, at the end of the experiment (1-way ANOVA, p≥0.05).

Before the exposure to the contaminants, fungal biomass in the microcosms was 1.225 ± 0.988 mg/g DM. Long term exposure to all toxicants affected fungal biomass in microcosms (1-way ANOVA, p≤0.01). The fungal biomass was highest in $AgNO_3$ treatment (Tukey test, p≤0.01), followed by AB treatments (Tukey test, p≤0.05). Tukey test showed that fungal biomass in AgNP treatments wasn't significantly different from the control treatments (p≥0.05), (Fig. 4.).

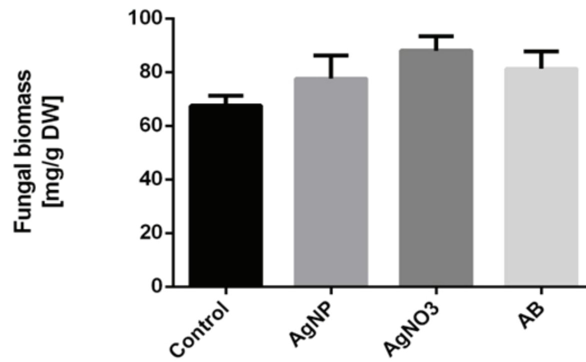


***Fig. 4.*** *Mean value for fungal biomass in different treatments, expressed in mg/g DM.*

There was no significant difference in sporulation rates between control and treatments with toxicants (1-way ANOVA, $p \geq 0.05$), (Fig. 5).

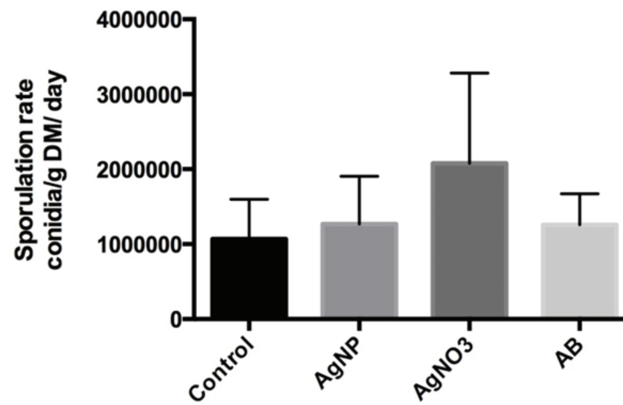


***Fig. 5.*** *Sporulation rate in different treatments, at the end of the experiment, expressed as number of conidia/g DM/day.*

***Table 3.***
*Mean values of decomposition rate, bacterial abundance, fungal biomass and sporulation rate (± standard deviation) at the end of the experiment*

| | Decomposition rate [$d^{-1}$] | Bacterial abundance [cells/g DM] | Fungal biomass [mg/g DM] | Sporulation rate [conidia/g DM/day] | Bacterial production [µg C/g litter/day] |
|---|---|---|---|---|---|
| **Control** | 0.037 ± 0.002 | $7.238 \cdot 10^{10} \pm 1.159 \cdot 10^{10}$ | 67.58 ± 3.69 | $1.067 \cdot 10^{6} \pm 5.324 \cdot 10^{5}$ | 280.5 ± 92.9 |
| **AgNP** | 0.032 ± 0.001 | $9.493 \cdot 10^{10} \pm 2.096 \cdot 10^{10}$ | 77.65 ± 8.57 | $1.269 \cdot 10^{6} \pm 6.365 \cdot 10^{5}$ | 252 ± 21.52 |
| **$AgNO_3$** | 0.032 ± 0.001 | $6.515 \cdot 10^{10} \pm 1.072 \cdot 10^{10}$ | 88.02 ± 5.32 | $2.078 \cdot 10^{6} \pm 1.203 \cdot 10^{6}$ | 229.5 ± 51.18 |
| **AB** | 0.022 ± 0.003 | $4.348 \cdot 10^{10} \pm 2.656 \cdot 10^{10}$ | 81.28 ± 6.53 | $1.258 \cdot 10^{6} \pm 4.145 \cdot 10^{5}$ | 318.75 ± 85.63 |
| **1-way ANOVA, p value** | ≤ 0.0001 | ≤ 0.01 | ≤ 0.01 | ≥ 0.05 | ≥ 0.05 |

*Fungal diversity*

The dominant species in control, AgNP and $AgNO_3$ treatment was *Flagellospora curvula*, followed by *Anguillospora filiformis*, while for the AB treatment the dominant species was *A. filiformis*, followed by *F. curvula*. The relative abundance of *F. curvula* in the control (3806.28 ± 847.43) was not significantly different from AgNP (2990.22 ± 251.7), (1-way ANOVA, $p \geq 0.05$) and AB treatment (5710.11 ± 3397.94), but was lower than in $AgNO_3$ treatment (13397.14 ± 7436.22), (Tukey test, $p \leq 0.05$). The relative abundance of *A. filiformis* was similar between control (2085.85 ± 1531.72) and AgNP (2558.64 ± 476.91), $AgNO_3$ (3352.89 ± 2505.39) and AB treatments (5885.56 ± 3565.91), (1-way ANOVA, $p \geq 0.05$). Some species, such as *Lemonniera pseudofloscula, Lemonniera terrestris* and *Tetracladium chaetocladium*, were present in control, but absent from AgNP, $AgNO_3$, AB treatments. *Clavariopsis aquatica, Mycocentrospora acerina* and *Anguillospora furtiva*, were present in AB treatment, but absent from other treatments (Fig. 6.).

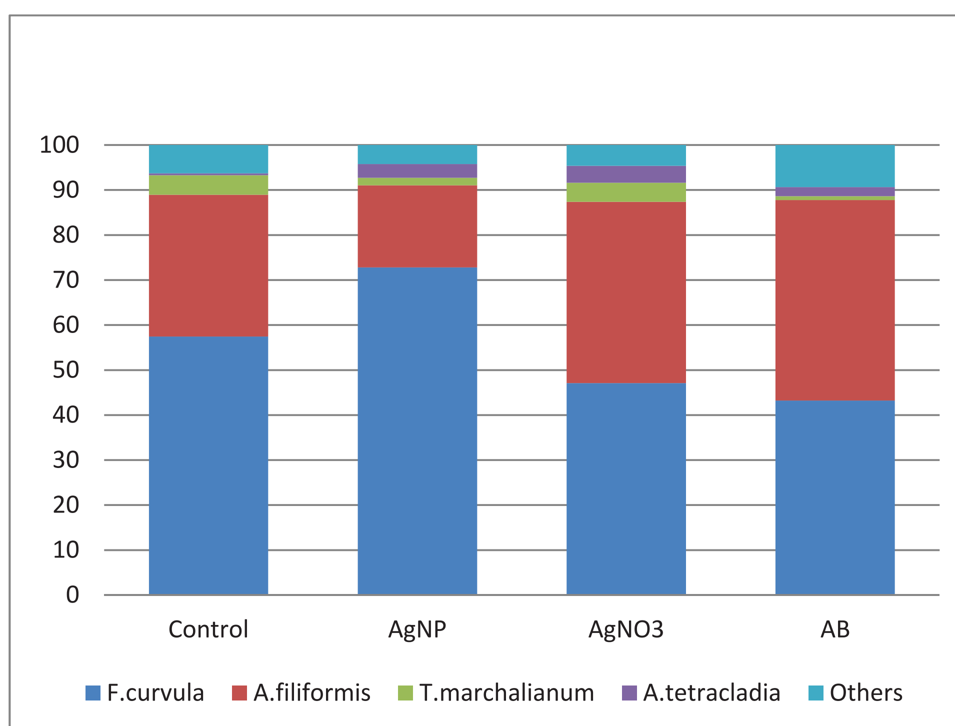


***Fig. 6.*** *Mean relative abundances (%) of aquatic hyphomycetes conidia on poplar leaves, incubated for 25 days, for different treatments.*

There was no significant difference between the treatments in Shannon indices values (1-way ANOVA, $p \geq 0.05$), (Table 4.).

There was no significant difference in species richness between treatments (1-way ANOVA, $p \geq 0.05$), (Table 4.).

Sørensen similarity index for control and AgNP communities is 0.78, for control and $AgNO_3$ communities is 0.83, while for control and AB communities is 0.82. Sørensen similarity index for AgNP and $AgNO_3$ communities is 0.84, for AgNP and AB communities 0.82 and for $AgNO_3$ and AB communities 0.7

***Table 4.***
*Values for Shannon index and species richness for different treatments*

| | Shannon indices | Species richness |
|---|---|---|
| **Control** | 0.853 ± 0.198 | 7.75 ± 1.5 |
| **AgNP** | 1.012 ± 0.04 | 6.75 ± 1.258 |
| **$AgNO_3$** | 0.803 ± 0.262 | 7.75 ± 1.708 |
| **AB** | 1.01 ± 0.046 | 8.0 ± 1.732 |

*Assessment of bacterial tolerance to AgNP, $AgNO_3$ and AB*

*Tolerance measurements*

Overall, in comparison to the control, pre-exposed bacterial communities to AgNP, $AgNO_3$ or AB showed significantly higher $EC_{50}$ values for AgNP (R=21.30, p≤0.05), $AgNO_3$ (R=4.01, p≤0.05), or AB (R=27.52, p≤0.05), respectively (Fig.7, Table 5.).

***Table 5.***
*$EC_{50}$ for AgNP, $AgNO_3$ and AB bioassays, for bacterial communities in different treatments, expressed in µg/l and CI 95 % values*

| | $EC_{50}$ AgNP [µg/l] | $EC_{50}$ $AgNO_3$ [µg/l] | $EC_{50}$ AB [µg/l] |
|---|---|---|---|
| **Control** | 21.96 (13.53-35.67) | 13.01 (10.45-16.18) | 113.5 (59.71-215.6) |
| **AgNP** | 467.8 (383.4-570.7) | 57.19 (46.37-70.53) | 714 (529.8-962.4) |
| **$AgNO_3$** | 407.3 (302.4-548.4) | 52.14 (35.66-76.24) | 824.7 (542.1-1255) |
| **AB** | 113 (49.75-256.7) | 69.12 (40.18-118.9) | 3124 (859-5252) |

*Co-tolerance measurements*

Pre-exposure to AgNP induced higher bacterial tolerance to $AgNO_3$ (R=4.40, p≤0.05) and AB (R=6.45, p≤0.05). Similarly, pre-exposure to $AgNO_3$ induced higher bacterial tolerance to AgNP (R=18.55, p≤0.05) and AB (R=7.27, p≤0.05). Pre-exposure to AB increased bacterial tolerance to AgNP (R=5.14, p≤0.05) and to $AgNO_3$ (R=5.31, p≤0.05), (Fig. 7.).

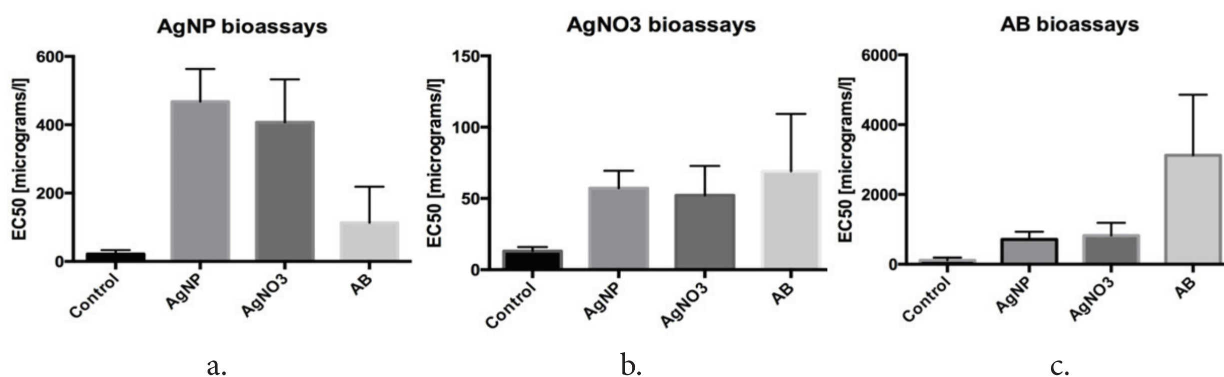


***Fig. 7.*** *Comparison between $EC_{50}$ values based on the bacterial production a) bioassays with AgNP, b) bioassays with the $AgNO_3$ c) bioassays with AB. Graph represents mean $EC_{50}$ values expressed in µg/l + standard deviation*

*Assessment of fungal tolerance to AgNP, $AgNO_3$ and AB*

Measurements of fungal sensitivities, based on fungal sporulation in bioassays, showed high variability between replicas. In order to reduce this variability, we identified outliers with ROUT test and excluded replicates with them from $EC_{50}$ calculation. In the AgNP bioassays, we excluded replica one replica from control treatment, in the $AgNO_3$ bioassays we excluded one replica from control and $AgNO_3$ treatments, while in AB bioassays we excluded one replica from control, AgNP and $AgNO_3$ treatments. In one replica in AB treatment, we didn't find any spores in the bioassays with concentration 0 of toxicant, so we excluded this replica, as well.

*Tolerance measurements*

No significant differences were observed between the $EC_{50}$ values for AgNP ($R=2.97$, $p \geq 0.05$), $AgNO_3$ ($R=1.16$, $p \geq 0.05$) or AB ($R=4.67$, $p \geq 0.05$) and control (Fig. 8, Table 6.).

***Table 6.***

*$EC_{50}$ for AgNP, $AgNO_3$ and AB bioassays, for fungal communities in different treatments, expressed in µg/l*

| | $EC_{50}$ AgNP [µg/l] | $EC_{50}$ $AgNO_3$ [µg/l] | $EC_{50}$ AB [µg/l] |
|---|---|---|---|
| **Control** | 321.5 (125.6-822.8) | 5.864 (0.528-1.009) | 3509 (968.6-12710) |
| **AgNP** | 954.5 (257.4-3539) | 10.27 (2.384-44.23) | 33537 (7216-155873) |
| **$AgNO_3$** | 820.4 (148.3-4538) | 6.835 (1.343-34.79) | 7923 (2044-30713) |
| **AB** | 124.4 (50.16-308.5) | 9.505 ($1.47 \cdot 10^{-7}$-895985) | 16402 (5523-48707) |

*Co-tolerance measurements*

Pre-exposure to AgNP didn't induce higher fungal tolerance to $AgNO_3$ ($R=1.75$, $p \geq 0.05$), nor AB ($R=9.56$, $p \geq 0.05$). Similarly, pre-exposure to $AgNO_3$ didn't affect fungal tolerance to AgNP ($R=2.55$, $p \geq 0.05$) and AB ($R=1.62$, $p \geq 0.05$). Pre-exposure to AB didn't affect fungal tolerance to AgNP ($R=0.38$, $p \geq 0.05$) and to $AgNO_3$ ($R=2.26$, $p \geq 0.05$), (Fig. 8.).

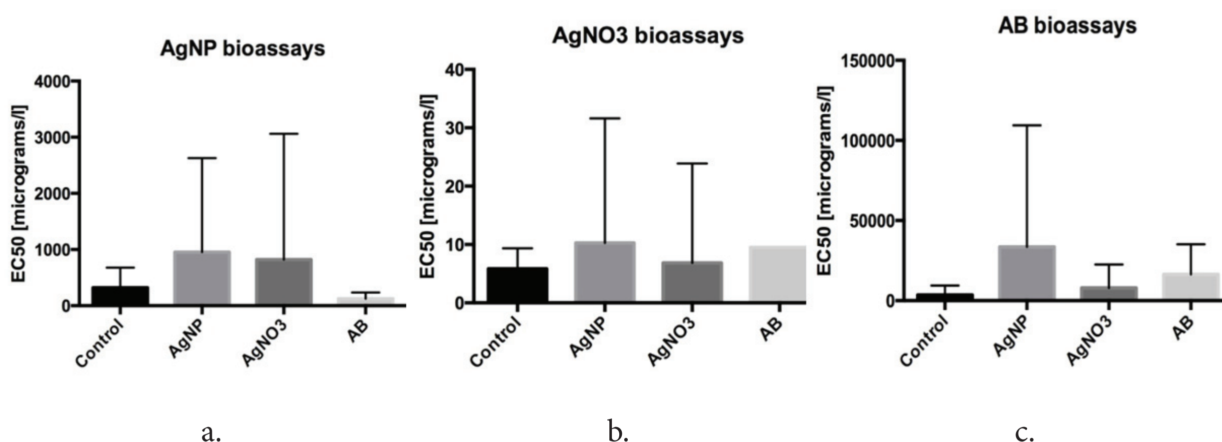


***Fig. 8.*** *Comparison between $EC_{50}$ values based on the fungal sporulation a) bioassays with AgNP, b) bioassays with $AgNO_3$, c) bioassays with AB. Graph represents mean $EC_{50}$ values for the treatment expressed in µg/l + standard deviation. In the $AgNO_3$ bioassays, standard deviation for AB treatment was too high to be presented in the graph, 457135.2.*

## 2.4. Discussion

As it has been known for a long time, Ag and AB are toxic to bacteria, while in the last century great antibacterial activity of AgNP was discovered. Silver is also known to exert direct toxicity on fungi, while antibiotics affect them only indirectly through trophic interactions with bacteria. Our results showed that $EC_{50}$ for control bacterial community is higher for antibiotics than for both AgNP and $AgNO_3$, which can be interpreted as a higher toxicity of silver toward bacteria. $EC_{50}$ for control fungal community was lowest for $AgNO_3$, followed with AgNP and highest for AB. This might indicate that toxicity of $AgNO_3$ was highest for fungi, while toxicity of AB the lowest. Here we have to take these results with caution, as variability between replicates was very high, especially in the bioassays with antibiotics.

By comparing the nutrient values within the treatments, at the end of the experiment, we can see that the concentration of DOC is lower in controls than other treatments, which implies a fast uptake of this nutrient in the first case. Also, the concentration of nitrate and nitrite was lower in controls, while the concentration of ammonium was higher in both controls and AB treatments. In natural conditions ammonium is nitrified to nitrate, and its rate depends on the availability of ammonium and dissolved oxygen, but also abundance of nitrifying bacteria (Kemp & Dodds, 2002; Bernot *et al.*, 2006). We can suppose that the abundance of nitrifying bacteria was lower in control and AB treatment, than in other two treatments.

Our results showed that the decomposition rate decreased in all treatments in the presence of toxicants, in comparison to the control. We can conclude that overall microbial community was affected by all the toxicants, and its ability to decompose leaf litter was reduced. Similar effects of AgNP and AgNO3 were found in a study by Pradhan and co-workers (2011), but in this study much higher concentrations of ionic (5 ppm, 20 ppm) and nano silver (100 ppm, 300 ppm) were used. However, in the study of Bundschuh *et al.* (2008), that were using the same mixture of antibiotics, no difference in a leaf mass loss between 200 µg/l of antibiotics, 2 µg/l of antibiotics and control was observed. Possible explanation for this difference, may be find in different leaves used in Bundschuh *et al.* (2008), black alder leaves, and present study, poplar leaves. It is known that leaf species may affect microbial community composition colonizing it (Aneja *et al.*, 2006). It is reasonable to assume that different fungal and bacterial species have different sensitivities to antibiotics.

After long-term exposure to toxicants we observed higher fungal biomass in AgNO3 and AB treatments, than in the control and AgNP treatments. The increase in fungal biomass in AB treatment, can be explained by the fact that AB impact bacteria, but not directly fungi. That would enable fungi to grow more, as some studies suggest antagonistic interactions between fungi and bacteria involved in leaf litter decomposition (e.g. Mille-Lindblom & Tranvik, 2003). Similarly, Bundschuh *et al.* (2008) found that fungal biomass increased by 38 % in AB treatment, with the same concentration like in our study. It is known that contrarily to bacterial biomass, fungal biomass is not very sensitive to moderate metal stress and declines only under high concentrations of toxicants (Duarte *et al.*, 2008; Niyogi *et al.*, 2002). Aquatic hyphomycetes are able to penetrate leaf litter tissues after colonization and remain inside the leaf (Krauss *et al.*, 2011). Leaf tissue may protect aquatic hyphomycetes from silver, because it is being retained by complexation sites on the leaf surface (Jang *et al.*, 2005). This is especially important for low concentrations of silver, like in our experiment, when not all complexation sites on the leaf surface are saturated. As the effect of silver would be higher on bacteria and their antagonistic effect would be absent, fungal growth might increase. Absence of fungal biomass increase in AgNP treatment, could be explained by low dissolution rate of AgNP that was observed, so it affected less bacteria, and they were able to manifest antagonistic effect on fungi.

Bacterial abundance decreased only in the treatment with antibiotics. However, bacterial production was similar between the treatments. Greater diversity can lead to higher abundances, probably due to interactive effects of co-occurring species. It is reasonable to assume, that antibiotics decreased bacterial diversity, and by that bacterial abundance. However, the function of bacterial production might remained unchanged due to functional redundancy. Functional redundancy suggests that there is some overlap in functioning among different species. Redundant species occupy broad range of niches and like that can co-exist. When a species disappear, redundant species can occupy this niche and continue the function (Walker, 1992). In order to confirm this theory, diversity analysis based on molecular tool, such as denaturing gradient gel electrophoresis (DGGE), of bacterial community should be performed.

Our results confirmed the hypothesis that pre-exposure to AgNP will increase tolerance of bacterial community on leaf litter to this toxicant. Indeed, in bacterial communities pre-exposed to AgNP, increased tolerance over 21-fold in comparison to the control, was observed. Pre-exposure of these communities to AgNO3 also induced increased tolerance for 4-fold, when compared to control. Similarly, pre-exposure of bacterial community to AB, increased its tolerance for 27-fold.

Development of tolerance to toxicant may happen in different ways. First, there is a possibility of horizontal gene transfer from the resistant bacteria to sensitive ones (Deshpande & Chopade, 1994). With gene transfer, sensitive bacteria are receiving the ability to develop resistance mechanisms toward toxicants. Resistance can be achieved by four basic mechanisms: reduction of membrane permeability, toxicant inactivation, efflux of the toxicant from the cell and mutation of cellular targets (Krulwich *et al.*, 2005). According to PICT, toxicants could exert selection pressure and change the community structure, with increased abundance of tolerant species and disappearance of sensitive ones (Tlili & Montuelle, 2011). In order to confirm shifts in community diversity, following long-term exposure to toxicants, a diversity analysis, such as DGGE, should be performed.

Our results, also confirmed our hypothesis that there is co-tolerance of bacterial community on leaf litter, between AgNP, AgNO3 and AB. Potential mechanisms for co-selection of antibiotics resistant bacteria by metal and vice versa are: co-resistance (different resistance genes are physically connected in a genetic element), cross-resistance (the same resistance gene brings resistance to different toxicants) and co-regulation (expression of resistance genes is under control of same regulatory pathway), (Baker-Austin *et al.*,2006). Also, there is a possibility that metals presence can increase recombination and horizontal gene transfer and to favor in that way spread of antibiotic resistance (Beaber *et al.*, 2004). Further investigations are needed in order to determine which mechanism is involved in antibiotics and silver co-tolerance.

Co-tolerance can be explained by horizontal transfer gene. There are plasmids that confers resistance to metals and antibiotics (Gupta *et al.*, 1999). It has been shown that metal and antibiotic resistance genes are linked, particularly on plasmids, and when the genes that are determining different specific resistance phenotype are located together on the same plasmid, transposon or integron, it is called co-resistance (Chapman, 2003). For example, Ghosh *et al.* found *Salmonella abortus* equi strains resistant to ampicillin, arsenic, cadmium, chromium and mercury. The plasmid removal resulted in strains sensitivity to these toxicants, while plasmid introduction to other bacteria made this bacteria resistant to these heavy metals and ampicillin.

As said before, according to PICT concept co-tolerance occurs when toxicants have similar mode of action, chemical structure or detoxifying mechanisms, and selected pressure selected the same, tolerant species for different toxicants. It is understandable why AgNO3 and AgNP showed co-tolerance, as they share the same mode of action. But silver and antibiotics used in study have different chemi-

cal structure and mode of action. There are several resistance mechanisms that were found for both metals and antibiotics: reduction in permeability, drug and metal alterations, drug and metal efflux, alteration of cellular targets, drug and metal sequestration (Baker-Austin *et al.*, 2006). It is reasonable to assume, that toxicants from the present studies share one of these detoxifying mechanisms.

$EC_{50}$ values, based on fungal sporulation, showed that hyphomycetes didn't gain tolerance to toxicants to which they were exposed during 25 days. Also, we didn't observe occurrence of co-tolerance in fungal community, between the different toxicants. Even if some species disappeared during the long term exposure to toxicants, species richness and Shannon index between the treatments did not change significantly and similarity between control and exposed communities remained high. The dominant species in control and silver treatments was *F. curvula*, this is in agreement with Funck *et al.* (2013), who found that this species is the most common under similar conditions to ours exposure to AgNP and AgNO3. Even if it was not dominant species in AB treatments, the abundance of *F. curvula* and *A. filiformis* was similar between AB treatment and control. It is possible that the selection pressure displayed by silver was not high enough to induce shift in fungal structure, as it is well known that fungi are more resistant to heavy metals than bacteria (Hiroki, 1992). It is a common knowledge that antibiotics don`t affect fungi. Because of this, exposed communities and their overall tolerance may remain unchanged. But looking at results from fungal communities, it is obvious that variability between replicas is very high. It is possible that due to this high variability, we couldn't observe tolerance, even if it was present. Whether is sporulation a reliable endpoint is still to be confirmed, because of high variability between replicates.

We confirmed that presence of AgNP in freshwaters can induce tolerance of bacterial community to this toxicant and AB. Also, exposure of bacterial community to AB will promote tolerance to both AB and AgNP.

# CHAPTER III

*Final remarks*

More and more often in general public, increased awareness about bacterial resistance to antibiotics can be noticed. Bacteria are also able to develop tolerance to silver and its nano form, which was considered as unlikely, because of broad range of mode of action manifest by silver. Bigger problem presents the occurrence of co-tolerance between these agents, which was observed in bacterial communities. This is direct threat to human health, but also to stream ecosystems. These toxicants affect microbial community directly, but indirectly higher trophic levels (macroinvertebrates, fishes...), and again through this indirect route, they affect humans.

What remained unclear in our work is tolerance of aquatic hyphomycetes toward silver nanoparticles, silver nitrate and antibiotics. Further investigation should be made with parameters other than sporulation, such as fungal production, in order to estimate fungal tolerance toward these toxicants with certainty. Also, it remained unclear whether increased tolerance, observed in bacterial community, is due to horizontal gene transfer or community shift. Further studies to determine microbial diversity, using molecular tools (e.g. DGGE) may clarify this issue, as it was done in some other studies that were analyzing effects of silver nano and ionic form on microbial decomposers (e.g. Pradhan *et al.*, 2011).

In order to understand observed co-tolerance in bacterial community, between silver nanoparticles, silver nitrate and antibiotics, more studies about their detoxifying mechanisms should be made.

Silver proved to induce tolerance of bacterial community, even in its nano form, and promote tolerance to antibiotics. Strong effort should be made in order to find an option that would not promote tolerance in bacteria and would not express co-tolerance with present antimicrobial agents. A good start would be a rational use of antibiotics and other antimicrobial agents in medicine, what would decrease occurrence of these substances in environment and consequently tolerance towards them.

# CHAPTER IV

*References*

**A**

Abelho M. (2001). From litterfall to breakdown in streams: a Review. The Scientific World 1: 656–680.

Allan J. D. (1995). Stream ecology, structure and function of running waters. Chapman & Hall, London.

Aneja M.K., Sharma S., Fleischmann F., Stich S., Heller W., Bahnweg G., Munch J.C. & Schloter M. (2006). Microbial Colonization of Beech and Spruce Litter- Influence of Decomposition Site and Plant Litter Species on the Diversity of Microbial Community. Microbial Ecology, 52 (1): 127-135.

**B**

Barbour M.T., Gerritsen J., Snyder B.D. & Stribling J.B. (1999). Rapid Bioassessment Protocols for Use in Streams and Wadable Rivers: Periphyton, Benthic Macroinvertebrates and Fish. US EPA, Washington, DC. 202

Baker-Austin C., Wright M.S., Stepanauskas R. & McArthur J.V. (2006). Co-selection of antibiotic and metal resistance. Trends. Microbiol. 14: 176–182.

Bärlocher F. (2005). Freshwater fungal communities. In: The fungal community: its organization and role in the ecosystem. Taylor and Francis, CRC Press: 39–60.

Beaber J.W., Hochhut B. & Waldor M.K. (2004). SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature 427(6969): 72-74.

Benda L., Hassan M.A., Church M. & May C.L. (2005). Geomorphology of steepland headwaters: The transition from hillslopes to channels. Journal of The American Water Resources Association 41: 835-851.

Bernot M.J., Tank J.L., Royer T.V. & David M.B. (2006). Nutrient uptake in streams draining agricultural catchments of the Midwestern United States. Freshwater Biology 51: 499–509.

Blanck H., Wänkberg S.A. & Molander S., 1988. Pollution-induced community tolerance- A new ecotoxicological tool. In Functional testing of aquatic biota for estimating hazards of chemicals: 219–230.

Boulton A. J. (1999). An overview of river health assessment: philosophies, practice, problems and prognosis. Freshwater Biology 41: 469–479.

Buesing N. & Gessner M.O. (2002). Comparison of detachment procedures for direct counts of bacteria associated with sediment particles, plant litter and epiphytic biofilms. Aquat. Microbial Ecol. 27: 29–36.

Bundschuh M., Hahn T., Gessner M.O. & Schulz R. (2009). Antibiotics as a chemical stressor affecting an aquatic decomposer–detritivore system. Environ. Toxicol. Chem. 28: 197–203.

**C**

Chapman J.S. (2003). Disinfectant resistance mechanisms, crossresistance and co-resistance. Int. Biodeterior. Biodegradation 51: 271–276.

Choi O., Deng K.K., Kim N.J., Ross L., Surampalli R.Y. & Hu Z.Q. (2008). The inhibitory effects of silvernanoparticles, silver ions, and silver chloride colloids on microbial growth. Water Res. 42: 3066–74.

Choi O. & Hu Z.Q. (2008). Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria. Environ. Sci. Technol. 42: 4583–4588.

Clarke A., Nally R.M., Bond N. & Lake P.S. (2008). Macroinvertebrate diversity in headwater streams: a review. Freshwater Biology 53: 1707-1721.

Cummins, K.W., 1974. Structure and function of stream ecosystems. Bioscience, 24, pp. 631-641.

**D**

Deshpande L.M. & Chopade B.A. (1994). Plasmid mediated silver resistance in Acinetobacter baumannii. Biometals 7: 49-56.

Duarte S., Pascoal C., Alves A., Correia A. & Cássio F. (2008). Copper and zinc mixtures induce shifts in microbial communities and reduce leaf litter decomposition in streams. Freshwater Biology 53: 91-102.

Duarte S., Pascoal C. & Cássio F. (2008). High diversity of fungi might mitigate the impact of pollution on plant litter decomposition in streams. Microb. Ecol. 56: 688-695.

Dudgeon D., Arthington, A. H., Gessner M.O., Kawabata Z. I., Knowler D. J., Lévêque C., Naiman R. J., Prieur-Richard A. H., Soto D., Stiassny M. L. J. & Sullivan C. A. (2006). Freshwater biodiversity: importance, threats, status and conservation challenges Biol. Rev. 81: 163–182.

**F**

Ferreira V., Graça M.A.S., Lima J. L. M. P. & Gomes R. (2006). Role of physical fragmentation and invertebrate activity in the breakdown rate of leaves. Archives Hydrobiology 165: 493–513.

Frossard A., Gerull L., Mutz M. & Gessner M.O. (2012). Disconnect of microbial structure and function:enzyme activities and bacterial communities in nascent stream corridors. The ISME Journal 6: 680–691.

**G**

Gessner M.O. & Chauvet E., 1993. Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. Appl. Environ. Microbiol. 59: 502-507.

Gessner M. O. & Chauvet E. (2002). A case for using litter breakdown to assess functional stream integrity. Ecological Applications 12: 498–510.

Gessner M.O., Dobson M. & Chauvet E. (1999). A perspective on leaf litter breakdown in streams. Oikos 85: 377-384.

Gessner M.O., Swan C.M., Dang C.K., McKie B.G., Bardgett R.D., Wall D.H. & Hättenschwiler S. (2010). Diversity meets decomposition. Trends Ecol. Evol. 25: 372–380.

Gessner M.O., Thomas M., Jean-Louis A. M. & Chauvet E. (1993). Stable successional patterns of aquatic hyphomycetes on leaves decaying in a summer cool sream. Mycological Research 97: 163–172.

Ghosh A., Singh A., Ramteke P. & Singh V. (2000). Characterization of large plasmids encoding resistance to toxic heavy metals in *Salmonella.abortus equi*. Biochem. Biophys. Res. Commun. 272: 6-11.

Gonçalves A.L., Gama M., Ferreira V., Graça M.A.S. & Canhoto C. (2007). The breakdown of Eucalyptus bark in a Portuguese stream. Fundamental and Applied Limnology 168: 307-315.

Gonçalves A. L., Graça M.A.S. & Canhoto C. (2013). The effect of temperature on leaf decomposition and diversity of associated aquatic hyphomycetes depends on the substrate. Functional Ecology 6: 546 – 553.

Graça M.A.S. & Canhoto C. (2006). Leaf litter processing in low order streams. Limnetica 25: 1-10.

Gulis V., Ferreira V. & Graça M. A. S. (2006). Stimulation of leaf litter decomposition and associated fungi and invertebrates by moderate eutrophication: implications for stream assessment. Freshwater Biology 51: 655–669.

Gupta A., Matsui K., Lo J.F. & Silver S. (1999). Molecular basis for resistance to silver cations in Salmonella. Nat. Med. 5: 183–188.

**H**

Hieber M. & Gessner M.O. (2002). Contribution of stream detritivores, fungi, and bacteria, to leaf breakdown based on biomass estimates. Ecology 83: 1026-1038.

Hiroki M. (1992). Effects of heavy metal contamination on soil microbial populations. Soil Sci. Plant Nutr. 38: 141-147.

Hirsch R., Ternes T., Haberer K. & Kratz K.L. (1999). Occurrence of antibiotics in the aquatic environment. Sci. Total Environ. 225: 109–118.

**J**

Jang A., Seo Y. & Bishop P.L. (2005). The removal of heavy metals in urban runoff by sorption on mulch. Environ. Pollut. 133 (1): 117–127.

**K**

Kaflon P., de Vaumas C., Samba D., Boulet E., Lefrant J., Eyraud D., Lherm T., Santoli F., Naija W. & Riou B. (2007). Comparison of silver-impregnated with standard multi-lumen central venous catheters in critically ill patients. Crit. Care Med. 35: 1032-1039.

Kemp M.J. & Dodds W.K. (2002). The influence of ammonium, nitrate, and dissolved oxygen concentrations on uptake, nitrification, and denitrification rates associated with prairie stream substrata. Limnology and Oceanography 47: 1380–1393.

Kim J. S., Kuk E., Yu K. N., Kim J. H., Park S. J., Lee H. J., Kim S. H., Park Y. K., Park Y. H., Hwang C. Y., Kim Y. K., Lee Y. S., Jeong D. H. & Cho M. H. (2007). Antimicrobial effects of silver nanoparticles. Nanomed. Nanotechnol. Biol. Med. 3: 95–101.

Krauss G.J., Sole M., Krauss G., Schlosser D., Wesenberg D. & Bärlocher F. (2011). Fungi in freshwaters: ecology, physiology and biochemical potential. FEMS Microbiol. Rev. 35 (4): 620–651.

Krulwich T.A., Lewinson O., Padan E. & Bibi E. (2005). Do physiological roles foster persistence of drug/multidrug- efflux transporters? A case study. Nat. Rev. Microbiol. 3: 566–572.

**L**

Lok C.N., Ho C.M., Chen R., He Q. Y., Yu W.Y., Sun H., Tam P. K., Chiu J.F. & Che C.M. (2006). Proteomic analysis of the mode of antibacterial action of silver nanoparticles. J. Proteome Res. 5: 916-924.

**M**

Malmquist B. & Rundle S. (2002). Threats to the running water ecosystems of the world. Environmental Conservation 29: 134–153.

Maurer-Jones M.A., Mousavi M.P.S, Chen L.D., Bühlmann P. & Haynes C.L. (2013). Characterization of dissolution from using fluorous-phase ion-selective electrodes and assessment of resultant toxicity to Shewanella oneidensis. Chemical Science 4: 2564-2572.

Martinez J.L. (2003). Recent advances on antibiotic resistance genes. In Recent Advances in Marine Biotechnology. Molecular Genetics of Marine Organisms, vol 10. Edited by Fingerman, Nagabhushanam: 13-32.

McArdell C.S., Molnar E., Suter M.J. & Giger W. (2003). Occurrence and fate of macrolide antibiotics in wastewater treatment plants and in the Glatt Valley watershed, Switzerland. Environ Sci Technol. 37: 5479–5486.

McArthur M.D. & Richardson J.S. (2002). Microbial utilization of dissolved organic carbon leached from riparian litterfall. Canadian Journal of Fisheries and Aquatic Sciences 59: 1668–1676.

Mille-Lindblom C. & Tranvik L.J. (2003). Antagonism between bacteria and fungi on decomposing aquatic plant litter. Microbiol. Ecol. 45: 173–182.

Molinero J., Pozo J. & Gonzalez E. (1996). Litter breakdown in streams of the Äguera catchment: influence of dissolved nutrients and land use. Freshwater Biology 36: 745–756.

Mueller N. C. & Nowack B. (2008). Exposure modeling of engineered nanoparticles in the environment. Environ. Sci. Technol. 42 (12): 4447–4453.

**N**

Navarro E., Piccapietra F., Wagner B., Marconi F., Kaegi R., Odzak N., Sigg L. & Behra R. (2008). Toxicity of Silver Nanoparticles to Chlamydomonas reinhardtii. Environ. Sci. Technol. 42 (23): 8959–8964.

Niyogi D. K. W. M. Lewis J.R. & McKnight D.M. (2001). Litter breakdown in mountain streams affected by mine drainage: biotic mediation of abiotic controls. Ecological Applications 11: 506–516.

Niyogi D. K. W. M., Lewis J.R. & McKnight D.M. (2002). Effects of stress on mine drainage on diversity, biomass and function of primary producers in mountain streams. Ecosystems 5: 554-567.

**P**

Pal S., Tak Y.K. & Song J.M. (2007). Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium Escherichia coli. Appl. Environ. Microbiol. 73: 1712–1720.

Posthuma L., Traas T.P. & Suter II G.W. (2002). General introduction to species sensitivity distributions. In: Posthuma L., Suter II G.W., Traas TP, editors. Species sensitivity distributions in ecotoxicology. Boca Raton, FL, USA: Lewis Publishers: 3-10.

Proia L., Lupini G., Osorio V., Pérez S., Barceló D., Schwartz T., Amalfitano S., Fazi S., Romaní A.M. & Sabater S. (2013). Response of biofilm bacterial communities to antibiotic pollutants in a Mediterranean river. Chemosphere 92: 1126–1135.

Robinson C. T. & Jolidon C. (2005). Leaf breakdown and the ecosystem functioning of alpine streams. J. N. Am. Benthol. Soc. 24: 495–507.

**S**

Schimel J., Balser T.C. & Wallenstein M. (2007). Microbial stress response physiology and its implications for ecosystem function. Ecology 88 (6): 1386-1394.

Seymour C. (2006). Audit of catheter-associated UTI using silver alloy-coated Foley catheters. Br. J. Nurs. 15: 598-603.

Soldo D. & Behra R. (2000). Long-term effects of copper on the structure of freshwater periphyton communities and their tolerance to copper, zinc, nickel and silver. Aquat.Toxicol. 47: 181–189.

Sondi I. & Salopek-Sondi B. (2004). Silver nanoparticles as antimicrobial agent: A case study on E. coli as a model for Gram-negative bacteria. J. Colloid Interface Sci. 275. 177-182.

Stepanauskas R., Glenn T.C., Jagoe C.H., Tuckfield R.C., Lindell A.H. & McArthur J.V. (2005). Elevated microbial tolerance to metals and antibiotics in metal-contaminated industrial environments. Environ. Sci. Technol. 39: 3671–3678.

Sudheer Khan S., Bharath Kumar E., Mukherjee A. & Chandrasekaran N. (2011), Bacterial tolerance to silver nanoparticles (SNPs): *Aeromonas punctata* isolated from sewage environment. J. Basic Microbiol. 51: 183–190.

**T**

Teitzel G.M. & Parsek M.R. (2003). Heavy metal resistance of biofilm and planktonic Pseudomonas aeruginosa. Appl. Environ. Microbiol. 69: 2313–2320.

Tlili A., Marechal M., Berard A., Volat B. & Montuelle B. (2011). Enhanced co-tolerance and co-sensitivity from long-term metal exposures of heterotrophic and autotrophic components of fluvial biofilms. Sci. Total Environ. 409: 4335-4343.

Tlili A. & Montuelle B. (2011). Microbial pollution-induced community tolerance. In: Amiart- Triquet C, Rainbow PS, Rom.o M, editors. Tolerance to environmental contaminants. CRCpress.

**U**

United States Geological Survey (USGS), Toxic Substances Hydrology Program website, Emerging Contaminants in the Environment, http://toxics.usgs.gov/regional/emc/index.html

**V**

Vannote R.L., Minshall G.W., Cummins K.W., Sedell J.R. & Cushing C.E. (1980). The River Continuum Concept. Can. J. Fish. Aquat. Sci. 37: 130-137.

**W**

Wallace J.B., Eggert S.L., Meyer J.L. & Webster J.R. (1997). Multiple trophic levels of a forest stream linked to terrestrial litter inputs. Science 277: 102–104.

Walker B.H. (1992). Biodiversity and Ecological Redundancy. Conserv.Biol. 6: 18-23.

Watkinson A.J., Micalizzi G.R., Bates J.R. & Costanzo S.D. (2007). Novel method for rapid assessment of antibiotic resistance in Escherichia coli isolates from environmental waters by use of a modified chromogenic agar. Appl Environ Microbiol. 73: 2224- 2229.

Webster J. R. & Benfield E. F. (1986). Vascular plant breakdown in freshwater ecosystems. Annu. Rev. Ecol. Syst. 17: 567–594.

Webster J.R., Benfield E.F., Ehrman T.P., Schaeffer M.A., Tank J.L., Hutchens J.J. & D'Angelo D.J. (1999). What happens to allochthonous material that falls into streams? A synthesis of new and published information from Coweeta. Freshw Biol. 41: 687–705.

Woo K.Y. (2009). Review: Silver-releasing dressings improve healing and quality of life in patients with non-healing chronic wounds. Evid. Based Nurs. 12: 117.

***Y***

Yoon K.Y., Byeon J.H., Park J.H. & Hwang J. (2007). Susceptibility constants of Escherichia coli and Bacillus subtilis to silver and copper nanoparticles. Sci. Tot. Environ. 373: 572-575.

Young J. C. (1995). Microwave-Assisted Extraction of the Fungal Metabolite Ergosterol and Total Fatty Acids. Journal of Agriculture and Food Chemistry 43: 2904-2910.

Young S., Juhl A. & O'Mullan G.D. (2013). Antibiotic-resistant bacteria in the Hudson River Estuary linked to wet weather sewage contamination. Journal of Water and Health 11 (2): 297–310.

**Z**

Zhou L.J., Ying G.G., Liu S., Zhao J.L., Yang B., Chen Z.F. & Lai H.J. (2013). Occurrence and fate of eleven classes of antibiotics in two typical wastewater treatment plants in South China. Sci. Total Environ. 452-453: 365-376.