**Universidade de Aveiro** Departamento de Biologia
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# Alteração das comunidades microbianas como medida de avaliação do impacto da contaminação do solo por nanopartículas.

# Changes of the microbial communities as a mean to evaluate the impact of soil contamination by nanoparticles.

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**2015**

**Sara Cristiana Lopes Peixoto**

# Alteração das comunidades microbianas como medida de avaliação do impacto da contaminação do solo por nanopartículas.

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Jacinta Oliveira (Investigadora em Pós-Doutoramento no Departamento de Biologia e CESAM da Universidade de Aveiro) e co-orientação da Doutora Susana Loureiro (Investigadora Auxiliar no Departamento de Biologia e CESAM da Universidade de Aveiro).

Dedico este trabalho aos meus pais.

**o júri**

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**palavras-chave**

Nanopartículas de prata, nitrato de prata, comunidades bacterianas do solo.

**resumo**

As nanopartículas de prata (AgNPs) são muito utilizadas na indústria devido às suas propriedades antibacterianas. A libertação de forma não controlada das AgNPs para o ambiente e a sua persistência no mesmo, aumentam a preocupação em relação à contaminação dos solos por AgNPs. Assim, o objetivo desta tese foi analisar os efeitos das AgNPs nas comunidades bacterianas do solo recorrendo a duas abordagens complementares: uma independente do cultivo [Polymerase Chain Reaction- Denaturing Gradient Gel Electrophoresis (PCR-DGGE)] e outra dependente do cultivo (método de difusão por discos para analisar a suscetibilidade bacteriana).

Para dar cumprimento a este objectivo, as comunidades bacterianas do solo LUFA 2.2 foram expostas a 5 µg/kg de AgNPs ou de catião prata ($Ag^+$, disponibilizado como $AgNO_3$) durante 56 dias.

A análise das comunidades bacterianas do solo por PCR-DGGE foi feita para 42 e 56 dias e na presença e ausência do *Porcellionides pruinosus*. Para os testes de suscetibilidade, apenas as comunidades bacterianas do solo para 56 dias foram analisadas.

O primeiro método possibilitou investigar os efeitos das AgNPs ou $Ag^+$ nas comunidades bacterianas do solo e compreender se estes efeitos alteravam com a presença do *P. pruinosus* ou ao longo do tempo de exposição. O segundo método permitiu avaliar a resposta de suscetibilidade das comunidades bacterianas do solo atendendo à forma (AgNPs ou $Ag^+$) e quantidade (10 µg, 1 µg ou 0.1 µg) da prata, à exposição conjunta e após reincidência da contaminação por prata.

Com o PCR-DGGE demostrou-se que a forma da prata (AgNPs ou $Ag^+$), o período de exposição (42 ou 56 dias) e a presença do *P. pruinosus* foram relevantes para a alteração da comunidade bacteriana do solo. O *P. pruinosus* poderá ser, provavelmente, útil para a bioremediação de catião prata para exposições perto dos 2 meses; ainda assim, mesmo na presença deste isópode, as AgNPs poderão constituir um risco para as comunidades bacterianas do solo. Através do método de difusão por discos foi possível disponibilizar uma análise da fracção viável das comunidades bacterianas do solo demonstrando que a forma, quantidade e combinação de ambas as formas de prata afectaram a suscetibilidade das comunidades bacterianas do solo. As bactérias mostraram ser mais suscetíveis $Ag^+$ do que às AgNPs sendo também possível observar uma resposta dependente da quantidade além do efeito aditivo para a exposição combinada.

Apesar dos efeitos das nanopartículas de prata nas comunidades bacterianas terem sido analisadas por dois métodos, foram identificados alguns padrões: as bactérias foram afetadas por ambas as formas de prata, mostrando que quer a estrutura da comunidade quer a suscetibilidade eram alteradas. É imperativa a análise de baixas quantidades em investigações futuras, além das testadas nesta tese, já que a resposta de suscetibilidade alterou-se para exposição prévia a contaminações com quantidades baixas (1 e 0.1 µg).

Comparativamente com o catião de prata, as AgNPs parecem afetar menos as comunidades bacterianas do solo. Assim, esta tese reforça o quão valioso é usar estas comunidades para avaliar os efeitos da contaminação no solo, já que provaram ser sensíveis à contaminação de prata pelas duas metodologias.

**keywords** Silver nanoparticles, silver nitrate, soil bacterial communities.

**abstract**

Silver nanoparticles (AgNPs) are broadly used in the industry due to AgNPs' antibacterial proprieties. Uncontrolled released of AgNPs and persistence in the environment might enhance the concern of soil contamination by AgNPs. Thus, the aim of this thesis was to evaluate the effects of AgNPs on the bacterial communities using two complementary approaches: a culture-independent method [Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE)] and a culture-dependent method (disc diffusion to analyze the bacterial susceptibility).

To explore this aim, the bacterial communities of the LUFA 2.2 soil was exposed to 5 µg/kg of AgNPs or silver cation ($Ag^+$, provided as $AgNO_3$) during 56 days.

The analysis of the soil bacterial communities by PCR-DGGE included those retrieved from soil at 42 and 56 days as well as those developed in the presence and absence of *Porcellionides pruinosus*. For the susceptibility tests, only the soil bacterial communities retrieved from soil at 56 days were analyzed.

The first method enabled to investigate the effects of AgNPs or $Ag^+$ on the bacterial community of soil and to understand if these effects changed with the presence of *P. pruinosus* or along the exposure period.

The second methodology provided an analysis of the viable soil bacterial community and allowed to assess the susceptibility responses of the soil bacterial communities according to the silver form (AgNPs or $Ag^+$), silver amounts (10 µg, 1 µg or 0.1 µg), joint exposure to AgNPs and $Ag^+$ and after previous exposure to silver.

By using PCR-DGGE, we demonstrated that the silver forms (AgNPs and $Ag^+$), exposure period (42 or 56 days) and the presence of *P. pruinosus* were relevant to alter the structure of soil bacterial community. *P. pruinosus* showed to be probably useful to minimize the effects of silver cation for exposures close to 2 months; yet, even in the presence of this isopod, the AgNPs might still be a risk for the soil bacterial communities. By using disc diffusion method we provided an analysis of the viable soil bacterial community demonstrating that the silver form, the amount and the combination of both silver forms affected the susceptibility of the soil bacterial communities. Bacteria were more susceptible to $Ag^+$ than to AgNPs and amount-dependent as well as addition effects were demonstrated.

Although the bacterial communities being analyzed by the two methods were different, overall patterns were identified: bacteria are affected by both silver forms, particularly by showing altered community structure and showing susceptibility through growth inhibition. Yet, lower concentrations than those herein tested are imperative to be considered in future investigations as we obtained most of the susceptibility changes for exposures with lower amounts (1 and 0.1 µg).

Compared to silver cation, the AgNPs appears to affect less the soil bacterial communities. Thus, this thesis strengthens how valuable is to use these communities to evaluate the effects of the soil contamination as they proved to be sensible to silver contamination by both methodologies.

# INDEX

## SCOPE, AIM AND STRUCTURE OF THE THESIS

1. Scope....1
2. Aim....2
3. Structure....3

## CHAPTER I – GENERAL INTRODUCTION

1. Nanotechnology....7
1.1. Nanoparticles *versus* nanomaterial: definition and distinction....7
1.2. Regulation....8
1.3. Source of nanoparticles....8
1.3.1. NPs from natural sources....9
1.3.1.1. Green nanoparticles (gNPs)....9
1.3.2. Anthropogenic sources....12
1.3.2.1. Engineered nanoparticles (ENPs)....12
2. ENPs: Silver nanoparticles (AgNPs)....14
2.1. AgNPs *versus* bulk form (Ag cation: $Ag^{+}$)....16
2.2. Emission and exposure scenarios of AgNPs into the environment....16
2.3. AgNPs interactions in soil compartment....19
2.4. AgNPs interaction with bacteria....22
2.5. Possible effects of the interaction between AgNPs and the microbiota from soil ...25
3. Ecotoxicology studies....26
3.1. Terrestrial ecotoxicological assays: standard soil. ....27
3.2. Evaluation of the toxicity of AgNPs to soil bacteria....28
3.3. Evaluation of the toxicity of AgNPs using terrestrial organisms....29
3.3.1. Model organism: *Porcellionides pruinosus* ....32
3.3.1.1. Characterization of the model organism ....33
4. Methods for analysis of the effects of AgNPs ....35
4.1. Culture-independent methods....35
4.1.1. Polymerase Chain reaction (PCR) ....36
4.1.2. Denaturing Gradient Gel Electrophoresis (DGGE) ....38
4.1.3. PCR-DGGE studies....40
4.2. Culture-dependent methods....41
4.2.1. Susceptibility tests....41
5. Comparison between culture-dependent and culture-independent methods ....44
6. References....45

## CHAPTER II - EFFECTS OF SILVER-NANOPARTICLES: PCR-DGGE PROFILES SHOW CHANGES ON SOIL BACTERIAL COMMUNITY

Abstract....57
Graphical abstract....58
1. Introduction....59
2. Material and methods....61
2.1. Experimental design ....61

2.2. Analysis of the soil bacterial community .....62
2.2.1. Total DNA extraction.....62
2.2.2. PCR-DGGE.....62
2.3. Statistical analysis .....63
3. Results.....64
4. Discussion.....71
5. Conclusions.....75
6. Acknowledgements.....75
7. References.....75

**CHAPTER III -** SUSCEPTIBILITY OF SOIL BACTERIAL COMMUNITY TO SILVER (NANOPARTICLE AND CATION)

Abstract.....81
Graphical abstract.....82
1. Introduction.....83
2. Material and methods.....84
2.1. Experimental design and sample preparation.....84
In the table: AgNPs – Silver nanoparticles; $Ag^{+}$– Silver cations supplied as $AgNO_3$. .....85
2.2. Disc diffusion method .....85
The disc diffusion method was used to analyze the susceptibility of the cultivable soil bacterial community to silver. The protocol was adapted from (Matuschek *et al*., 2013; Saeb *et al*., 2014) and will be briefly described. .....85
2.3. Statistical analysis .....87
3. Results.....87
3.1. Non-exposed soil microbial communities .....87
3.2. Soil microbial communities previously exposed to silver.....90
3.3. Bacterial groups representative of soil bacterial community .....92
4. Discussion.....93
5. Conclusions.....98
6. Acknowledgements.....98
7. References.....98
8. Supplementary data.....102

**CHAPTER IV –** FINAL DISCUSSION AND CONCLUSION

1. Final discussion and conclusions.....105
2. References.....109

**ANNEXES**

ANNEX 1. Properties of LUFA 2.2 soil .....115
ANNEX 2. Electrophoresis of the PCR products obtained prior to DGGE. .....116
ANNEX 3. Culture media used in the susceptibility tests: preparation .....118
ANNEX 4. Susceptibility of bacterial strains to antibiotics.....119
ANNEX 5. Images of the resulting plates from the susceptibility test at 25ºC. .....120
ANNEX 6. Susceptibility tests at 37ºC .....123
ANNEX 7. Images of the resulting plates from the susceptibility test at 37ºC. .....129

# LIST OF FIGURES

**SCOPE, AIM AND STRUTURE OF THE THESIS**

**Figure 1.** Experimental design: bacterial community from LUFA 2.2 soil was analyzed by two methods, disc diffusion (culture-dependent method) and PCR-DGGE (culture-independent method). .....3

**CHAPTER I –** GENERAL INTRODUCTION

**Figure 2.** Silver nano-based products in different commercial products (Adapted from: Muller & Nowack, 2008) .....15

**Figure 3.** Life cycle stages of nanomaterials (NMs) and their environmental fate (Adapted from: Som *et al*., 2010).....17

**Figure 4.** Predicted concentrations (PEC) of AgNPs (ng/kg dry weight soil) in Europe (expecting 57% of agricultural land used, minimum NPs transfer to sludge and a 25 cm ploughing depth) (Faust & Backhaus, 2011). In the figure: highest PEC was determined for England (> 10 ng, represented in red); the most abundant PEC rounds 5-10 ng/kg dry weight of soil in central and south of Europe (represented in orange) while the lowest PEC (0-5 ng/kg dry weight of soil) is represented in yellow (Faust & Backhaus, NanoFATE) .....19

**Figure 5.** Key processes in soil related to the transformation and potential risk driven by manufactured nanoparticles. In the figure: (1) dissolution; (2) sorption/aggregation; (3) plant accumulation; (4) invertebrate accumulation and toxicity; (5) microbial toxicity; (6) direct particle uptake/toxicity; (7) particle migration (Klaine *et al*., 2008).....20

**Figure 6.** Interactions between silver nanoparticles and environment. The figure shows the major characteristics of AgNPs (green) and the environmental conditions [pH, DO (dissolved oxygen), organic matter and salt concentration] (red) affecting the AgNPs toxicity (grey) to the biota (microorganisms and organisms) (Adapted from: Dinesh *et al.*, 2012).....21

**Figure 7.** Some mechanisms of action of NPs against microorganisms (Choi *et al*., 2012).....23

**Figure 8.** Schematic representation of possible responses of microbial communities to a disturbance (Adapted from Sharma, 2010) .....25

**Figure 9.** Ecotoxicological studies focused on different ENPs (B) and group of organisms (A) (Adapted from Kahru & Dubourguier, 2010).....26

**Figure 10.** Hazard of AgNPs (a) and bulk form ($AgNO_3$) for different organisms. Ranging was obtained using the median of $LC_{50}$ (concentration causing mortality for 50% of the organisms) or $EC_{50}$ (concentration causing effect for 50% of the organisms)

values for all organisms except for bacteria for which the MIC (minimum inhibitory concentration). (Adapted from Bondarenko *et al*., 2013).....27

**Figure 11.** Internal morphology of the digestive system of an adult isopod (I); in the figure: head (ST), midgut glands-hepatopancreas (MG), anterior region of the hindgut (ANT), typhlosole (TI), papillate region of the hindgut (PAP), sphincter (SP), rectum (RE), anus (AN) (Adapted from: Kostanjsek *et al*., 2002). *Porcellionides pruinosus* - external morphology (II). Hepatopancreas attached to isopod head (III).....34

**Figure 12.** Molecular methods used to detect and quantify environmental microbial communities (EMC) (Adapted from: Gao & Tao, 2012). In the figure: denaturing gradient gel electrophoresis (DGGE); terminal restriction fragment length polymorphism (T-RFLP); fluorescence in situ hybridization (FISH); phospholipid fatty acid (PLFA); restriction fragment length polymorphism (RFLP); microarray analysis (Geochip); stable isotope probing (SIP) and automated rRNA intergenic spacer analysis (ARISA) .....36

**Figure 13.** PCR-DGGE approach for bacterial community analysis (Adapted from: Nakatsu, 2007).....39

**CHAPTER II** - EFFECTS OF SILVER-NANOPARTICLES: PCR-DGGE PROFILES SHOW CHANGES ON SOIL BACTERIAL COMMUNITY

**Figure 1**. PCR-DGGE profile obtained for the soil bacterial community exposed to AgNPs or $Ag^{+}$, during 42 days (A) and 56 days (B). Please see **Table 1** for sample identification details. Triplicates of each sample are ordered and grouped. Lane M refers to DGGE marker: I - RAI 70; II - RAN 60; III - RAI 3; IV - RAI 43; V - RAN 18; VI - RAN 12; VII - RAN 140; VIII - RAI 76 (Henriques *et al*., 2004).....66

**Figure 2**. Cluster (A) and PCoA analysis (B) of DGGE profiles obtained from the soil bacterial community exposed to AgNPs or $Ag^{+}$ during 42 days. Please see **Table 1** for sample identification. For Figure 2A: the cluster mentioned in the text as cluster 1 is delimited by solid line and represents the controls, cluster 2 is delimited by dashed line and represents silver contaminated samples in the absence of *P. pruinosus*; the remaining samples are grouped in cluster 3 which represents silver contaminated samples in the presence of *P. pruinosus*; clusters and PCoA groups were defined at 4% similarity.....67

**Figure 3.** Cluster (A) and PCoA analysis (B) of DGGE profiles obtained from the soil bacterial community exposed to AgNPs or $Ag^{+}$ during 56 days. Please see **Table 1** for sample identification. For Figure 3A: the cluster mentioned in the text as cluster 4 is delimited by solid line, the cluster 5 is delimited by dashed line and the remaining samples are grouped in cluster 6;clusters were qualitatively defined for better description in the text; groups in PCoA were defined at 15% similarity .....70

**CHAPTER III** - SUSCEPTIBILITY OF SOIL BACTERIAL COMMUNITY TO SILVER (NANOPARTICLE AND CATION)

**Figure 1.** Susceptibility of soil bacterial communities (CT0 and CT56) not exposed to silver: (A) dBC (directly plated bacterial communities) and (B) pBC (pre-enriched bacterial communities). Please see **Table 1** and **2** for sample identification. Data represent diameters mean ± standard deviation (n=4). (*) Statistical differences between soil treatments (CT0 *vs.* CT56), for the same silver amount, according to one-way ANOVA (*Dunnett's* test) for $p<0.05$. Different letters (*e.g.* a and a') corresponds to significant differences, for the same silver amount, within soil treatments (*e.g.* within CT0 or within CT56) and between silver forms (AgNPs *vs.* $Ag^{+}$) $p<0.05$ (two-way ANOVA) .....89

**Figure 2.** Susceptibility of soil bacterial communities previously exposed to silver forms (NP56 or Ag56): (A) dBC (directly plated bacterial communities) and (B) pBC (pre-enriched bacterial communities). Data relative to non-exposed soil bacterial communities from the end of the experiment (CT56 for dBC and CT56 for pBC) was maintained for comparison purposes. Please see **Table 1** and **2** for sample identification. Data represent diameters mean ± standard deviation (n=4). (*) Statistical differences between soil treatments (CT56 *vs.* NP56 or CT56 *vs.* Ag56), for the same silver amount, according to one-way ANOVA (*Dunnett's* test) for $p<0.05$. Different letters (*e.g.* a and a') corresponds to significant differences, for the same silver amount, within soil treatments (*e.g.* within NP56 or within Ag56) and between silver forms (AgNPs *vs.* $Ag^{+}$) $p<0.05$ (two-way ANOVA) .....91

**Figure 3.** Susceptibility of bacterial strains to silver exposure. In the figure: *Escherichia coli* ATCC 25922 was only used as control and is separate from the tested bacterial strains using a dashed line; Antibiotic imipenem (IMI) was only used for *Pseudomonas putida*. Please see **Table 2** for sample identification. Data represent diameters mean ± standard deviation (n=4). (*) Statistical differences among bacterial strains (*Bacillus sphaericus* ATCC 29726 *vs. Arthrobacter arilaitensis* GCNP1_I *vs. Pseudomonas putida* NCIMB 10432), for the same silver amount, according to one-way ANOVA (*Dunnett's* test) for $p<0.05$. Different letters (*e.g.* a and a') corresponds to significant differences, for the same silver amount, within each bacterial strain (*e.g.* within *Bacillus sphaericus* ATCC 29726 or within *Arthrobacter arilaitensis* GCNP1_I or within *Pseudomonas putida* NCIMB 10432) and between silver forms (AgNPs *vs.* $Ag^{+}$), $p<0.05$ (two-way ANOVA).....93

**Figure S1.** Plates of cultured bacterial communities not exposed to silver (CT56) after testing response to silver forms and amounts: (A) dBC (directly plated bacterial communities) and (B) pBC (pre-enriched bacterial communities). Please see **Table 1** and **2** for sample identification. See also **ANNEX 5**.....102

**ANNEXES**

**Figure A2.1**. Electrophoresis in agarose gels (1.5% in TAE 1x; 120V; 40 min) of the PCR products obtained from controls samples (not exposed soil) at 0 (CT0) and 56 days (CT56). Triplicates are grouped. In the figure: Cp - Positive control (consists of all reagents used during sample processing but contains a DNA that you know it will

amplify instead of sample); Cn - Negative control (consists of all reagents used during sample processing but contains water instead of sample; it can also be a DNA that you know it will not amplify); B - Reagent blank (consists of all reagents used during sample processing but contains no sample) control; Pv - Empty well; MK - Thermo Scientific™ FastRuler Middle Range DNA Ladder (#SM1113) [composed of the following DNA fragments (in base pairs): 5000, 2000, 850, 400 and 100].....116

**Figure A2.2.** Electrophoresis in agarose gels (1.5% in TAE 1x; 120V; 40 min) of the PCR products obtained from soil samples after exposure to AgNPs during 56 days in the absence (NP56) and presence (NP56Pp) of *P. pruinosus*. Triplicates are grouped. In the figure: Cp - Positive control; Cn - Negative control; B - Reagent blank control; Pv - Empty well; MK - Thermo Scientific™ FastRuler Middle Range DNA Ladder (#SM1113) [composed of the following DNA fragments (in base pairs): 5000, 2000, 850, 400 and 100].....116

**Figure A2.3.** Electrophoresis in agarose gels (1.5% in TAE 1x; 120V; 40 min) of the PCR products obtained from soil samples after exposure to $Ag^+$ during 56 days in the absence (Ag56) and presence (Ag56Pp) of *P. pruinosus*. Triplicates are grouped. In the figure: Cp - Positive control; Cn - Negative control; B - Reagent blank control; Pv - Empty well; MK - Thermo Scientific™ FastRuler Middle Range DNA Ladder (#SM1113) [composed of the following DNA fragments (in base pairs): 5000, 2000, 850, 400 and 100].....117

**Figure A2.4.** Electrophoresis in agarose gels (1.5% in TAE 1x; 120V; 40 min) of the PCR products obtained from non-contaminated soil samples at the beginning (CT0) and at 42 days in the absence (CT42) and presence (CT42Pp) of *P. pruinosus* as well as contaminated soil samples (with AgNPs in the absence (NP56) and presence (NP56Pp) of *P. pruinosus* or to Ag+ in the absence (Ag56) and presence (Ag56Pp) of the isopod). Triplicates are grouped. In the figure: Cp - Positive control; Cn - Negative control; B - Reagent blank control; Pv - Empty well; MK - Thermo Scientific™ FastRuler Middle Range DNA Ladder (#SM1113) [composed of the following DNA fragments (in base pairs): 5000, 2000, 850, 400 and 100].....117

**Figure A5.1.** Susceptibility assays of the soil bacterial communities [directly plated soil bacterial communities: dBC; non-exposed (at 0 and 56 days) and exposed to AgNPs or $Ag^+$] by disc diffusion method at 25ºC. Please see **Table 2** (**Chapter III**) for disc identification details. Quadruplicate assay (R1, R2, R3 and R4).....121

**Figure A5.2.** Susceptibility assays of the soil bacterial community (after recover to TSB) to AgNPs or $Ag^+$ exposures, by disc diffusion method at 25ºC. Please see **Table 2** (**Chapter III**) for sample identification details. Quadruplicate assay (R1, R2, R3 and R4).....121

**Figure A5.3.** Susceptibility assays of the bacterial strains (*P. putida; A. arilaitensis; B. sphaericus* and *E. coli*) to AgNPs or $Ag^+$ exposures, by disc diffusion method at 25ºC. Please see **Table 2** (**Chapter III**) for sample identification details. Quadruplicate assay (R1, R2, R3 and R4).....122

**Figure A6.1.** Susceptibility of soil bacterial communities previously not exposed to silver forms (CT0 and CT56) at 37ºC: **(A)** dBC (directly plated bacterial communities) and **(B)** pBC (pre-enriched bacterial communities). Please see **Table 1** and **2** (**Chapter**

**III**) for sample identification. Data represent diameters mean ± standard deviation (n=4). (*) Statistical differences between soil treatments (CT0 *vs.* CT56), for the same silver amount, according to one-way ANOVA (*Dunnett's* test) for p<0.05. Different letters (*e.g.* a and a') corresponds to significant differences, for the same silver amount, within soil treatments (*e.g.* within CT0 or within CT56) and between silver forms (AgNPs *vs.* $Ag^{+}$), p<0.05 (two-way ANOVA).....124

**Figure A6.2** Susceptibility of soil bacterial communities previously exposed to silver forms (NP56 or Ag56) at 37ºC: (A) dBC (directly plated bacterial communities) and (B) pBC (pre-enriched bacterial communities). Data relative to non-exposed soil bacterial communities from the end of the experiment (CT56 for dBC and CT56 for pBC) was maintained for comparison purposes. Please see **Table 1** and **2** (**Chapter III**) for sample identification. Data represent diameters mean ± standard deviation (n=4). (*) Statistical differences between soil treatments (CT56 *vs*. NP56 or CT56 *vs*. Ag56), for the same silver amount, according to one-way ANOVA (Dunnett's test) for p<0.05. Different letters (e.g. a and a') corresponds to significant differences, for the same silver amount, within soil treatments (e.g. within NP56 or within Ag56) and between silver forms (AgNPs *vs.* $Ag^{+}$), p<0.05 (two-way ANOVA) .....126

**Figure A6.3.** Susceptibility of bacterial strains to silver exposure at 37ºC. In the figure: *Escherichia coli* ATCC 25922 was only used as control and is separate from the tested bacterial strains using a dashed line; Antibiotic imipenem (IMI) was only used for *Pseudomonas putida*. Please see **Table 2** (**Chapter III**) for sample identification. Data represent diameters mean ± standard deviation (n=4). (*) Statistical differences among bacterial strains (*Bacillus sphaericus* ATCC 29726 *vs. Arthrobacter arilaitensis* GCNP1_I *vs. Pseudomonas putida* NCIMB 10432), for the same silver amount, according to one-way ANOVA (*Dunnett's* test) for p<0.05. Different letters (*e.g.* a and a') corresponds to significant differences, for the same silver amount, within each bacterial strain (*e.g.* within *Bacillus sphaericus* ATCC 29726 or within *Arthrobacter arilaitensis* GCNP1_I or within *Pseudomonas putida* NCIMB 10432) and between silver forms (AgNPs *vs.* $Ag^{+}$), p<0.05 (two-way ANOVA).....128

**Figure A7.1.** Susceptibility assays of the soil microbiota to AgNPs or $Ag^{+}$ exposures, by disc diffusion method at 37ºC. Please see **Table 2** (**Chapter III**) for sample identification details. Quadruplicate assay (R1, R2, R3 and R4).....129

**Figure A7.2.**Susceptibility assays of the soil microbiota (after recover to TSB) to AgNPs or $Ag^{+}$ exposures, by disc diffusion method at 37ºC. Please see **Table 2** (**Chapter III**) for sample identification details. Quadruplicate assay (R1, R2, R3 and R4).....130

**Figure A7.3.** Susceptibility assays of the bacterial collections (*P. putida; A. arilaitensis; B. sphaericus* and *E. coli*) to AgNPs or $Ag^{+}$ exposures, by disc diffusion method at 37ºC. Please see **Table 2** (**Chapter III**) for sample identification details. Quadruplicate assay (R1, R2, R3 and R4) .....131

# LIST OF TABLES

**CHAPTER I –** GENERAL INTRODUCTION

**Table 1.** Silver nanoparticles (AgNPs) synthetized using the green method.....12

**Table 2**. ENPs mentioned in the literature as causing environmental concern.....14

**Table 3.** OECD and ISO regulations to evaluate the soil quality using the microorganisms (Adapted from: Kuperman *et al*., 2014).....29

**Table 4.** Ecotoxicological tests to analyse the effect of silver nanoparticles in terrestrial organisms.....31

**Table 5.** Scientific classification of *Porcellionides pruinosus* (Adapted from: Geoffrey, Fauna Europaea).....32

**Table 6**. Bacterial susceptibility tests applied to AgNPs.....43

**CHAPTER II -** EFFECTS OF SILVER-NANOPARTICLES: PCR-DGGE PROFILES SHOW CHANGES ON SOIL BACTERIAL COMMUNITY

**Table 1**. Sample identification of soil experimental setup.....62

**Table 2.** ANOSIM statistical analysis (*R* value; *P*=0.1%) generated from the patterns of bands obtained by DGGE of the soil bacterial community (42 days).....68

**Table 3.** Richness (*S*) and shannon diversity (*H*') and evenness (*J*') indexes of the soil bacterial community (42 days).....68

**Table 4.** ANOSIM statistical analysis (*R* value; *P*=0.1%) generated from the patterns of bands obtained by DGGE of the soil bacterial community (56 days).....71

**Table 5**. Richness (*S*) and **s**hannon diversity (*H*') and evenness (*J*') indexes of the soil bacterial community (56 days).....71

**CHAPTER III -** SUSCEPTIBILITY OF SOIL BACTERIAL COMMUNITY TO SILVER (NANOPARTICLE AND CATION)

**Table 1**. Sample identification of soil experimental setup (n=3).....85

**Table 2.** Identification of the discs used in the susceptibility testing. .....86

**ANNEXES**

**Table A1.1** Main properties of LUFA 2.2 soil .....115

**Table A3.1.** Composition on the medium TSB and MHA (MERK, Germany) .....118

**Table A4.1**. Susceptibility patterns to the two antibiotics Imipenem and Ticarcillin - Clavulanic Acid [Adapted from: CLSI document M100-S23 (M02-A11): "Disc diffusion supplemental tables"].....119

# SCOPE, AIM AND STRUTURE OF THE THESIS

# 1. Scope

Nowadays, one of the environmental challenges in global society is to maintain and improve the quality of soil, water and air. "Pollutants of emerging concern" are chemicals or materials that have the potential to threat or that represent a real threat to human/biota health or to the environment and to which legislation is lacking (Stuart & Compton, 2015). Concentrations of these recent pollutants in the environment are uncertain. This increases the difficulty in monitoring the quality of the environment (Ditta *et al*., 2015). Silver nanoparticles (AgNPs) fall into this category.

These nanoscale particles (1-100 nm) have been produced for years but large-scale production began only in past two decades with high impact in the industry and economy (Yu *et al*., 2013; Bour *et al*., 2015).

Due to large production (near 500 tons of AgNPs per year worldwide) and widespread use (Yu *et al*., 2013), the inevitable release of AgNPs from industry sources into the environment increased in the last years. Yet, the impact of AgNPs discharge is not fully known and there is a lack of legislation concerning their use in the industrial processes (Tourinho *et al*., 2012). Therefore, it is necessary to conduct studies to assess the potential effects of AgNPs on systems, particularly in the terrestrial compartment which is highly affected by AgNPs contamination (Tourinho *et al*., 2013).

AgNPs have highly appreciated antibacterial properties in a commercial point of view; yet these properties raise concern about AgNPs' potential risk to the environment, in particular to the natural soil microbiota (Mirzajani *et al*., 2013). As AgNPs can interact with organisms and microorganisms (bacteria included) (Engelke *et al*., 2014) and because of bacterial communities' ubiquity and their essential role in soil quality and function (Holden *et al*., 2014), the possible effects in the microbiota as a consequence of exposure/interactions with AgNPs must be investigated.

## 2. Aim

Changes in the bacterial communities of the soil due to AgNPs' presence might induce bacterial imbalance and pose a risk to the terrestrial ecosystem. In order to verify this hypothesis, this thesis aimed to evaluate the possible effects of the AgNPs in the bacterial communities of the soil using the standard LUFA 2.2 soil.

In line with this goal, both molecular approaches and culture-dependent methods were used in a complementary perspective. Thus, to achieve the main aim, the experimental design (**Figure 1**) was divided into two integrated tasks which detailed description follows:

1. Evaluation of the changes in soil bacterial community using Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE):

The effects of AgNPs and $Ag^+$ (in the form of $AgNO_3$) on structure, richness, diversity and evenness of the soil bacterial community were evaluated. In this case, the bacterial community tested was the bacterial community from LUFA 2.2 soil after exposure to AgNPs or $Ag^+$ to 5 µg/kg during 42 or 56 days and when the isopod *Porcellionides pruinosus* was included as a biota element.

2. Evaluation of the soil bacteria susceptibility using the disc diffusion method:

The effects of AgNPs and $Ag^+$ (in the form of $AgNO_3$) on the susceptibility of the soil bacterial communities were evaluated by measuring the growth inhibition zones around the discs loaded with each of the mentioned contaminants, in different amounts or when combined. In this case, the viable fraction of the bacterial community of LUFA 2.2 soil was tested (1) without previous exposure to silver and (2) after exposure to 5 µg/kg of AgNPs or $Ag^+$ during 56 days.

| SOIL EXPOSURE | AgNPs or $Ag^+$ at 5 µg/Kg soil | |
|---|---|---|
| **DURATION EXPOSURE** | 56 days | |
| **METHOD** | PCR-DGGE | Disc diffusion |
| **OUTPUT** | Structure<br>Richness<br>Diversity<br>Eveness | Zones of growth inhibition |
| **TARGET SOIL BACTERIAL COMMUNITIY** | Bacterial DNA from both viable and non-viable soil bacterial cells | Viable soil bacterial cells |
| **TESTED VARIABLES** | 1. Time of exposure (42 or 56 days)<br>2. Silver form (AgNPs or $Ag^+$)<br>3. *Porcellionides pruinosus* presence | 1. Time of exposure (56 days)<br>2. Silver form (AgNPs or $Ag^+$)<br>3. Silver amount (0.1 µg; 1 µg and 10 µg)<br>4. Combined exposure (5 µg AgNPs + 5 µg Ag+)<br>5. Recurrence of exposure<br>6. Soil community *vs.* soil bacterial groups<br>7. Plating method<br>8. Temperature (25ºC or 37ºC) |

**Figure 1.** Experimental design: bacterial community from LUFA 2.2 soil was analyzed by two methods, disc diffusion (culture-dependent method) and PCR-DGGE (culture-independent method).

## 3. Structure

This thesis is organized in four chapters with annexes.

The first chapter (**CHAPTER I**) pretends to introduce the problematic of soil pollution by silver nanoparticles (AgNPs) and the potential influence of this nanoparticles to bacterial communities including a literature review.

The second chapter (**CHAPTER II**) is structured as a scientific paper describing the effects of AgNPs on the soil bacterial community using PCR-DGGE.

The third chapter (**CHAPTER III**) is also structured as a scientific paper, describing the susceptibility of soil bacterial community to AgNPs using the disc diffusion method.

The last chapter (**CHAPTER IV**) is a global discussion providing also the main conclusions about the work along with the perspective for future work.

The **ANNEXES** include relevant additional information (e.g. data from other pratical studies done along with the work presented in this thesis).

# CHAPTER I
GENERAL INTRODUCTION

# 1. Nanotechnology

Since the nineties, and particularly in the last few years, nanotechnology became a fast-growing sector (Fajardo *et al*., 2014), particularly in the USA, Europe and East Asia (Nel *et al*., 2006). This science has occupied the center stage in scientific research, in terms of number of research investigations and the quantum of research funding (Pratap, 2015). It has grown into a billion dollar research enterprise with a rapid commercial deployment. Global market for products based on nanotechnology is predicted to grow from $147 billion in 2007 to $3.1 trillion in 2015, according to the research firm Lux Research (Schmidt, 2009).

Nanotechnology is defined as: "*the application of scientific knowledge to control and utilize matter in the nanoscale, where properties and phenomena related to size or structure can emerge"* - this definition was published by the International Organization for Standardization (ISO) in the ISO/TS 80004-1 from 2010 which lists terms and definitions related to core terms in the field of nanotechnologies. Overall, nanotechnology includes the production, manipulation and use of nanoscale materials which include nano-size particles: the nanoparticles (NPs) (Suresh *et al*., 2013). The major aim of this science is to use bulk materials to obtain nanoscale (1-100 nm; 1 meter = 1 000 000 000 nanometers) materials with new and different properties. This process includes structural and atomic arrangements to lower the size to the nanoscale (Dinesh *et al*., 2012).

## 1.1. Nanoparticles *versus* nanomaterial: definition and distinction

In general, NPs are singular small objects that behave as a whole unit with respect to its transport and properties. Nanomaterial is simply a physical object with at least one dimension in the nanoscale (Cornelis *et al*., 2014). However, these definitions have been changed in the last years, to reach consensus in the scientific community. In 2008 nanoparticles were defined by ISO/TS 27687 as "*nano object with all three external dimensions at the nano scale*". Recently, the ISO modified the list and definitions related to NPs and particles in the field of nanotechnologies (ISO/TS 80004-2:2015). In this case, the nanoparticles were redefined as "*nano-object with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the nano-object do not differ significantly"*. On the other hand, the nanomaterial definition was

established in 2011, by the EC (European Commission): "*Nanomaterial means a natural, incidental or manufactured material containing particles, in an unbound stage or as an aggregate or as an agglomerate and where for 50% or more external dimensions is in the size range 1 nm-100nm*".

## 1.2. Regulation

Since 2004, the regulation and overview of environmental, health and safety (EHS) risks associated with nanotechnology have been undergoing significant developments in the European Union; many were linked with adaptations or recast of existing regulatory frameworks (Justo-Hanani & Dayan, 2015).

Currently, three European regulations incorporate the definition of nanomaterial to enable their use in cosmetics (EC/1223/ 2009), food labelling (EU/1169/ 2011) and the biocidal products (EU/528/ 2012) (Justo-Hanani & Dayan, 2015). After that, in 2012, the EC published the Second Regulatory Review on Nanomaterials. This year, some definitions present in the ISO/TS 27687:2008 was reviewed (ISO/TS 80004-2:2015) to facilitate the communications between industry and external organizations.

Despite all the improvements, the current regulations still needs further information and general rules are required, especially regarding ENPs production, use and disposal. Another gap in regulation consists of the inexistence of a CAS (Chemical Abstracts Service) register number for ENPs. Today, this number is the same for the bulk form and for the respective ENPs. Though they both might seem identical, differences in chemical properties and consequently in the potential environmental toxicity are evident. There is still the necessity to recognize ENPs as a new class of chemicals (Bondarenko *et al*., 2013).

## 1.3. Source of nanoparticles

NPs exist in the environment since the beginning of Earth's history and have distributed in earth's atmosphere, ocean surface, soil and even living organisms (Smita *et al*., 2012), thus NPs can be classified as having natural source. However, other sources of NPs exist, namely due to unintentional and/or intentional anthropogenic activities (Smita *et al*., 2012; Rana & Kalaichelvan, 2013).

### 1.3.1. NPs from natural sources

Natural silver nanoparticles (AgNPs) were discovered in the San Miguel Tenango mining area from Texas (Gomez-Caballero *et al*., 2010).

Several million tons per year of natural NPs are estimated to be released into the environment. Natural NPs can have different sizes and can be released into the atmosphere as a result of forest fire, weathering and volcanic activity, among others. They can be transported over thousands of kilometers and remain suspended in the air for several days (Rana & Kalaichelvan, 2013). On the other hand, plants might absorb these natural NPs or the respective released metal ions (Gardea-Torresdey *et al*., 2003).

The natural occurrence of NPs drive attention into a new field of research: the green synthesis of NPs involving environmental friendly reducing agents and nontoxic stabilizing agents (Yu *et al*., 2013).

#### 1.3.1.1. Green nanoparticles (gNPs)

Biosynthetic processes of obtaining NPs, called the green synthesis method, and where plant extract or microorganisms (bacteria or fungi) are used for the synthesis of NPs, is receiving much attention as an alternative for the production of metal NPs (Bindhu & Umadevi, 2015).

Green synthesis method is simple, fast and inexpensive. This method is based in reducing salts (e.g. silver nitrate) but dependent on several factors: the type of plant extract, the organic content, the concentration of the salt used, the temperature, among others (Mohanpuria *et al*., 2008; Abdel-Aziz *et al*., 2013).

Furthermore, this method can potentially eliminate the adverse effects of chemical agents in products' application because no chemical ingredients are added therefore making nanoparticles more biocompatible. Thus, this technique enables the production of ecofriendly NPs, possible nontoxic to the environment (Park, 2014; Bindhu & Umadevi, 2015).

**Table 1** summarizes some research works with green synthesis of AgNPs showing a good antibacterial activity against several bacteria (Savithramma *et al*., 2011; Park, 2014).

Considering the above mentioned advantages, this method show promise in the application of gNPs in the food industry (Makarov *et al*., 2014). Nevertheless, this methodology did not yet attract the interest of industries because the obtained NPs are still highly diverse in their shape and size (Makarov *et al*., 2014). This is comprehensible as size is one of the most critical criteria: the range in size should be as narrow as possible to target specific applications (Dipankar & Murugan, 2012). Consequently, studies for the implementation of this methodology in industries are still necessary.

Overall, the "green synthesis" of NPs is not yet incorporated in daily products and engineer NPs still leads nanotechnology processes.

**Table 1.** Silver nanoparticles (AgNPs) synthetized using the green method.

| Synthetized AgNPs | | | | | Antibacterial susceptibility | | Main achievements: antibacterial potential[#] | References |
|---|---|---|---|---|---|---|---|---|
| **Bulk material** | **Preparation** | **Characterization[†]** | **Size/shape** | **Concentration/Amount** | **Method[§]** | **Tested bacteria** | | |
| Bacteria<br>*Bacillus megaterium*<br>(NCIM 2326) | Cell filtrates were mixed with $AgNO_3$ (1 mM). | UV-vis<br>FTIR<br>TLC<br>AFM | Size: 10-12 nm. | 5, 10, 15, 20 µl/disc | DD | *Streptococcus pneumoniae*<br>*Salmonella typhi* | The synthesized Ag-BNPs have high antibacterial activity against *S. pneumoniae* and moderate activity against *Salmonella typhi.* | Saravanan *et al*., 2011 |
| Leaf aqueous extracts<br>*Iresine herbstii* | Leaf (10 mL) was added to aqueous solution of $AgNO_3$ (90 mL, 1 mM). | UV-vis<br>XRD<br>SEM<br>EDX<br>FTIR | Size: 1.2 nm. | 50, 100, 150, 200, 250 µg/mL | WD | *Staphylococcus aureus*<br>*Enterococcus faecalis*<br>*Escherichia coli*<br>*Klebsiella pneumoniae*<br>*Pseudomonas aeruginosa* | AgNPs exhibited strong antibacterial activity against all bacteria but maximum effect was agaisnt *E. coli*: $ZoI_{E.\ coli}$=15.7±0.6 mm. *K. pneumoniae* only showed growth inhibition at 100 g/mL. | Dipankar & Murugan, 2012 |
| Leaf extract<br>*Chenopodium murale* | Leaf extract (1 mL, 0.2 g/mL) was mixed with aqueous solution of $AgNO_3$ (50 mL, $5x10^{-3}$ M). | UV-vis<br>TEM | Size: 30-50 nm. | 0.1 mL/well | WD | *Staphylococcus aureus* | AgNPs showed higher antimicrobial activity against *S. aureus* than $AgNO_3$ (5 mM). | Abdel-Aziz *et al*., 2013 |
| Pine mushroom extract<br>*Tricholoma matsutake* | Mushroom extract was treated with an aqueous solution $AgNO_3$ (1 mM). | UV-vis<br>XRD<br>FTIR | Size: 10-5 nm.<br>Shape: spherical, homogeneous. | 5 µg/disc | DD | *Escherichia coli*<br>*Bacillus subtilis* | Excellent antimicrobial activity: $ZoI_{E.coli}$=21.00 mm; $ZoI_{B.\ subtilis}$=18.0 mm. | Anthony *et al.,* 2014 |
| Fungus<br>*Curvularia tuberculata* | The fungal freecell (10 g mycelia mixed with 100 mL deionized water) was added to $AgNO_3$ (1 mM). | UV-vis<br>TEM<br>FTIR | Size: 20-70 nm.<br>Shape: spherical, disperse or aggregate. | 50, 100 µL/mL | WD | *Escherichia coli* (ATCC 25922)<br>*Staphylococcus aureus* (NCTC 6571)<br>*Proteus mirabilis*<br>*Salmonella typhi* | The bacterial growth inhibition at 50 µL/mL of AgNPs was slightly lower (ZoI=13-24 mm) than at 100 µL/mL (ZoI=15–26 mm). For both concentrations of AgNPs, *P. aeruginosa* showed the highest ZoI while *P. mirabilis* showed the lowest ZoI. | Muhsin & Hachim, 2014 |
| Flower extract<br>*Chrysanthemum indicum* | Flower extract (5 mL) mixed with $AgNO_3$ (500 mL, 1 mmol). | UV-vis<br>XRD<br>TEM<br>EDX | Size: 37.71-71.99 nm;<br>In average: 52.9±4.6 nm.<br>Shape: spherical, smooth surface, poly-dispersed. | 25 µg/disc | DD | *Bacillus subtilis* (MTCC 121)<br>*Staphylococcus aureus* (MTCC 96)<br>*Staphylococcus epidermidis* (MTCC 435)<br>*Escherichia coli* (MTCC 433)<br>*Klebsiella pneumoniae* (MTCC 109)<br>*Pseudomonas aeruginosa* (MTCC 1934) | Highest activity: $ZoI_{E.\ coli}$=13.00±0.90 mm; $ZoI_{K.\ pneumoniae}$=19.10±0.50 mm<br>Moderate activity: $ZoI_{P.\ aeruginosa}$=9.60±0.51 mm; $ZoI_{S.\ aureus}$=8.33±0.57 mm<br>Nule activity: *B. subtilis* and *S. epidermidis*<br>Antibacterial activity of $AgNO_3$ only occured for: $ZoI_{K.\ pneumoniae}$=7.00±0.50 mm | Arokiyaraj *et al.,* 2014 |
| Lemon<br>*Citrus limon* | Lemon extract was mixed with $AgNO_3$ ($10^{-2}$ M to $10^{-4}$ M). | UV-vis<br>SEM | Size: 58.7 nm. | 15 µL/disc (5 mg of AgNPs) | DD | *Aeromonas hydrophila*<br>*Edwardsiella tarda*<br>*Pseudomonas aeruginosa*<br>*Flavobacterium branchiophilum*<br>*Staphylococcus aureus*<br>*Bacillus cereus*<br>Vibrio and Citrobacter species | Highest zone of inhibition against *E. tarda* and *S.aureus.* $AgNO_3$ exhibited highest inhibitory zone against all bacteria compared to the synthetized nanoparticle. | Swain *et al*., 2014 |
| Vasaka leaf extract<br>*Justicia adhatoda L.* | Vasaka leaf extract (0.2 mL) was mixed with $AgNO_3$ (20 mL, 1 mM). | UV-vis<br>TEM | Size: 5-50 nm;<br>In average: 20 nm.<br>Shape: spherical, agglomerated. | DD=50 µL/disc; WD=50 µL/well;<br>SD=50 µL bacteria in 1mL of AgNPs | DD<br>WD<br>SD | *Pseudomonas aeruginosa* (MTCC 741) | DD: AgNPs caused $ZoI_{P.\ aeruginosa}$=7–9 mm being as efective as $AgNO_3$ ($ZoI_{P.\ aeruginosa}$=8–10 mm).<br>WD: strong efficiency to inhibit *P. aeruginosa* (average ZoI=1.36–1.4 cm).<br>SD: inhibit the growth of *P. aeruginosa* at the concentration of 10-5 M. | Bose & Chatterjee, 2015 |
| Beetroot extract | Beetroot extract (1 mL) mixed with aqueous solution of $AgNO_3$ (50 mL, 3 mM). | UV-vis<br>XRD<br>TEM<br>EDX | Size: 15 nm.<br>Shape: spherical, monodispersed. | 50 µg/mL | DD | *Escherichia coli* (ATCC 25922)<br>*Pseudomonas aeroginosa* (ATCC 27853)<br>*Streptococcus aureus* (ATCC 12384)<br>*Staphylococcus aureus* (ATCC 25923) | Moderate antibacterial activity: $ZoI_{E.\ coli}$=7mm<br>Good antibacterial activity:<br>$ZoI_{P.\ aeruginosa}$=11 mm; $ZoI_{S.\ aureus\ (ATCC\ 12384)}$=16 mm; $ZoI_{S.\ aureus\ (ATCC\ 25923)}$=19 mm. | Bindhu & Umadevi, 2015 |

[†] Methods used for characterization of AgNPs: UV-visible spectroscopy (UV-vis); Fourier transform infrared spectroscopy (FTIR); Thin Layer Chromatography (TLC); Atomic Force Microscopy (AFM); X-ray diffraction (XRD); Scanning electron microscope (SEM); Energy-dispersive X-ray spectroscopy (EDX); Transmission electron microscopy (TEM). [§] Methods used for antimicrobial testing: Disc diffusion (DD); Agar Well-diffusion (WD); Serial dilution (SD). [#] ZoI - Zone of Inhibition.

### 1.3.2. Anthropogenic sources

NPs might appear accidentally in the environment due to anthropogenic activities for instance when NPs are originated from vehicles exhaustion or derivate from different industrial processes (Smita *et al*., 2012). Additionally manufactured or engineered NPs (ENPs) might also be released into the environment (air, water and soil) after use of the industrial products containing ENPs (Fajardo *et al*., 2014).

The industrial production of ENPs has increased their concentrations in the environmental compartments (Rana & Kalaichelvan, 2013). The major difference and the one that raises concern, between naturally-occurring and anthropogenic resulting nanoparticles, is that the latest are not biodegradable meaning that they can persist in the environment and interact with organisms and microorganisms with unknown consequences (Fajardo *et al*., 2014). Probably, the ENPs might cause more adverse effects in the biota than those naturally-occurring NPs because the organisms never contacted before with these ENPs.

#### 1.3.2.1. Engineered nanoparticles (ENPs)

Large numbers of ENPs have been produced for different industrial and biochemical applications (Fajardo *et al*., 2014). Due to reduced dimension and high superficial area ratio, ENPs acquired novel and unexpected properties relative to their bulk material, such as physic-chemical, opto-electronic, magnetic and biological properties (Suresh *et al*., 2013).

These properties become economically attractive due to their advantage after incorporated into daily products or industrial processes. Nowadays, ENPs are present in several daily products of various sectors: energy (catalysis), materials (paints, sportswear), electronics (chips and screen), optics, remediation (disinfection, water filters), food (additives), cosmetics (skin lotions), medicine (catheters, diagnostics, drug delivery), agriculture products, among others (Pulit-Prociak *et al*., 2015).

ENPs can be characterized according to their structure (Suresh *et al.*, 2012; Sharma *et al.*, 2014): shell can be classified as inorganic (e.g. silver sulfide, silver chloride) or organic (e.g. citrate, polyvinyl-pyrrolidone) while the core includes various classifications, such as quasi-spherical, nanotubes, rods, triangular nanoplates, among others (Sharma *et al*., 2014).

The composition of the surface of NPs is very important to maintain their stability. Many NPs lose their properties when they aggregate or precipitate in a suspension which is solved by adding a surface coating that facilitates dispersion. All these characteristics will influence the behaviour and transformations that NPs undergo in the environment (Levard *et al*., 2012).

The biological reactivity, the toxicity and the antimicrobial activity, is dependent on NPs' characteristics (Suresh *et al.,* 2012; Sharma *et al.*, 2014).

The toxicity of ENPs as well as AgNPs, is highly dependent on its physical properties such as size, class, shapes, superficial area and surface coating (which acts as a stabilizer), dose of exposure, on their biocompatibility, reactivity, method of synthesis, among others (Suresh *et al*., 2012; Savithramma *et al.*, 2011; Sharma *et al.*, 2014).

Compared to NPs', the bulk form releases ions in a moderate way for a longer period of time while higher amounts are briefly released from the superficial area of NPs. Due to this, the toxicity of the compound is dependent on the release of their ions (Thiéry *et al.,* 2012).

Studies by Suresh and their collaborators (2012) showed that the toxicity is different between shapes and that the spherical shape is more toxic than rod shape (Suresh *et al*., 2012). In the case of AgNPs, the triangular shape has more reactivity to bacteria than the rod or spherical shapes (Pal *et al*., 2007).

The biological reactivity of NPs means that surface atoms are labile and can easily change its redox state (Thiéry *et al*., 2012) to distinctly affect the biota. In general, when ENPs decrease in size, their biological reactivity and, consequently, their toxicity increases (this has been reported for AgNPs) (Suresh *et al.,* 2012). Therefore, ENPs dimensions have an inverse proportionality in relation to toxicity, meaning that high dimension might cause low toxicity (Sharma *et al*., 2014).

The antimicrobial activity is also dependent on the type of ENPs (metal nano-based) and on the microorganisms with which interact (Sharma *et al*., 2014).

Some of the ENPs which are causing environmental concern due to toxicity risk are mentioned in **Table 2** (Klaine *et al.*, 2008; Rana & Kalaichelvan, 2013).

**Table 2.** ENPs mentioned in the literature as causing environmental concern.

| Nanoparticles | Examples | References |
|---|---|---|
| Fullerenes | Fullerenes rings, carbon nanotubes and nanocones. | Rana & Kalaichelvan, 2013 |
| Metal nanoparticles | Elemental silver (Ag), gold (Au) and iron (Fe). | Sharma *et al*., 2014 |
| Oxides | Binary compounds when including carbides, nitrides (e.g $NO_3$). | Sharma *et al*., 2014 |
| Complex compounds | Alloys, composites, nanofluids, (consisting of two or more elements; e.g. cobalt, zinc and iron oxide). | Rana & Kalaichelvan, 2013 |
| Quantum dots (or q-dots) | CdSe. | Klaine *et al*., 2008 |
| Organic polymers | Dendrimers, polystyrene. | Pal *et al*., 2007 |

## 2. ENPs: Silver nanoparticles (AgNPs)

Among all the manufactured NPs, the AgNPs plays a major role in the field of nanotechnology (Rana & Kalaichelvan, 2013) due to their broad application as a result of their antimicrobial activity (Losasso *et al*., 2014).

According to the Silver Institute, approximately 5% of the global silver production is used for production of AgNPs. The global production of AgNPs is estimated in 55 tons per year reaching 11 million Euros in 2015 therefore, being among the most used ENPs (Mueller & Nowack, 2008; Piccinno *et al*., 2012).

In a total of 1854 nano-base products (containing ENPs), the AgNPs are incorporated in more than 410 commercialized products (Woodrow Wilson Database of 2014) distributed by different sectors as **Figure 2** shows. Most nano-based products are targeted to paint industries (35%) and cosmetics (25%) (Muller & Nowack, 2008).

Products based in AgNPs are particularly attractive owing to anti-inflammatory activity (Vaidyanathan *et al*., 2009) and protection of the skin against unwanted microbial action. For instance, the incorporation of AgNPs in soap is a slightly change in the soap formulation that adds value to the previous existing product by: (1) adding new properties (inhibition of acne) and (2) selecting a distinct target consumer. AgNPs coatings have been commonly used to treat infected wounds and the prevention of biofilm formation on home appliances (Sheng & Liu 2011).

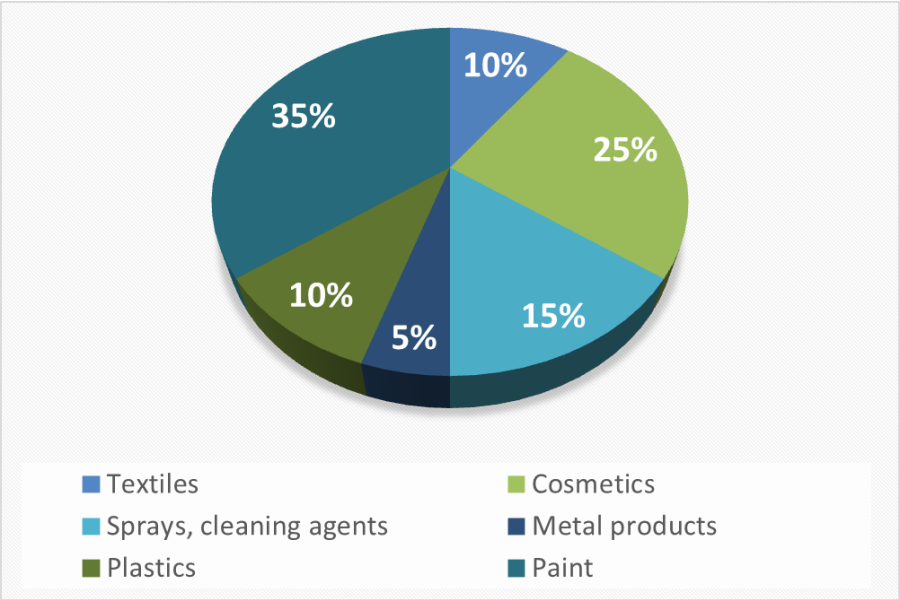


**Figure 2.** Silver nano-based products in different commercial products (Adapted from: Muller & Nowack, 2008).

Another application of AgNPs based products is dental implants and toothpaste or tooth cleaning gels. AgNPs reduce the attachment of bacteria to the surfaces of dental implants preventing the formation of biofilms on the implant surface that may cause infection. In tooth paste, odors and dental cavities are reduced or eliminated due to the antibacterial effect of this nanoparticle (Sivolella *et al*., 2012).

AgNPs are also impregnated into the structures of socks, trousers and other textile products. Nanoparticles incorporation prevents the penetration of bacteria into hard-to-reach places inhibiting bacterial growth: odors as well as prejudicial bacteria responsible for disease (as *Staphylococcus aureus* and *Escherichia coli*) were eliminated (Vaidyanathan *et al*., 2009). Doors, windows and paints of new building materials have also AgNPs. AgNPs also offer a wide range of possibilities for the use of polyurethane foam characterized by its biocidal properties (Pulit-Prociak *et al*., 2015).

This nanoparticle can be also incorporated in food packaging enhancing protection of food products against the surrounding environment, inhibiting or retarding microbial growth and extending food shelf life (Azeredo, 2013).

## 2.1. AgNPs *versus* bulk form (Ag cation: $Ag^+$)

Silver metal has been used in different applications due to high silver electrical and thermal conductivity properties (Panyala *et al*., 2008) as well as in medicine to fight infections and in food industry to prevent spoilage (Rai *et al*., 2009). However, because of silver metal toxicity to human cells (Echavarri-Bravo *et al*., 2015), its use in medicine was discouraged. In this line of though, AgNPs have opened the range of applicability providing new properties that confer advantage particularly because AgNPs is not toxic for human cells (Echavarri-Bravo *et al*., 2015) as comproved using skin *in vivo* and keratinocytes *in vitro* (Samberg *et al*., 2010).

Comparison between silver metal and the nanoparticle forms shows that the main difference is that the surface/volume ratio is higher in the nanoparticle thus leading to higher biological reactivity (contact with the microorganisms is improved) (Choi *et al*., 2008; Losasso *et al*., 2014). Also, in the bulk form, the rapid release of ions is linked to high toxicity of silver bulk forms while for AgNPs, the release of ions occurs in a longstanding manner being more persistent in the environment (Fajardo *et al*., 2014).

## 2.2. Emission and exposure scenarios of AgNPs into the environment

AgNPs can be released into the environment in different states: uncoated, functionalized, aggregated or embedded in a matrix (Gottschalk & Nowack, 2011).

During the life cycle of the NMs containing AgNPs, which covers production, use and disposal, the AgNPs might represent hazard to the technosphere, to the distinct environmental compartments and to the associated biota (humans included) (Som *et al*., 2010) (**Figure 3**).

Considering the long duration of NMs life cycle and the opportunities they represent for novel applications, it becomes clear how extensive can be the exposure scenarios and the potential adverse effects (Som *et al*., 2010).

The highest probability of direct exposure to NMs is during "production" stage. NMs containing AgNPs might be directly released into the environment at this life cycle stage. Ideally the AgNPs and $Ag^+$ released at production stage should undergo treatment inside Waste Water Treatment Plants (WWTP). However, the synthesis process often involves mixing, centrifugation and ultrafiltration steps to remove impurities and the

waste is directly discharged into the environment or into WWTP without proper prior treatment (Yu *et al.*, 2013).

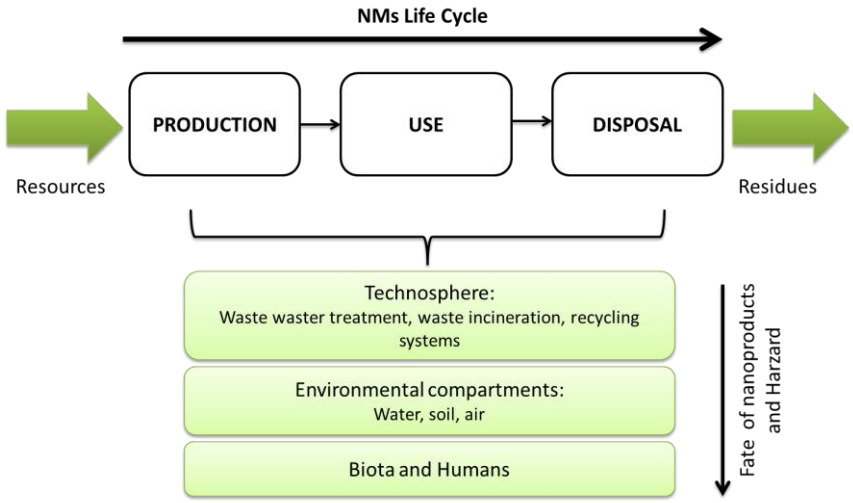


**Figure 3**. Life cycle stages of nanomaterials (NMs) and their environmental fate (Adapted from: Som *et al*., 2010).

The uncontrolled release of silver during the use (including recycling) and disposal stages gives rise to additional public concern (Yu *et al*., 2013).

Exposure to NMs during the "use" life cycle stage occur from intended applications (e.g. using sunscreen containing NMs) but also from unintended sources (e.g. release of NMs from nano-textiles). Cleaning products, creams and cosmetics have AgNPs contained in liquid phases which are easily released within hours after application, whereas NPs embedded into a solid matrix (paints) are gradually released and are supposed to remain in the soil matrix for years (Som *et al*., 2010).

The "disposal" life cycle stage includes the release of NMs into the environment, which can either occur at the end-of-life of NMs or when they are disposed into landfills or burned in waste incineration plants (Gottschalk & Nowack 2011; Som *et al*., 2010). Little is known about the clearing efficiency of nanoparticles in WWTP. Benn and Westerhoff (2008) showed that WWTP biomass is able to largely reduce the metallic nanoparticles in the effluent stream. The retention of Ag in WWTP may strongly depend on Ag speciation (e.g. $Ag_2S$) in the influent and it does alter along different treatment stages. Also, different Ag removal efficiencies and transformation processes may control the retention of metallic AgNPs within WWTP (Kaegi *et al*., 2011).

Unfortunately, in many countries untreated sewage sludge is often used as agricultural additive or fertilizer in a landfill (Keller *et al*., 2013). So it is expected that in these hazardous substances may be released into the soil, ultimately reaching the groundwater. Because of this, the "disposal" life cycle stage is an important source of environmental contamination by AgNPs (Gottschalk & Nowack 2011).

As a result, it is guaranteed and inevitable that AgNPs are being released into the environment (soil, water and air) (Tourinho *et al*., 2012), at an unknown rate or concentrations. Nonetheless some predictions of AgNPs concentrations were made. Modelling the Predicted Environmental Concentrations (PEC) is very difficult because the production volumes are continuously increasing (Rana & Kalaichelvan, 2013). Still, Gottschalk and collaborators (2009) demonstrated that, in Europe, the values of PEC for AgNPs in soil could reach 0.227 µg/Kg per year. These environmental concentrations were calculated as probabilistic density functions and were compared to data from ecotoxicological studies. Also, Muller and Nowack (2008) suggested that the PEC for AgNPs in soil could reach 0.02 µg/Kg or 0.1 µg/Kg in a realistic or in a high-emission scenario, respectively.

Studies by Tønning and collaborators (2012) pointed that the contamination of nanosilver, only analyzing textiles' production on the Danish market, involved the release into the soil of an amount of 0.1 mg Ag/kg soil (dry weight).

Boxall and collaborators (2007) estimated that the penetration of AgNPs into the soil was 4.26 µg/Kg. This value was corroborated by Faust and Backhaus investigation (NanoFATE) suggesting it as a worst case scenario estimation for each environmental compartment. The PEC of AgNPs in Europe soil was determined by NanoFATE project conceived to link methodological gaps to environmental risks by ENPs and is represented in **Figure 4**.

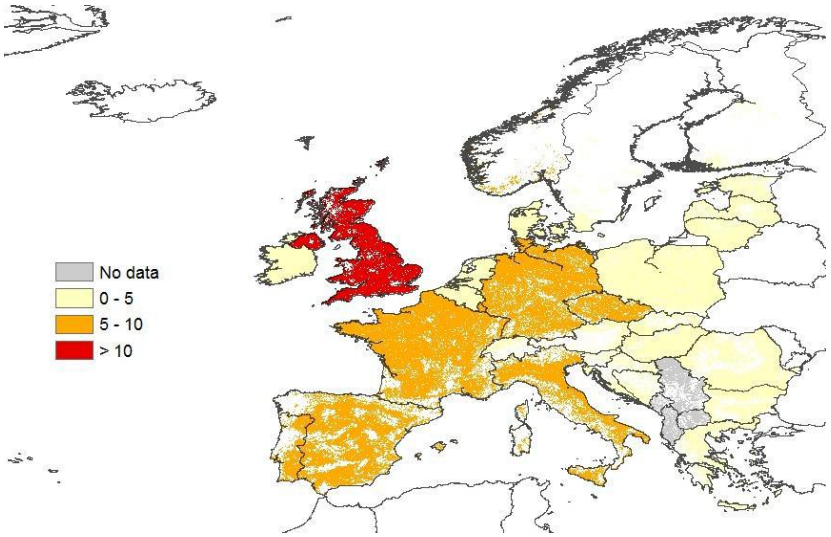


**Figure 4.** Predicted concentrations (PEC) of AgNPs (ng/kg dry weight soil) in Europe (expecting 57% of agricultural land used, minimum NPs transfer to sludge and a 25 cm ploughing depth). In the figure: highest PEC was determined for England (> 10 ng, represented in red); the most abundant PEC rounds 5-10 ng/kg dry weight of soil in central and south of Europe (represented in orange) while the lowest PEC (0-5 ng/kg dry weight of soil) is represented in yellow (Faust & Backhaus, NanoFATE).

## 2.3. AgNPs interactions in soil compartment

The soil compartment is a dynamic and complex system supporting the habitat for microorganisms, organisms and humans, which interacts with water and air compartments (van Gestel, 2012).

The interaction between AgNPs and environmental matrices is complex. Both abiotic and biotic factors may influence the transformation and bioavailability of AgNPs in the environment (Dwivedi *et al*., 2015).

AgNPs contamination in soil can undergo distinct routes. AgNPs may be partially degraded and migrate into other ecosystems (Klaine *et al.*, 2008) for instance it can be mobilized into groundwater or be biomagnified (through the food chain and ultimately affecting human health) (Dror *et al*., 2015).

The AgNPs that remain in the soil matrix can interact with the surrounding environment and biota in different ways (**Figure 5**), either directly or indirectly (Klaine *et al.*, 2008; Dinesh *et al*., 2012). AgNPs can directly interact with the biota (e.g. microorganisms, plants and invertebrates) or indirectly when dissolved in the interstitial water or aggregated into the soil pores (Klaine *et al.*, 2008).

AgNPs may experience aggregation, dissolution, redox reactions, photo-transformations, among others (Fabrega *et al.* 2011). These processes are dependent on

the AgNPs characteristics (as size, shape, surface coating), on water occurrence and on the presence of contaminants, among other factors (Klaine *et al*., 2008).

Aggregation reduces the surface area, which affects the transport of the AgNPs in porous, the sedimentation, the reactivity, the bioavailability and the toxicity to the biota (Klaine *et al*., 2008).

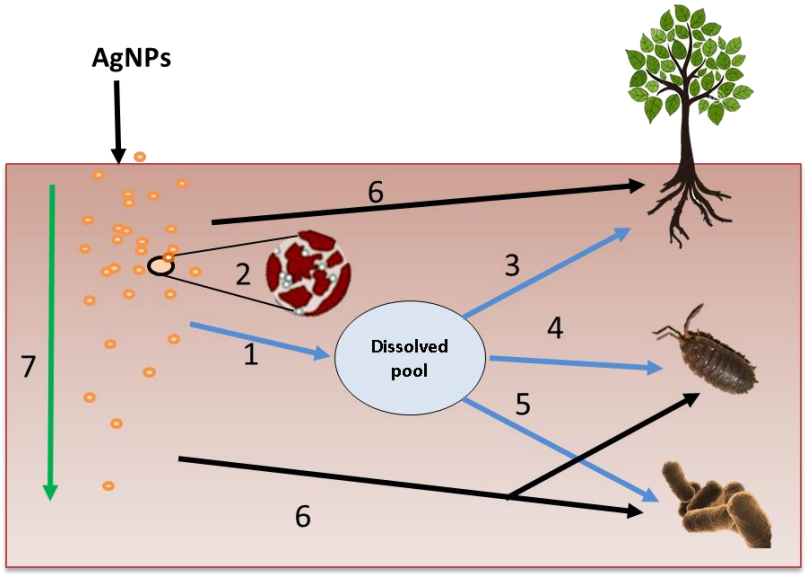


**Figure 5.** Key processes in soil related to the transformation and potential risk driven by manufactured nanoparticles. In the figure: (1) dissolution; (2) sorption/aggregation; (3) plant accumulation; (4) invertebrate accumulation and toxicity; (5) microbial toxicity; (6) direct particle uptake/toxicity; (7) particle migration (Klaine *et al*., 2008).

The soil intrinsic characteristics such as porosity, organic matter, pH water content can influence the toxicity to AgNPs (Drobne *et al*., 2002). Also, the AgNPs behaviour, bioavailability into the soil can be decisive to the toxicity (Tourinho *et al*., 2012) (**Figure 6)**.

When dissolved in soil, the pH, strength and ionic composition, the presence of natural organic and inorganic suspended colloids are determinant for the behaviour of AgNPs (Dror *et al*., 2015). Dissolved humic substances can form an amorphous domain coating the suspended minerals (Wilson *et al*., 2008). The precipitation of dissolved humic substances and the dissociation of functional groups of humic acids are controlled by ionic strength and pH. So, the negative charges of the AgNPs may change as it interacts with these functional groups of humic acids (Dror *et al*., 2015).

When in contact with dissolved organic matter, the AgNPs may be adsorbed or even change their original surface properties thus altering their interactions with the solid phase of soil (Dror *et al*., 2015).

Several factors influence the transport and fate of AgNPs in soils. The AgNPs are less mobile in soils with high content of organic matter (Coutris *et al*., 2012). It was proved that organic materials, such as sewage sludge, interact with AgNPs with sorption rates greater than 90% for uncoated particles (Kiser *et al*., 2010).

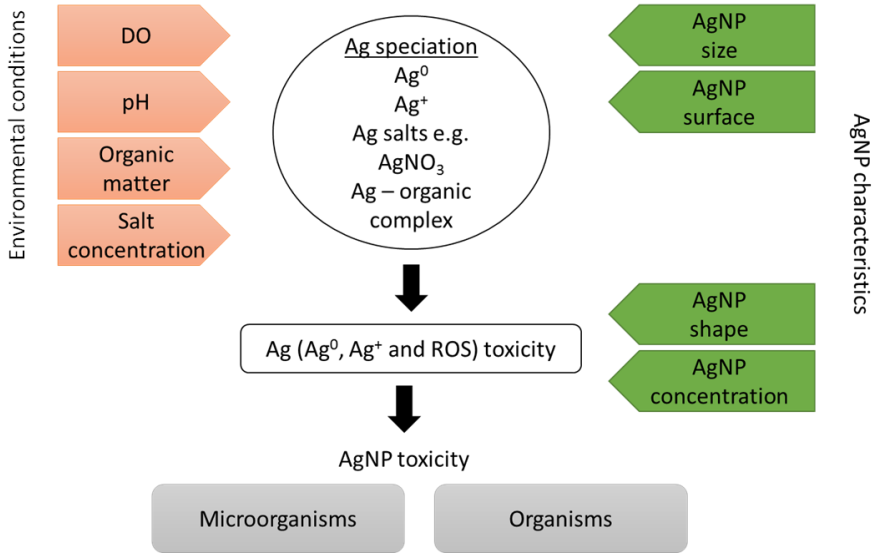


**Figure 6.** Interactions between silver nanoparticles and environment. The figure shows the major characteristics of AgNPs (green) and the environmental conditions [pH, DO (dissolved oxygen), organic matter and salt concentration] (red) affecting the AgNPs toxicity (grey) to the biota (microorganisms and organisms) (Adapted from: Dinesh *et al*., 2012).

Hund-Rinke and Schlich (2014) observed an evident relationship between the low grain size distribution of the soil and the high toxicity of AgNPs towards ammonia oxidizing bacteria.

Shah and collaborators (2014) showed, using pyrosequencing analysis, that the AgNPs effects on the bacterial communities were not significant. The same authors suggested that the behaviour, migration of AgNPs into the soil and changes in the soil bacteria diversity due to AgNPs exposure were dependent on chemical transformation (oxidation and ionization) as well as on the properties of the soil. Environmental parameters influence the rate of chemical transformation of NPs (e.g. release ions) as

well as their fate, thus altering their toxicity of AgNPs to the soil microorganisms (Dinesh *et al*., 2012).

For AgNPs, the soil pH and parameters affecting the sorption behaviour (organic carbon content) are important but the toxicity of AgNPs cannot be attributed to a single soil parameter (Dinesh *et al*., 2012). Furthermore, the bioavailability of AgNPs in natural soils is difficult to predict because of the interactions between different biological processes, physicochemical properties and environmental conditions (Cornelis *et al*., 2013).

## 2.4. AgNPs interaction with bacteria

There is not a consensus about the interactions between the bacteria and the AgNPs neither the mechanism of action of AgNPs in relation to the microorganisms is well clarified.

Tenover (2006) suggested that the mechanism of the bactericidal effect of silver colloid particles against bacteria could be similar to the ones of antimicrobial agents against bacterial infections and include four different mechanisms of action:

(1) interference with cell wall synthesis;

(2) inhibition of protein synthesis;

(3) interference with nucleic acid synthesis, and

(4) inhibition of a metabolic pathway.

However, other mechanisms were proposed and the most acceptable at the moment are schematized in **Figure 7**.

The first direct contact between AgNPs and bacteria is through the membrane. This interaction occurs either by disrupting the membrane (Rai, 2009) or changing the membrane potential charge (Klaine *et al*., 2008). Thus, the differences in the membrane composition of the Gram-positive and Gram-negative bacteria are important to AgNPs toxicity. Gram-positive bacteria are more affected and/or susceptible to AgNPs having higher toxicity potential than Gram-negative (Losasso *et al*., 2014) because of the composition of the Gram-negative membrane (consisting of an additional membrane) which enhances impenetrability (Losasso *et al*., 2014).

Still, some Gram-negative bacteria were reported to be affected by AgNPs namely: *Acinetobacter baumannii* (Niakan *et al*., 2013), *Escherichia coli* (Li *et al*.,

2010), *Pseudomonas aeruginosa* (Klapiszewski *et al*., 2015) and *Salmonella serovars* (Losasso *et al*., 2014). The efficacy of AgNPs against Gram-positive bacteria was also confirmed for the following bacteria: *Bacillus* (isolated from waste water) (Shahrokh & Emtiazi, 2009), *Enterococcus faecalis* (Lotfi *et al*., 2011), *Listeria monocytogenes* (Zarei *et al*., 2014), *Staphylococcus aureus* (Ahangaran *et al*., 2012) and *Streptococcus pyogenes* (Cheng *et al*., 2014).

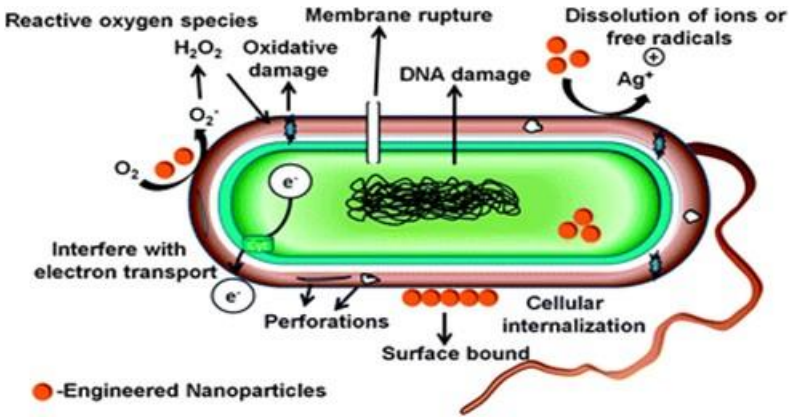


**Figure 7.** Some mechanisms of action of NPs against microorganisms (Suresh *et al*., 2012).

Silver ions might react with electron donor groups (N, O, or S atoms) which are present in bacteria (e.g. in amino, imidazole, phosphate, carboxyl, or thiol groups of proteins or even in DNA) (Gordon *et al*., 2010). The silver ions released from AgNPs surface react with the thiol and sulfidril (-SH) groups present in the membrane proteins causing a decrease in the membrane permeability leading to perforations of the membrane and, consequently, causing the bacterial cell lysis (Dinesh *et al.*, 2012). Furthermore, the thiol groups were identified as the main targets for disruption of bacterial activity by silver compounds because the addition of silver ions immediately inactivate the enzymes succinate dehydrogenase (involved in the electron transport chain) and aconitase (Fe-S proteins functioned as electron-transfer agents) (Gordon *et al*., 2010).

Another AgNPs interaction with the membrane is through the oxidative dissolution of $Ag^+$ (Kim *et al.*, 2007). The oxidative dissolution of ions in superficial area consists of ions release and their interaction with the cell membrane leading to ions

inclusion into the cell; then, ions interrupt several signals regarding DNA translation and protein production (Klaine *et al*., 2008; Prabhu & Poulose, 2012).

Studies about the toxicity of AgNPs to *E. coli* and to nitrifying bacteria have shown that the antibacterial activity was dominated by the release of $Ag^{+}$ rather than by the AgNPs themselves (Li *et al*., 2011).

AgNPs can also affect the production of reactive oxygen species (ROS) (Choi *et al.*, 2008; Klaine *et al*., 2008). Generally, ROS species can be subdivided into two types: radical ROS (hydroxide radicals or nitric oxide) and non-radical ROS (hydrogen peroxide). Joshi and collaborators investigation (2015) indicated that oxidative stress (ROS production) and dissolved ionic species play an important role in AgNPs toxicity, which may also involve membrane damage and protein oxidation. ROS can be generated, in acidic environment, by NPs interactions with cell surface receptors.

Gordon and their collaborators showed that the bactericidal activity of silver ions is due to the inactivation of key enzymes by thiol group binding, hydroxyl radical formation, and subsequent DNA damage (Gordon *et al*., 2010). The AgNPs repress the expression of several main functional proteins of the bacteria *Nitrosomonas europaea* including ATP synthase, ammonia monooxygenase and hydroxylamine oxidoreductase (Yuan *et al*., 2013).

Despite these mechanisms of action, distinct nanomaterials (NMs) usually exhibit different mechanisms depending on their composition, surface modification, intrinsic properties and depending also on the type of bacterial species being affected (Hajipour *et al*., 2012).

Thought silver resistance was previsiously referred regardingenvironmental and clinical isolates, investigations up to now have been limited to the resistance evolution mechanism encoded by the conjugally transferable plasmid pMG101 (which confers resistance to mercury, tellurite and several antibiotics) (Vasileiadis *et al*., 2015). Althought a “resistance mechanism” associated to bacteria isolates need more research, bacteria as a community are able to protect from AgNPs as demonstrated by Gambino (*et al*., 2015) which observed that the presence of the AgNPs can induce quorum-sensing related genes in *Bacillus subtillis*.

## 2.5. Possible effects of the interaction between AgNPs and the microbiota from soil

The degree to which a community is susceptible to a perturbation is dependent on the composition and diversity of the original community inducing a response of resistance, resilience or tolerance and functional redundancy (Allison & Martiny, 2008) (**Figure 8**).

After a perturbation on the initial bacterial community, the microbiota might show resistance which means that bacterial community is not affected thus it does not change their composition but becomes more capable of defend itself in case of similar future stress (Sharma, 2010).

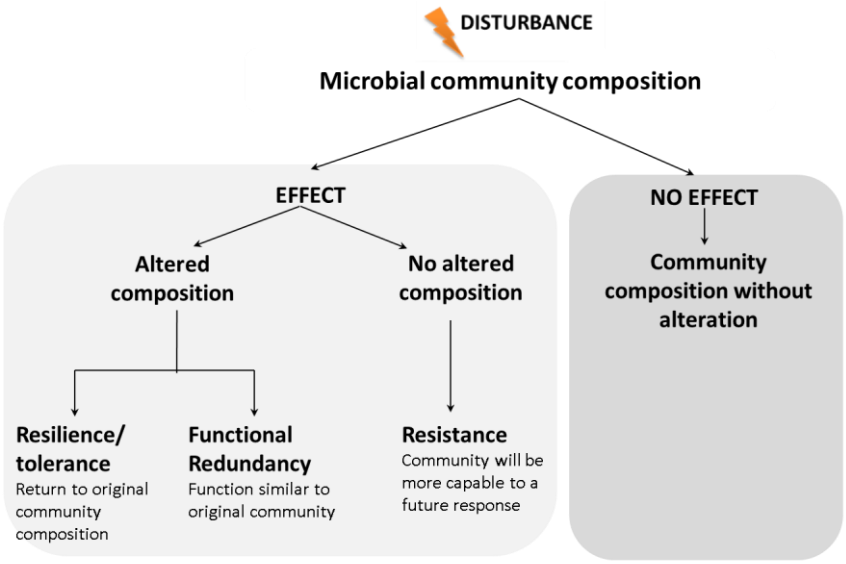


**Figure 8.** Schematic representation of possible responses of microbial communities to a disturbance (Adapted from Sharma, 2010).

When resilience response occurs, the microbial community show sensitivity to the perturbation but quickly returns to the initial composition (Allison & Martiny, 2008). Herein, microorganisms tolerate the stress and grow rapidly having high potential to recover from the disturbance showing high degree of physiological flexibility (Allison & Martiny, 2008). A resilience response also appears to be true for ENPs exposure though long-term exposure (not higher than 2 months) (Colman *et al.*, 2014) thus,

further research is needed to evaluate whether ecosystem composition is permanently disturbed or not (Simonin & Rishaume, 2015).

Functional redundancy occurs when microbiota recovers after a stress that eliminates part of the microbial community (Sharma, 2010). The remaining microbiota can quickly return to a "stable" state by substituting the functions which were attributed to the eliminated microbiota; thus, the composition is changed (Allison & Martiny, 2008). The larger the initial microbial diversity, the better functional redundancy will be supported (Sharma, 2010).

## 3. Ecotoxicology studies

The ecotoxicological studies focused on AgNPs increased in last decade. Nowadays, these studies represent 13% of all ecotoxicological data (**Figure 9**) (Kahru & Dubourguier, 2010). Ecotoxicological assays focusing in ENPs comprise mainly crustaceans and bacteria (**Figure 9 A**) representing 33% and 27% of overall studies, respectively.

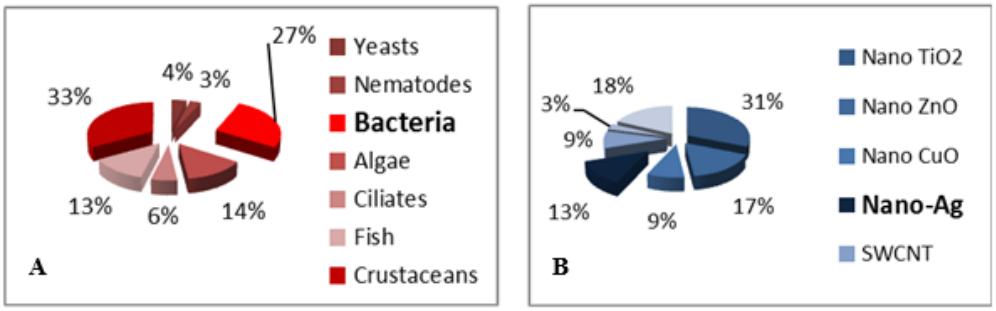


**Figure 9.** Ecotoxicological studies focused on different ENPs (B) and group of organisms (A) (Adapted from Kahru & Dubourguier, 2010).

Silver cation ($Ag^+$) appears to be more toxic than AgNPs for crustaceans, algae and fish (**Figure 10**). AgNPs exhibited the highest toxicity for crustaceans with a $LC_{50}$ and $EC_{50}$ of 0.01 mg/L (Bondarenko *et al.*, 2013).

The toxicity of AgNPs for algae was slightly lower ($LC_{50}$ and $EC_{50}$ were 0.36 mg/L) followed by fishes with $LC_{50}$ and $EC_{50}$ of 1.36 mg/L (Bondarenko *et al.*, 2013) (**Figure 10**). When considering the minimum inhibitory concentration (MIC) values for comparison of the effects between the silver forms in bacteria: Ag salt (3.3 mg/mL)

shows a larger hazard range (harmful to extremely toxic) than AgNPs (7.10 mg/mL) ("not classified" to "very toxic") (Bondarenko *et al.*, 2013) (**Figure 10**).

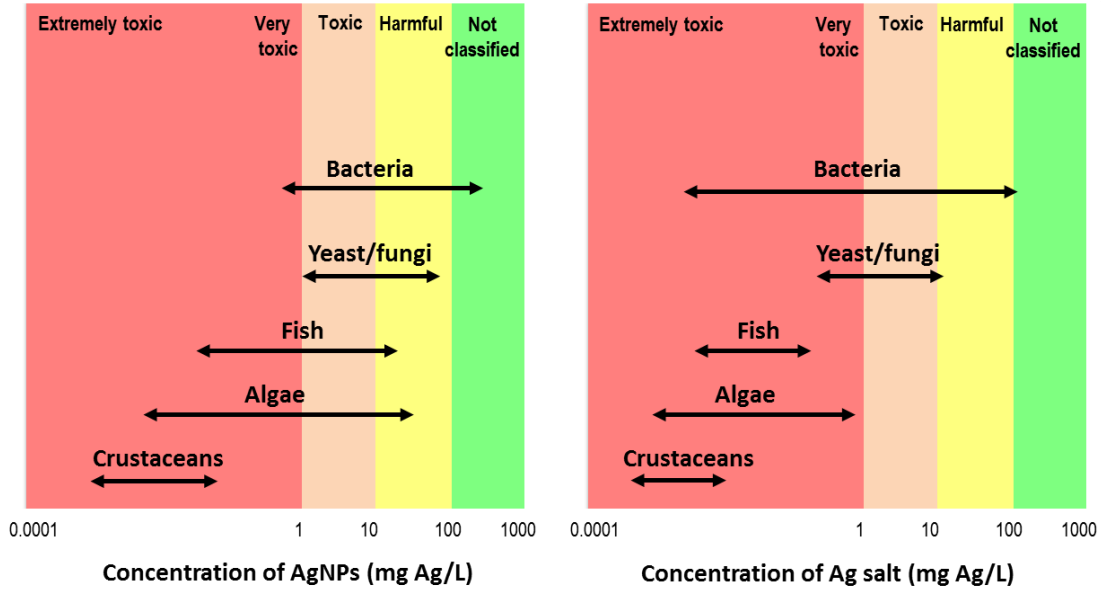


**Figure 10.** Hazard of AgNPs (a) and bulk form ($AgNO_3$) for different organisms. Ranging was obtained using the median of $LC_{50}$ (concentration causing mortality for 50% of the organisms) or $EC_{50}$ (concentration causing effect for 50% of the organisms) values for all organisms except for bacteria for which the MIC (minimum inhibitory concentration). (Adapted from Bondarenko *et al.*, 2013).

## 3.1. Terrestrial ecotoxicological assays: standard soil.

The type of soil used to determine the toxicity of chemicals/contaminants changed along with the increasing knowledge about the concept of bioavailability: only a fraction of the total amount of chemical in the soil is available for uptake by organisms and therefore only that fraction is of relevance for the risk assessment (van Gestel, 2012).

The soil used to evaluate the toxic effect can be either natural or artificial. In ecotoxicological assays the OECD soil artificial, the European natural soils (EUROSOILS) and the LUFA 2.2 natural soil are the most used standard soils (Bastos *et al.*, 2014). The LUFA 2.2 soil is commercially available from the Landwirtschaftliche Untersuchungs und Forschungsanstalt (LUFA) in Speyer, Germany. Since that time, LUFA 2.2 standard soil seems to become more usually used for toxicity tests with soil invertebrates (van Gestel, 2012) due to their ecological relevance, because interfere with decomposition process, nutrient cycling and influence on soil core structure and texture

(Fernandez & Tarazona, 2008). Also, several test species (e.g. collembolan *Folsomia candida* or nematode *Caenorhabditis elegans*) seem to perform as good in this natural soil as they do in artificial soil and/or in the environment (Fajardo *et al*., 2014).

LUFA 2.2 soil has other advantages such as: availability for long time, enabling the comparison of tests; the properties of the soil, which are well described and well standardized; data concerning location, history, treatment and sampling are provided; this soil is also supplied with field fresh active microbiota (van Gestel, 2012).

## 3.2. Evaluation of the toxicity of AgNPs to soil bacteria

Microorganisms have key functions in the global biogeochemical cycling of nutrients (carbon, nitrogen and phosphorus) and biomass decomposition of the soil (Suresh *et al*., 2013). Even very low concentrations of AgNPs may induce modifications of the microbial activities (decrease in respiration and enzymatic activities) affecting the biogeochemical cycles (Simonin & Richaume, 2015). It was proven that AgNPs have some potential toxic effects on bacteria in soil, including the beneficial bacteria that are essential for nitrogen fixation (Panyala *et al*., 2008; Mirzajania *et al*., 2013).

Microorganisms are sensible to chemical stress (Sharma, 2010). Among the microorganisms that are affected by AgNPs are those considered growth promoters in plants like *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas fluorescens* and *Bacillus subtilis* as well as bacteria involved in nitrification (*Nitrosomonas europaea*) and denitrification (*Paracoccus desnitrificans*) processes (Dinesh *et al*., 2012). Thus, ecotoxicological studies involving bacteria are advantageous due to their ubiquity and high diversity (i.e. important and distinct functions in several habitats), small size and short generation times allowing fast tests (Rana & Kalaichelvan 2013). However, because the microbial communities tend to be highly responsive, short generation might also constitute a problem for the interpretation of results (Kuperman *et al*., 2014).

The OECD and ISO published standardized methodologies to evaluate the impact of chemical exposure to microorganisms enabling an additional way of evaluating the soil quality (**Table 3)**.

**Table 3.** OECD and ISO regulations to evaluate the soil quality using the microorganisms (Adapted from: Kuperman *et al*., 2014).

| Organization | Number designation | Regulation |
|---|---|---|
| OECD | 209 | Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation) |
| | 216 | Soil microorganisms nitrogen transformation test OECD |
| | 217 | Soil microorganisms carbon transformation test OECD |
| ISO | 16702:2002 | Laboratory methods for the determination of soil microbial respiration |
| | 10381-6:2009 | Soil quality -Sampling -Part 6: Guidance on the collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory |
| | 29843-2:2011 | Soil quality -Determination of soil microbial diversity -Part 2: Method by phospholipid fatty acid analysis (PLFA) using the simple PLFA extraction method |
| | 15685:2012 | Soil quality - Determination of potential nitrification and inhibition of nitrification -- Rapid test by ammonium oxidation |
| | 22939:2010 | Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates |

These regulations (**Table 3)** are very useful to evaluate the AgNPs effects in soil microorganisms providing an important understanding of the microbial processes and their potential role in soil health. Most studies examined the biomass, respiration rates and enzyme activities (Peyrot *et al*., 2014) of the soil bacteria and less attention has been given to the analysis of the soil bacterial community (Hill *et al*., 2000). On the other hand, the microbial community changes rapidly and across very short spatial scales (Sharma, 2010). Using the microbiota as a measurement endpoint might be difficult as it can easily change with variations in temperature, moisture, oxygen, among others (Kuperman *et al*., 2014). In addition, the analysis of community microbial interactions is complex due to interaction among different species. Nonetheless, quantitative and qualitative changes in the composition of the microbial communities are an important and sensitive indicator of short and long changes in the soil health (Hill *et al*., 2000).

## 3.3. Evaluation of the toxicity of AgNPs using terrestrial organisms

Several methodologies of assessing the toxicity of contaminants have been standardized for different organisms including microorganisms, plants, vertebrates and invertebrates or for systems (e.g. mesocosms) (Rana & Kalaichelvan 2013).

Regarding AgNPs, most studies suggest that this contaminant has effect in the reproduction rate, mortality, biomass and feeding behaviour of the organisms (Loureiro

*et al.*, 2009; Tourinho *et al.*, 2015; Morgado *et al*., 2015) (**Table 4**). Compared to survival rates, reproduction seems to be a more relevant and sensitive endpoint for analyzing the effects at population level (van Gestel, 2012). Also, the reproduction parameter enables to evaluate the possible long term effects of the contaminant as the results reveal future reproduction capacities of the species. However the main disadvantage of the reproduction tests that include soil species is that reproduction cannot be directly observed (Hopkins, 1997).

Overall, studies summarized in **Table 4** shown that AgNPs have a significant impact in organism activities and AgNPs are less toxic than the respective ionic form.

**Table 4.** Ecotoxicological tests to analyse the effect of silver nanoparticles in terrestrial organisms.

| | Organisms | Exposure (duration) | Nanoparticles | Concentration of Nanoparticles | Main achievement | References |
|---|---|---|---|---|---|---|
| Plants | Seeds of *Phaseolus radiatus* (mung bean) and *Sorghum bicolor* (sorghum) | 24h (Agar), 5 days (soil) | AgNPs | Agar (30 mL/petri dish): 0, 5, 10, 20, and 40 mg/L.<br><br>Soil medium: 0, 100, 300, 500, 1000, and 2000 mg/kg dry soil. | AgNPs have a negligible effect on the plant growth. | Lee *et al.,* 2012 |
| | Seeds of *Glycine max* (soybean) and *Triticum aestivum* (wheat*)* | 14 days | AgNPs | 5 mg/L | $Ag^+$ exhibited strongest effects on plant growth at equivalent nominal concentrations. Once Ag was taken up by plant roots, the upward translocation of Ag from roots to shoots depended upon both Ag properties and plant species. | Quah *et al.,* 2015 |
| Soil invertebrates | *Porcellionides pruinosus*(isopod) | 14 days | AgNPs | 50, 100, 200, 400, and 800 mg Ag/kg for AgNPs.<br>12.5, 25, 50, 100, and 200 mg Ag/kg for $AgNO_3$ | The range of concentrations for AgNPs and $AgNO_3$ were distinct and used to determine the $LC_{50}$ and $EC_{50}$ on consumption ratio, biomass change and avoidance behavior of the isopod *P. pruinosus*.In the feeding trials for both soil and dietary exposures, AgNPs were found generally to be less toxic than $AgNO_3$. Following soil exposure, ionic Ag caused greater biomass losses and mortality, while AgNPs caused no mortality and had less effect on biomass. | Tourinho *et al*., 2015 |
| | *Caenorhabdtis elegans*(nematode) | 96 h | $Al_2O_3$ nanoparticles and AgNPs. | 0.5 mg/L of AgNPs and 5 g/L of $Al_2O_3$ | The test with chemicals not demonstrated the toxicity to the endpoints for growth, survival and reproduction. Only observed differences in growth as a result of using the different soils (LUFA 2.2 and 2.4). | Fajardo *et al*., 2014 |

### 3.3.1. Model organism: *Porcellionides pruinosus*

*Porcellionides pruinosus* is a terrestrial isopod of the family *Oniscidea* (**Table 5**) with a cosmopolitan distribution (Paoletti & Hassall, 1999). The origin of this isopod occurred in Asia Minor (Broly *et al*., 2013) and it has colonized the entire world as the result of human activities (Drobne *et al*., 2008).

**Table 5.** Scientific classification of *P. pruinosus* (Adapted from: Geoffrey, Fauna Europaea).

| **Scientific classification** | |
|---|---|
| **Kingdom** | *Animalia* |
| **Phylum** | *Arthropoda* |
| **Subphylum** | *Crustacea* |
| **Class** | *Malacostraca* |
| **Order** | *Isopoda* |
| **Sub-order** | *Oniscidea* |
| **Family** | *Porcellionidae* |
| **Genus** | *Porcellionides* |
| **Species** | *P. pruinosus* |

The terrestrial isopods are macroinvertebrates (Ferreira *et al*., 2015) that play an important role in the decomposition processes, in the vegetal litter fragmentation and in the re-cycling process of nutrients (carbon and nitrogen) (Kostanjsek *et al*., 2002; Loureiro *et al*., 2006; Ferreira *et al*., 2015). This is mainly due to their diet consisting mostly of decayed plant material composed of cellulose and other polysaccharides (Kostanjsek *et al*., 2002).

Nearly 5000 species distributed worldwide from forests, agro ecosystems, rangelands, up to mountains and in subterranean caves are included in this sub-order (Paoletti & Hassall, 1999) being represented by small to middle sized organisms (1.2-30 mm).

Several characteristics of these important detritivorous organisms support their use as a model organism (Loureiro *et al*., 2005). In particular, *P. pruinosus* has been described as a good test-organism (Loureiro *et al*., 2006; Calhôa *et al*., 2006) to evaluate changes in their habitat as well as soil contamination (Ferreira *et al*., 2015) and other environmental changes (e.g UV radiation) (Morgado *et al*., 2015).

Isopods respond quickly to the environmental contamination. Heavy levels of pollution were reported to cause increased mortality and loss of biomass (Paoletti & Hassall, 1999). Exposure to AgNPs, via soil and food, caused avoidance and change in the feeding activity of the isopods (Tourinho *et al*., 2015) probably because isopods are able to detect pollutants through chemoreceptors located in the tegument or choose food according to their microbial component (Lapanje *et al.*, 2007; Zimmer, 2002). Besides quick response, isopods are capable of accumulating high levels of heavy metals, especially copper and silver (Loureiro *et al*., 2006), in special organelles (granules) located on the hepatopancreatic tissue (Zimmer, 2002; Tkalec *et al*., 2011).

In addition, isopods have small body size being easy to manipulate, are easy to maintain, have low food/environmental requirements and its physiology/morphology and ecology is well known (Jansch *et al*., 2005). The isopods can contribute for decomposition process through the enhancing of the soil microbiota (Zimmer, 2002). The major disadvantage of this organism was explained by difficulty in reproduction evaluation due to its long reproductive cycle and the fact that females can retain sperm for long periods and use it to get pregnant in several occasions (Tourinho *et al*., 2015).

#### 3.3.1.1. Characterization of the model organism

Isopods are protected by a sclerotized tegumental cover (**Figure 11**), a cuticle or exoskeleton (constituted by an organic matrix containing chitin and sclerotized proteins). This is the most important barrier against environmental threats and also an important way of controlling the body permeability to water (Paoletti & Hassall, 1999).

Terrestrial isopods molt frequently throughout their life to grow (Hornung, 2011). Molting has two phases: in the first, the posterior part of the cuticle is changed; one day after, the second phase occurs when the other half is changed, the exoskeleton's anterior (Hornung, 2011).

The digestive system of the isopods is a simple tube covered with a cuticle which is renewed at molting (Kostanjšek *et al*., 2002).

The digestive system tract (**Figure 11**) consists of the foregut (esophagus and stomach) and hindgut (sub-divided by anterior chamber, papillate region and rectum) and endodermal digestive glands (hepatopancreas or midgut glands) (Kostanjsek *et al*., 2006). The most important compartments for the digestion are the foregut (including

grinding, filtration, transport and absorption of food, and ion transport) and the hindgut (involved in water uptake), in which is a site for food channeling (Kostanjsek *et al*., 2002). The digestive process begins in the foregut where the food is briefly masticated and filtrated into the hindgut. Then, the food is mixed with secretions from the hepatopancreas cells (**Figure 11**) before passing into the hindgut (Kostanjsek *et al*., 2002).

Absorptive process has been attributed to the anterior hindgut and to the hepatopancreas (containing digestive enzymes) whereas water and ions are reabsorbed in the papillate region (Zimmer, 2002). Dry fecal pellets are formed and excreted through the anus.

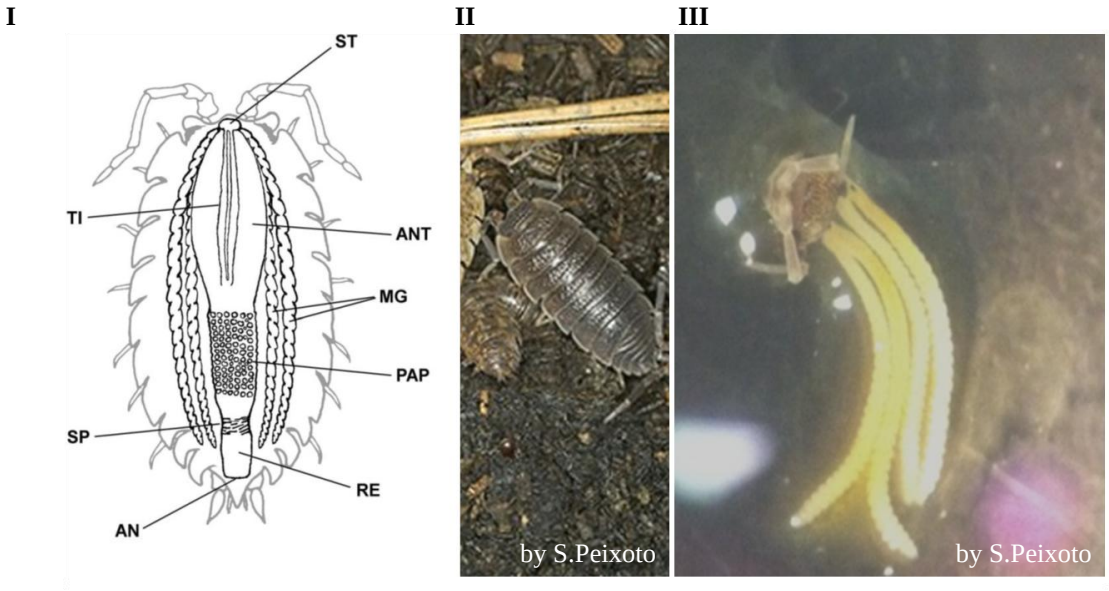


**Figure 11.** Internal morphology of the digestive system of an adult isopod (I); in the figure: head (ST), midgut glands-hepatopancreas (MG), anterior region of the hindgut (ANT), typhlosole (TI), papillate region of the hindgut (PAP), sphincter (SP), rectum (RE), anus (AN) (Adapted from: Kostanjsek *et al*., 2002). *Porcellionides pruinosus* - external morphology (II). Hepatopancreas attached to isopod head (III).

Kostanjšek and their collaborators (2006) showed that the hindgut of *P. scaber* is colonized by a diverse bacterial community possibly introduced into the isopods digestive system along with the food or some might be indigenous to the organisms (Zimmer & Topp, 1998; Kostanjšek *et al*., 2002). Ingested microorganisms might be used as a source of nutrients and vitamins. However, the digestive system of the isopods harbours suitable conditions for microbial colonization and proliferation. The microbiota altered through this process can be expelled via feces and enhance the microbial activity in soil, which is important for fragmentation of the substrate. Thus, in

this process, isopods can contribute to the higher distribution of distinct microbiota in the soil (Zimmer, 2002) enhancing the decomposition process.

# 4. Methods for analysis of the effects of AgNPs

## 4.1. Culture-independent methods

Molecular approaches represent an alternative to the culture-dependent techniques but may also complement each other because those microorganisms (e.g. bacteria from soil microbiota) that are not possible to culture can be detected using molecular methodologies (Muyzer, 1999).

In the past 20 years, several methods based on the direct amplification, using Polymerase Chain Reaction (PCR), and analyses of the small subunit ribosomal RNA gene (16S rRNA gene) have been developed to directly study environmental microorganisms (Oliveira *et al*., 2013). The target gene for the molecular analysis of bacterial communities is the 16S rRNA gene because several copies are present inside the cell, it is a highly conserved gene but with hypervariable regions as well that provide species-specific signature sequences essential for the identification of the bacteria (Woo *et al*., 2008).

In 2012 the most used molecular methods to analyze the microbial community was the denaturing gradient gel electrophoresis (DGGE) (Gao & Tao, 2012) (**Figure 12**). Thought nowadays this methodology is highly used, pyrosequencing have gained more attention than DGGE because it provides more detailed information yet is one of the most expensive molecular methods (Vaz-Moreira *et al*., 2011).

The other methods commonly used included PLFA analysis (Echavarri-Bravo *et al*., 2015). In terms of bacterial diversity in sediments, this technique has the lowest resolution in comparison to other PCR-based techniques, as DGGE. FISH can have the same problem yet, it is more specific in providing microorganism's identification (by using specific probes) (Echavarri-Bravo *et al*., 2015), but requires more labor (direct microscopic counting) and it is more expensive than DGGE.

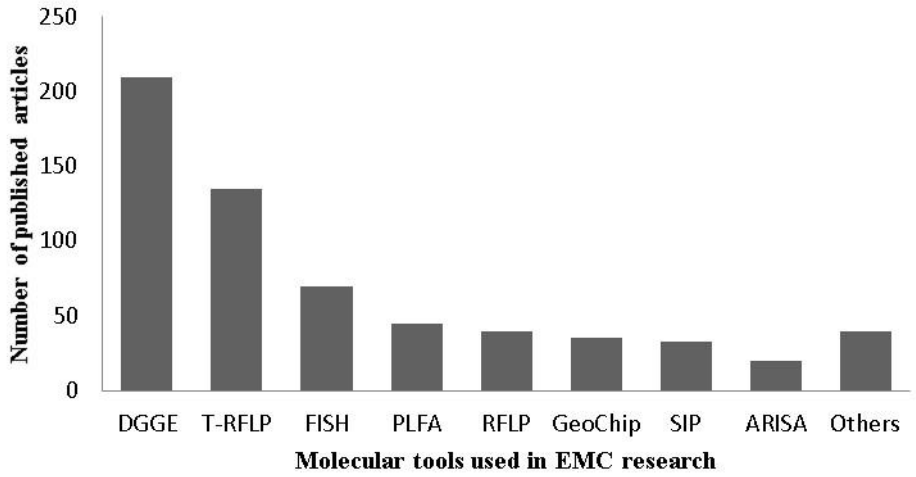


**Figure 12.** Molecular methods used to detect or quantify environmental microbial communities (EMC) (Adapted from: Gao & Tao, 2012). In the figure: denaturing gradient gel electrophoresis (DGGE); terminal restriction fragment length polymorphism (T-RFLP); fluorescence in situ hybridization (FISH); phospholipid fatty acid analysis (PLFA); restriction fragment length polymorphism (RFLP); microarray analysis (Geochip); stable isotope probing (SIP) and automated rRNA intergenic spacer analysis (ARISA).

### 4.1.1. Polymerase Chain reaction (PCR)

The principle of PCR is the amplification of a specific DNA fragment from a complex DNA sample (Burton, 1996).

Practical procedure of PCR includes mixing the DNA template with the universal primers, the deoxynucleoside triphosphates (dNTPs), a buffer solution, a solution with bivalent cations (e.g. $MgCl_2$) and a DNA polymerase (e.g. *Taq* polymerase - a thermostable enzyme isolated by Thomas Brock in 1965 from the bacterium *Thermus aquaticus*) (Green *et al*., 2010). A set of two complementary primers to the DNA target are used to establish the limits of the bacterial sequence of interest to be amplified (Green *et al*., 2010). The buffer solution is necessary to maintain chemically stable the PCR reaction and protect the functioning of the *Taq* polymerase. If the buffer is not present, the pH can become more acidic and can inhibit the action of this enzyme (Gyllensten, 1989). The bivalent solution of $MgCl_2$, in particular the $Mg^{2+}$, is required for polymerase activity and to promote the bound of dNTPs. Without this bound, the polymerase will not recognize the dNTPs as a substrate and will not work. Under DNA elongation the $Mg^{2+}$ is essential in removing the phosphate groups (Saiki, 1989). DNA *Taq* polymerase is responsible for putting together the dNTPs and amplifying the segments of DNA (Green *et al*., 2010).

The PCR reaction can also include the Dimethlysulfoxide (DMSO) which is a PCR-enhancing agent since it improves the DNA amplification by loosen secondary structures that may prevent the *Taq* polymerase from attaching to the template strand (Gelfand, 1989). It is also useful when amplifying GC-rich sequences where lowering of the melting temperature (Tm) is needed (Baskaran *et al*., 1996). Due to their third H-bonds the strength of these bonds requires high energy. DMSO binds to DNA at the cytosine residue and changes its conformation thus making the DNA more lable for heat denaturation. Since most of the primers are GC rich, DMSO indirectly facilitates the annealing of primers to the template and this enhances the amplification (Baskaran *et al*., 1996). PCR is a semi-conservative process because the new double DNA strand is constituted of a new strand complementary to the original and a conserved strand which was used as a mold). PCR consists of three steps: denaturing, annealing and extension (Gasser, 2006).

Denaturing consists of the separation of the double original strand of DNA by increasing the temperature to 95ºC or 94ºC resulting in two single strands (Muyzer, 1999).

In the next step (annealing) specific primers (oligonucleotides, consisting of a simple chain of DNA of around 20 to 30 nucleotides long) detect and bind to the DNA target sequence. For the DNA target and the primers to bind it is necessary a decrease in the temperature near 50ºC up to 70ºC. Each annealing temperature must be chosen and optimized according to the primer set, based on the Tm of each primer (Kawasaki & Wang, 1989). After primers' annealing, the DNA polymerase can bind to the sequence of interest and DNA synthesis can initiate. The DNA polymerase synthesizes the new strand by adding dNTPs in a complementary way to the target DNA region; this step occurs at a temperature dependent on the DNA polymerase used and is designed extension (Gasser, 2006).

Millions of DNA strands can be generated by PCR (Green *et al*., 2010) by means of several cycles (e.g. 20-30 cycles). In each cycle, each DNA strand is doubled into two new strands, thus the copy number can be estimated by $2^n$ (with n being the number of cycles).

Finally, horizontal/vertical electrophoresis of the PCR products in agarose gel stained with ethidium bromide or other staining reagent enables to verify if the PCR process run correctly (Green *et al*., 2010) and it was obtained a PCR product with the expected size (Bayley & Scott's, 2007).

A positive and negative control which does not have the DNA of interest is included during the PCR process (Green *et al*., 2010). The negative control consists of substituting the DNA of interest by water; it provides information on possible contamination of the reagents or of the water (Green *et al*., 2010). The positive control consists of substituting the DNA of interest by a distinct DNA capable of being amplified by the same set of primers (Green *et al*., 2010).

### 4.1.2. Denaturing Gradient Gel Electrophoresis (DGGE)

Muyzer and collaborators (1993) published the first scientific article about denaturing gradient gel electrophoresis (DGGE) for microbial communities in ecology (Muyzer *et al.*, 1993). However, it was previsiously used to identify sequence variations in a number of genes from several different organisms (Muyzer *et al.*, 1993). Nowadays, the DGGE is a frequently used molecular technique for fingerprint analysis of microbial community composition (Green *et al*., 2010) and microbial structure of different habitats (Bekaert *et al*., 2015). This method is suitable for comparison of several samples from different treatments which has made this technique a common practice across laboratories (Green *et al*., 2010).

The molecular fingerprint can be obtained by coupling the PCR-amplified targets with the sequence dissimilarities analyzed by DGGE. Indeed, many researchers used the method of combining PCR and DGGE to the study of microbial communities of various environments (Muyzer & Smalla, 1997). Through PCR, small DNA fragments of the same length are obtained but with different nucleotide compositions. Vertical electrophoresis is then used to separate the PCR-amplified fragments of genes coding for 16S rRNA (Muyzer *et al*., 1993). In this electrophoresis, the mobility within the acrylamide gel occurs due to partially melted double-strand DNA according to the linear denaturing gradient which comprises a mixture of urea and formamide (Justé *et al*., 2008). A solution of 100% chemical denaturant consists of 7 M urea and 40% formamide (Green *et al*., 2010).

In the gel, different bands are observed which represent specific sequences of the 16S rRNA gene, which might vary, in theory, by a single nucleotide. The bands movement on the gel is dependent of the melting properties which depend on nucleotide sequence and GC content (Green *et al*., 2010) (**Figure 13**).

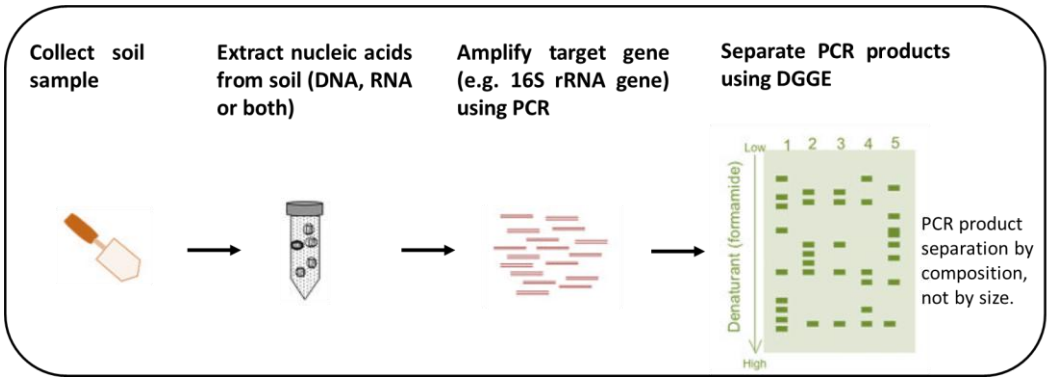


**Figure 13.** PCR-DGGE approach for bacterial community analysis (Adapted from: Nakatsu, 2007).

The band separation obtained on the final acrylamide gel is then analyzed. Distinct quantitative measures can be used to analyze the bacterial community, for instance, richness and the diversity and evenness indexes (Green *et al*., 2010).

Richness (*R*) reveals how many different species there are in the sample, which in the DGGE gel refers to the number of bands in each lane (Vaz-Moreira *et al*., 2011).

The Shannon-Weaver index (*H'*) demonstrates the entropy of the sample, which means how many different species exist in the sample and how evenly the individuals are distributed among all the existing species of the population. It is calculated according to:

$$H' = -\sum_{i=1}^{S} p_i \ln p_i$$

Where: *H'*= Shannon diversity index; *Pi*= proportion of individuals belonging to the entire population made up of $i^{th}$ species; *S*= numbers of species.

The Pielou's evenness index (*J'*) reflects the species evenness which means how close in numbers each species are in a sample and is calculated following:

$$J = \frac{H'}{\ln(S)}$$

Where: *J'*= evenness index; *S*= number of species

Pielou's evenness index might vary between 0 and 1: the higher the variation in communities between the species, the lower evenness there is and so, the lower is *J'*index.

DGGE is a laborious technique that requires specific equipment. Sequences with more than 400 pb are not accurately analyzed by this technique (Myers *et al*., 1989). Also, the primers are expensive because a GC tail must be included in one of the primers. In addition to these disadvantages, this method only enables to analyze around 1% of the microbial community (Muyzer *et al*., 2004; Muhling *et al*., 2008). Also, several problems may hamper the analysis: different sequences may display similar migratory behaviour in the gel, thus giving coinciding bands; the bands may appear fuzzy on gel, because of the presence of multiple melting domains [longer PCR product often melt in multiple transitions, and have different stability (Kisand & Wikner, 2003) with the same molecule]; microheterogeneity can also occur (Kisand & Wikner, 2003).

### 4.1.3. PCR-DGGE studies

Despite all limitations, the PCR-DGGE is being continuously used as an adequate approach to compare microbial communities and to infer the influence of environmental conditions or of contaminants (Vaz-Moreira *et al*., 2011; Gao & Tao, 2012). Regarding the assessment of AgNPs effects on the soil microbial communities using PCR-DGGE, there are only few studies. Carbone and their collaborators (2014) evaluated the influence of AgNPs on soil bacterial community from forest soils using PCR-DGGE along with plate counts. In this study, the soil was treated with AgNPs (10 and 100 μg $g^{-1}$ dry weight) during 30, 60 and 90 days. These authors observed that the time was determinant for the change of the bacterial community showing a significant influence of the AgNPs on the soil microbial community, after 60 days of incubation.

Kumar *et al*., (2011) evaluated the microbiota from arctic soil collected and exposed to AgNPs during 176 days. The main results indicate that the microbial populations were affected by this contaminant.

Das and collaborators (2012) observed changes in the structure of natural bacterioplankton communities exposed (5 days) to carboxy-funcionalized AgNPs (0.01, 0.02, 0.1 and 1.0 mg/L). Lower doses to carboxy-funcionalized AgNPs (0.01 and 0.02

mg-Ag/L) caused less severe effects than those submitted to higher doses (0.1 and 1.0 mg Ag/L).

Overall these studies showed that though the AgNPs affect the structure of bacterial communities, these effects can be influenced by the time of exposure and type of contaminants as well as by the distinct properties of soils.

## 4.2. Culture-dependent methods

Methods to analyze the microbiota include traditional microbiological techniques such as microcopy and cultivation (Muzer, 1999). These methods based on a large number of phenotypic, morphologic, and biochemical characteristics, on motility and on antibiotic sensitivity to characterize the microbiota (Rastogi & Sani, 2011).

When looking at the effects of contaminant on the microbiota, culture-dependent techniques such as antibacterial susceptibility tests are useful (Reller *et al*., 2009). These tests were used for clinical diagnose to detect possible drug resistance in common pathogens and to assure susceptibility to drugs of choice for particular infections. Though these techniques are not expensive, they are laborious and the results are difficult to interpret due to the low discriminatory power (Muzer, 1999). Also, several microorganisms are not suitable or are difficult to grow in the existing media (Reller *et al.*, 2009).

Due to the disadvantages associated to the culture-dependent methods, the culture-independent methods have gained interest in the scientific community (Rastogi & Sani, 2011).

### 4.2.1. Susceptibility tests

The main aim of the bacterial susceptibility test is to understand if bacteria are affected thus being susceptible to the chemical being tested. The initial approaches were used for antibiotics tests but now they are also used for testing the microbial susceptibility to AgNPs (Anthony *et al*., 2014; Swain *et al*., 2014). In **Table 6** are described several works where the susceptibility of microorganisms to AgNPs was tested. Thought different methodologies were used, the results showed, in general, that: the AgNPs have antimicrobial properties and (2) that the silver bulk form (usually $AgNO_3$) was less toxic than the nanoparticle form.

There are several methods to perform antibacterial susceptibility tests (Shameli *et al*., 2012). The most used methods include: disc diffusion method, drop plate method,

cut plate method, diffusion on plate and 96 well and broth dilution (macrodilution and microdilution), agar dilution and E-test method. Using these methods the susceptibility can be assessed through the proliferation or inhibition of growth (e.g. existence of zones of inhibition) or the colony forming unit (CFU) counts (Shameli *et al*., 2012).

Among the earliest antimicrobial susceptibility tests was the macrobroth or tube-dilution method (Bose and Chatterjee, 2015) which provided a quantitative result (the MIC) however it was laborious to prepare the solutions, several reagents were required and large space per test was needed (Reller *et al*., 2009).

Prasad and Elumalai (2011) showed that the disc diffusion method has higher resolution than cut plate method. Bose and Chatterjee (2015) reported that although the agar-cup assay method and serial dilution turbidity measurement assays were more sensitive than disc diffusion method, this method was easy and procedures as well as the interpretation of the zones of the inhibition were standardized enabling comparison. Indeed, the standard zones of inhibition have been determined for susceptible and resistant values and are well documented for antibiotics (Matuschek *et al*., 2013). Nowadays, it's possible to found breakpoint tables by Committee on Antimicrobial Susceptibility Testing (EUCAST). The method, medium, conditions for each antimicrobial agent and bacteria as well as the interpretation of the inhibition zones are well described. The most used medium is the Müeller-Hinton agar (MHA) since it shows good bacteria culture reproducibility and gives satisfactory growth for most nonfastidious organisms as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* (Shameli *et al*., 2012).

Being one of the oldest approaches to assess the antimicrobial susceptibility to antibiotics, the disc diffusion method is the most used in routine clinical microbiology laboratories being denominated of Kirby-Bauer disk diffusion method (Matuschek *et al*., 2013). It was first described by Bauer *et al*. (1966) and later modified by the National Committee for Clinical Laboratory Standards (NCCLS) from USA. It is a versatile, reliable, low cost, simple and reproducible method requiring low tech equipment (Matuschek *et al*., 2013). On the other hand, this method lacks mechanization or automation and slow growing bacteria cannot be accurately tested by this method due to culture difficulties (Reller *et al*., 2009).

**Table 6.** Bacterial susceptibility tests applied to AgNPs.

| Antibacterial susceptibility | | Tested bacteria | | | | Nanoparticles | | | Main achivements | References |
|---|---|---|---|---|---|---|---|---|---|---|
| Method § | Temperature; duration | Denomination | Culture preparation (Temperature; Time; Agitation) | Growth media | Initial concentration (CFU/mL) | Characterization† | Size/shape | Concentration/Amount | | |
| DP | 31ºC; 24 h. | Heterotrophic bacteria | 25.5ºC; 30 h; 100rpm. | R2A agar | $10^7$ | ZS | Size: 33-59 nm. | 0, 1, 50, 200 mg (Ag/L) | Bacteria isolated from the wastewater biofilms were highly vulnerable. | Sheng & Liu, 2011 |
| WD | | *E. coli* (RGR13)<br>*P. aeruginosa* (PS1)<br>*Bacillus circulans* (D1)<br>*Bacillus aerius* (SPT2) | 37ºC (*Bacillus* and *E.coli*) or 28ºC (*P. aeruginosa*); 4 h; 120 rpm. | MHA | $10^7$ | XRD<br>FTIR<br>TG-DTG-DTA | n.d. | 10, 25, 50 mg ($Ag_2O$ NP) | Inhibition zone of $Ag_2O$ nanoparticles increased with the increasing concentrations from 10 to 50 mg per well. | Negi *et al*., 2013 |
| FD | 37ºC; 24 h. | *E. coli* (ATCC 11303)<br>*S. aureus* (ATCC 29737) | 37ºC; 24 h; 150 rpm. | MHA | $10^8$ | FTIR-ATR<br>AFM<br>FE-SEM<br>AFM<br>AAS | In average: 50 nm.<br>Shape: round. | AgNPs (coated LDPE films) | The nanocomposite of silver/LDPE indicated higher bactericidal activity against *S. aureus* than *E. coli*. | Sadeghnejad *et al*., 2014 |
| DD | 35-37ºC; 24-48 h. | *E. coli*<br>*S. aureus* | *n.d. | AM | $10^8$ | SEM<br>EDX<br>FTIR-ATR | Shape: spherical.<br>Size: 2-30 nm | 49, 100 ppm (AgNPs coated cotton fabrics) | Fabrics coated with a solution containing 50 ppm AgNPs in presence of binder retain excellent antibacterial action. | El.Rafie *et al*., 2014 |
| DD | 35-37ºC; 24-48 h. | *E. coli*<br>*S. aureus*<br>*P. aeruginosa* | n.d. | AM | $10^8$ | FTIR<br>SEM | Shape: spherical.<br>Size (100 ppm): 1-4 nm;<br>Size (50 ppm) n.d. | 50, 100 ppm (AgNPs-alginate composite) | Using 50 ppm of AgNPs–alginate composite is sufficient to exhibit excellent antibacterial activity against *E. coli*, *P. aeruginosa* and *S. aureus*. | Zarhan et al., 2014 |
| WD | 35ºC; 24 h. | *E. coli* (ATCC 25923)<br>*P. aeruginosa* (ATCC 15442)<br>*S. aureus* (ATCC 10536)<br>*B. subtilis*<br>*K. pneumoniae* | n.d. | MHA | $10^6$ | TEM<br>FTIR<br>EDS<br>AAS | Size: 30–36 nm. | 500, 1000, 1250, 1500 µg (Lignin/silica–AgNPs ) | The strongest antimicrobial effect was observed for *P. aeruginosa*.<br># $ZOI_{B.\ Subtilis}$=*28.67±0.58 mm;*<br>$ZOI_{S.\ aureus}$=28.67±0.58 mm<br>$ZOI_{P.\ aeruginosa}$=11.00±1.00 mm<br>$ZOI_{E.\ coli}$=26.33±0.58 mm<br>$ZOI_{K.\ pneumoniae}$=26.00±1.00 mm | Klapiszewski *et al*., 2015 |
| MD | 37ºC; 24 h. | *E. coli*<br>*S. aureus* | n.d. | TSB | $10^6$ | SEM<br>XPS | n.d. | 1, 2, 5, 10 mg/mL (AgNPs non-woven polyethylene terephthalate) | Silver treated fabrics were more effective against *E. coli* than to *S. aureus*: showing 100% and 99.7% in the reduction rates at 5 mg/ml AgNPs, respectively. | Deng *et al*., 2015 |
| DD | 25ºC; 24 h. | *Aeromonas salmonicida* (KCTC 2766) | 25ºC; 16 h; 160 rpm. | MA | $10^3$ - $10^4$ | FTIR<br>FE-SEM<br>ICP–AES | Size: 15-35 nm.<br>Shape: spherical | 0, 12.5, 25, 50, 75 µg/disc (CAgNCs) | CAgNCs inhibited the growth of *A. salmonicida* in a concentration-dependent manner and showed the highest growth arrest at 75 µg. | Dananjaya *et al*., 2016 |

§ Methods used for antimicrobial testing: Drop plate (DP); Agar Well-diffusion (WD); Macrodilution (MD); Agar Diffusion caused by NP film (FD); Disc diffusion (DD). † Methods used for characterization of AgNPs: Malvern zetasizer nano-ZS (ZS); X-ray diffraction (XRD); Fourier transform infrared spectroscopy (FTIR); simultaneous differential thermal analysis (TG-DTG-DTA); Fourier transform infrared-attenuated total reflection (FTIR-ATR); Field emission scanning electron microscope (FE-SEM); Atomic force microscopy (AFM); Atomic absorption spectrometry (AAS); Scanning electron microscope (SEM); Energy-dispersive X-ray spectroscopy (EDX); Transmission electron microscopy (TEM); Energy dispersive X-ray spectroscopy (EDS); X-ray photoelectron spectroscopy (XPS); Inductively coupled plasma-atomic emission spectrometer (ICP-AES). # ZoI - Zone of Inhibition. *n.d. - not described.

## 5. Comparison between culture-dependent and culture-independent methods

Culture-dependent and culture-independent techniques are very different, each one having their one advantages and drawbacks, nonetheless both should be used as complementary methodologies (Vaz-Moreira *et al*., 2011).

Regarding the initial community being analyzed: (1) culture-dependent methods enables the analysis of the cultivable and viable organisms; (2) culture-independent techniques, particularly DGGE, focuses on the total DNA of the bacterial population (which represents around 1 % of the entire original soil community) which means that screened individuals include those live, death, viable and/or not viable.

Culture-independent methods are relevant considering the output information despite being time consuming. Despite DGGE disadvantages, this technique is still considered an adequate approach to compare microbial communities (Vaz-Moreira *et al*., 2011). Indeed, microorganisms in environment might be efficiently detected by culture-independent methods which may gain advantage during the DNA extraction and PCR amplification stages. On the other hand, culture-dependent methods are easier to perform but give a worst screening of the community. Standard culture techniques involve isolation and characterization of microorganisms to characterize the microbial community (Rastogi & Sani, 2011).

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**CHAPTER II**

# EFFECTS OF SILVER-NANOPARTICLES: PCR-DGGE PROFILES SHOW CHANGES ON SOIL BACTERIAL COMMUNITY

# EFFECTS OF SILVER-NANOPARTICLES: PCR-DGGE PROFILES SHOW CHANGES ON SOIL BACTERIAL COMMUNITY

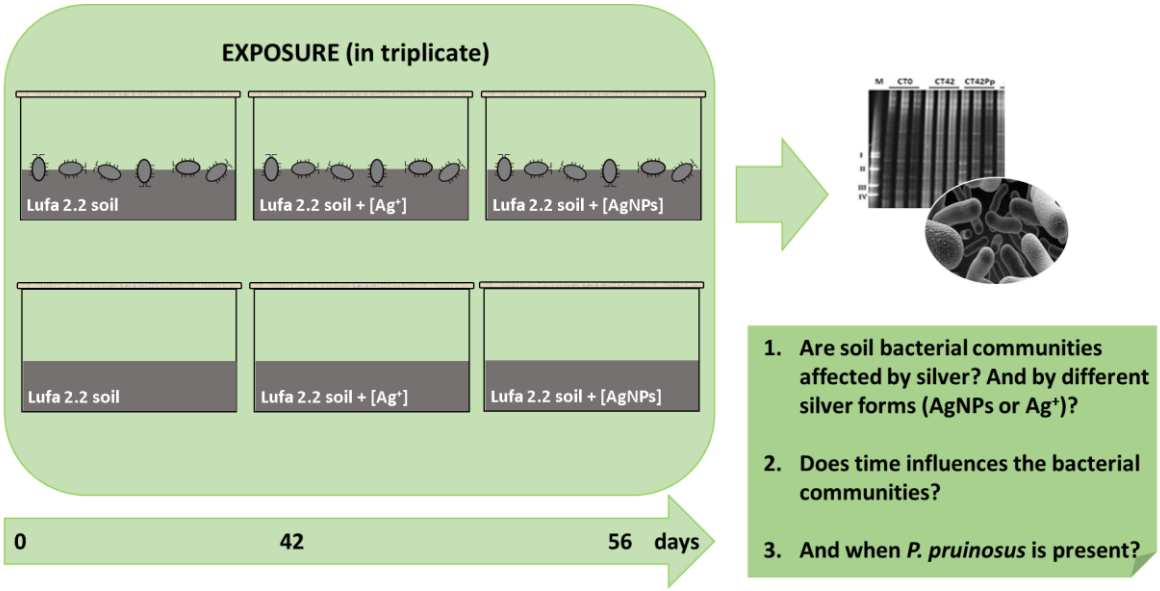
## Abstract

Silver nanoparticles (AgNPs) with antibacterial properties are broadly used in daily products. Therefore, contamination of terrestrial compartment is inevitable probably leading to effects on soil bacterial community, which plays a fundamental ecological role. Thus, assessing the effects of AgNPs on soil bacterial community is of upmost importance. Therefore, the present study aimed to investigate the effects of AgNPs or $Ag^{+}$ (provided as $AgNO_3$) on the bacterial community of soil and to understand if these effects changed with the presence of *Porcellionides pruinosus* or along the exposure period. LUFA 2.2 soil was separately exposed to the two forms of silver (5 µg of Ag/Kg of soil) during 42 and 56 days. The same setup was used including the isopod *P. pruinosus*. Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) was used to analyze the bacterial communities and results were interpreted in terms of the structure, richness, diversity and evenness.

PCR-DGGE results revealed that depending on the time of exposure, distinct results were achieved. After 42 days, silver contamination (regardless of the silver form) and the presence of *P. pruinosus* were determinant factors deciding the structural changes. On the other hand, after 14 additional days (at day 56), time gained relevance in defining the structure of the soil bacterial communities; moreover, the silver form showed distinct impacts on the structure of the soil bacterial community and *P. pruinosus* showed to distinctly impact the soil bacterial community depending on the silver form. *P. pruinosus* might be useful to minimize the effects caused by silver cation for long exposures (close to 2 months); yet, even in the presence of this isopod, the AgNPs might still be a risk for the soil bacterial communities.

**Keywords:** DGGE, AgNPs, soil bacterial community, *Porcellionides pruinosus*.

# Graphical abstract



# 1. Introduction

Silver nanoparticles (AgNPs) are embedded in several daily products as textiles, laundry additives, cosmetics, paintings, medical devices (Rai, 2009; Foldbjerg *et al*., 2013; Tran *et al*., 2015).The interest for this nanoparticle in the industrial market has increased due to their antimicrobial activity (Zhou *et al*., 2015).

Being non-biodegradable, contamination by AgNPs rises environmental concern (Fajardo *et al*., 2014). Untreated sewage from wastewater treatment plants (Som *et al*., 2010; Rana & Kalaichelvan, 2013) which is further used as agricultural fertilizer (Schlich *et al*., 2013) increases soil exposure to this contaminant turning it into the most affected environmental compartment (Tourinho *et al*., 2013). Once in soil, AgNPs can compromise the terrestrial ecosystem productivity (Carbone *et al*., 2014) as well as the biogeochemical cycling (Colman *et al*., 2014; Yang *et al*., 2014) ultimately impacting the soil quality.

Bacterial communities play an essential role in maintaining the quality of soil (Vasileiadis *et al*., 2015). Thus, it is important to evaluate the effects that AgNPs might have on the soil bacterial community in a perspective of soil quality and risk assessment as already indicated by Sharma *et al.*, (2010).

Even in healthy soils, natural bacterial community constantly adapts to several environmental factors (pH, temperature, humidity, among others). Contamination might constitute and additional pressure leading to a bacterial response to all these disturbances and ultimately changing over time (Sharma, 2010; Sierra *et al*., 2015).

Besides the type of contaminant, the effects on soil bacterial community resulting from contamination is influenced by the dose/concentration (Carbone *et al*., 2014), duration of exposure (Beddow *et al*., 2014) and properties of the soil (Fajardo *et al*., 2014), among other variables.

The NanoFATE research project predicted the concentration of engineered nanoparticles in different environmental compartments. For the soil compartment, the predicted environmental concentration (PEC) for AgNPs was 5 ng/Kg (Faust & Backhaus, NanoFATE). Massive production of products containing AgNPs and their continuous and uncontrolled released for environment makes this prediction difficult being foreseeable an increase. Because of this, our study considered the worst case scenario indicated by Faust & Backhaus (NanoFATE) of 5 µg Ag/Kg of soil (100 x PEC).

Previous attempts of assessing the effects of AgNps on soil bacterial communities used concentrations of AgNPs much higher than 100 x PEC probably hampering further extrapolations into a real scenario. Besides, these studies considered little studied soils. Kumar *et al*., (2011) reported changes on bacterial communities of artic soil contaminated after 176 days exposed to AgNPs [0.066% (w/w)]. Carbone *et al*., 2014 investigated the effects of AgNPs (10 and 100 μg $g^{-1}$ dw) in forest soil after incubation for 30, 60 and 90 days and observed a marked shift in soil bacterial communities after 60 days of exposure. Moreover, for all of these studies, the effect of the AgNPs treatment was as strong as or stronger than with $AgNO_3$. Another study by Colman *et al*. (2013) showed that soils contaminated by AgNPs (0.14 mg Ag $kg^{-1}$ soil) via sewage during 50 days did not significantly affect the community composition. Such contradictory results might be explained based on distinct properties of the soils, on distinct time of exposure and concentrations of contaminants thus requiring further investigations.

Considering the use of different soils, because soil properties are determinant for AgNPs effects (Dwivedi *et al*., 2015), the use of the standard natural soil LUFA 2.2 provides a good alternative in a perspective of further comparison of results and because it has been broadly used in ecotoxicological tests (Bastos *et al*., 2014). This soil was already used by Fajardo *et al.* (2014) who also included the LUFA 2.4 soil to assess effects of $Al_2O_3$ or Ag nanoparticles (0.5 mg/L and 5 g/L, respectively) in soil microbial communities using Fluorescence *In Situ* Hybridization (FISH). Fajardo *et al.* (2014) showed that differences in the structure of microbial community occurred for both contaminants and for both soils.

The soil ecosystem is complex and is one of the most important environments regarding its function and the taxonomic diversity and trophic relations that harbours (Ferreira *et al*., 2015). Soil function and quality can be controlled by many factors, in which the edaphic organisms are included. Thus, when looking at the changes in the soil bacterial community, including an edaphic organism might modify the overall effect of the contaminants. The terrestrial invertebrate *Porcellionides pruinosus*, Brandt 1833 (Crustacea: Isopoda) is considered a good model to evaluate the presence of pollutants in soil (Loureiro *et al*., 2005; Calhôa *et al*., 2006) as AgNPs (Tourinho *et al*., 2015). Furthermore isopods are important for nutrient cycles (Kostanjsek *et al*., 2002). Additionally, isopods have an intrinsic relation with the soil microbiota (Zimmer, 2002). The isopods' digestive tract is a suitable environment for bacterial colonization

(Lapanje *et al*., 2010) therefore, while feeding and decomposing the organic matter, isopods augment the soil microbial activity as well as the spreading of bacteria in the soil (Zimmer, 2002). Though the inclusion of one other edaphic organism (the nematode *Caenorhabditis elegans*) has already been addressed (Fajardo *et al*., 2014), to our knowledge, *P. pruinosus* was not yet used.

Considering the above mentioned, we can hypothesize that AgNPs might cause changes on the soil bacterial communities, that might alter during time of exposure (probably with less impact than the silver cation) and that *P. pruinosus* presence may act antagonistically lowering the pressure of the silver contaminant on the soil bacterial community. Thus, this study aims to investigate the effect of AgNPs, time and *P. pruinosus* on the bacterial communities from the standard LUFA 2.2 soil.

## 2. Material and methods

### 2.1. Experimental design

Exposures were made using LUFA 2.2 soil (Speyer, Germany) with the following properties, according to the manufacturer: pH of 5.5 ± 0.2 (0.01 M $CaCl_2$), 40 ± 3.0% water holding capacity (WHC), 1.61 ± 0.2% organic C, 0.17 ± 0.02% nitrogen, 7.3 ± 1.2% clay; 13.8 ± 2.7% silt and 78.9 ± 3.5% sand (**ANNEX 1**).

Soil was spiked using the AgNPs (AMEPOX, 3-8 nm, 1000 mg/L) or $Ag^+$ (in the form of $AgNO_3$; Sigma-Aldrich; CAS 7761-88-8; Germany; 99.0% pure). Stock aqueous solutions of 1 mg/mL of each silver form (AgNPs or $Ag^+$) were prepared and protected from light due to $Ag^+$ photosensivity (Rana & Kalaichelvan, 2013; Starnes *et al*., 2015).

The soils were moistened up to the 40% of WHC and left to equilibrate for three days before the exposure test (McLaughlin, 2002). Silver was included in the soil at 5 µg/Kg (100 x PEC for AgNPs). For comparison purposes, the same concentration for both silver forms ($Ag^+$ and AgNPs) was used. The same procedure was done including 30 isopods (*Porcellionides pruinosus* Brandt, 1833) per replicate. Isopods were acquired from cultures maintained at the Department of Biology of the University of Aveiro (25 ± 2ºC and 16:8 h light/dark photoperiod) (Loureiro *et al*., 2005). Only health male adults and non-pregnant females (>15-28 mg) were used and juveniles (Ferreira *et al.*, 2015), isopods with abnormalities or without antenna (Tourinho *et al*., 2015) or moulting, were excluded.

The experiment was performed in plastic boxes with 100 g of LUFA 2.2 soil during 56 days in triplicate design. Humidity was restored every 14 days. In the boxes containing *P. pruinosus*, food supply was also guaranteed (10 discs of *Alnus glutinosa* leaves every 14 days). Samples was collected at 0, 42 and 56 days and included non-contaminated soil (control), soil contaminated with each silver form as well as these exposures considering the presence of *P. pruinosus*.

**Table 1**. Sample identification of soil experimental setup.

| Sample ID | Soil treatment | Presence of *P. pruinosus* | Collection time (days) |
|---|---|---|---|
| **CT0** | Non-treated | - | 0 |
| **CT42** | Non-treated | - | 42 |
| **CT42Pp** | Non-treated | + | |
| **NP42** | AgNPs | - | |
| **Ag42** | $Ag^+$ | - | |
| **NP42Pp** | AgNPs | + | |
| **Ag42Pp** | $Ag^+$ | + | |
| **CT56** | Non-treated | - | 56 |
| **CT56Pp** | Non-treated | + | |
| **NP56** | AgNPs | - | |
| **Ag56** | $Ag^+$ | - | |
| **NP56Pp** | AgNPs | + | |
| **Ag56Pp** | $Ag^+$ | + | |

In the table: AgNPs – Silver nanoparticles; $Ag^+$ – Silver cations supplied as $AgNO_3$; "-"- *P. pruinosus* absent; "+"- *P. pruinosus* present.

## 2.2. Analysis of the soil bacterial community

### 2.2.1. Total DNA extraction

Soil (0.25 g) was collected from each replicate and transferred into UltraClean® bead tubes (MoBio Laboratories, Inc., Carlsbad, CA). DNA was extracted using the UltraClean® Power Soil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) following the instruction of the manufacturer.

### 2.2.2. PCR-DGGE

The V3 region of the 16S rRNA gene was amplified using the primers: 338f-GC (5'-GACTCCTACGGGAGGCAGCAG-3') and 518r (5'-ATTACCGCGGCTGCTGG-

3') (MBI Fermentas, Vilnius, Lithuania). The amplification reaction mixture (final volume of 25 μL) contained, besides the DNA template: 0.02 mM dNTPs (MBI Fermentas, Vilnius, Lithuania), 3 mM $MgCl_2$ (Promega, Wisconsin, USA), 1X Green GoTaq® Flexi Buffer (Promega, Wisconsin, USA), 1 U/μL GoTaq® Flexi DNA polymerase (Promega, Wisconsin, USA), 0.25% DMSO (dimethyl sulfoxide; Sigma-Aldrich, for molecular biology, 99.9% purity, Germany), 0.3 μM of each primer (Sigma-Aldrich, Germany) and sterile Mili-Q water. Temperature profile included 5 min at 94°C, followed by 30 cycles of 30 sec at 92°C, 30 sec at 55°C and 30 sec at 72°C, and a final extension for 7 min at 72°C. The reaction was performed in a thermal cycler (TProfessional basic, Biometra, Germany). Negative and positive controls were included in all reactions (**ANNEX 2**).

PCR amplification products were loaded into 8% (w/v) polyacrylamide gels (37.5:1, acrylamide:bisacrylamide) gels with denaturing gradient ranging from 35% to 62.5% [100% denaturant corresponded to 7 M urea and 40% (v/v) formamide]. A DGGE marker composed by 8 bands was included in the extremities of each gel (Henriques *et al*., 2004). The electrophoresis was performed on a D-Code Universal Mutation Detection System (Bio-Rad) with in 1X TAE buffer (Sigma-Aldrich, Germany) at 60ºC in two steps, (1) for 15 min at 20 V and (2) 70 V for 16 h. Gels were stained in a solution of Ethidium bromide (0.5 μg/mL) during 5 minutes and rinsed in water distilled (20 min) and their images captured in a UV transillumination with Molecular Imager FX system (Bio-Rad).

## 2.3. Statistical analysis

GelComparII (AppliedMaths, Sint-Martens-Latem, Belgium) was used to analyse the DGGE profiles. The biological matrix resulting from the analysis of the DGGE gel was produced using Bionumerics v7.1 software (Applied Maths, Belgium). Primer v6 & Permanova+ software (PRIMER-E Ltd., Plymouth, UK) (Clarke and Gorley, 2006) was used to obtain the richness (*S*), diversity (*H'*) and evenness (*J'*) indexes and to construct the dendrogram and the Principal Coordinate Analysis (PCoA) after transforming the data [Log(x+1)] and applying Bray-Curtis similarity.

SigmaPlot v12.0 software was used for statistical analysis of the indexes by one-way analysis of variance (one-way ANOVA, *Dunnett's* method) assuming variances at $p<0.05$.

A similarity analysis (ANOSIM) providing an *R* value was used to test statistical differences on the structure of the bacterial community (*R*=0, no separation of communities' structure occurs; *R*=1, no similarity: separation of communities' structure (Ramette, 2007).

## 3. Results

DGGE analysis of 16S rDNA fragments was used to investigate the effect of AgNPs on soil bacterial community in the presence or absence of isopods at two distinct periods of exposure (42 or 56 days), as depicted in **Figure 1**.

After 42 days, regardless of the presence (CT42Pp) or absence (CT42) of *P. pruinosus*, the soil bacterial community remained structurally similar to that of the beginning of the experiment (CT0) (**Figure 2**). All non-silver exposed bacterial communities (CT0, CT42Pp and CT42) grouped (cluster 1) (**Figure 2**) showing that the time did not affected the structure of the soil bacterial community (ANOSIM, CT0 *vs.* CT42: *R*=0.48; CT0 *vs.* CT42Pp: *R*=0.22; *P*=0.1%; **Table 2**). Even though the presence of *P. pruinosus* caused a slight change in the structure of the soil bacterial community, it was not enough to originate a new cluster (ANOSIM, CT42 *vs.* CT42Pp: *R*=0.63; *P*=0.1%; **Table 2**).

Statistical significant differences were found in the diversity of soil bacterial community after 42 days (CT42) of exposure when compared to the beginning of the experiment (CT0) (one-way ANOVA, CT0 *vs*. CT42; $P=0.02<0.05$, $\alpha=0.05$; **Table 3**).

After 42 days, all silver exposures caused effect in the structure of the bacterial community [all exposed samples were structurally distinct from CT42 (ANOSIM, *R*=1; *P*=0.1%; **Table 2**) and well separated from CT0 (ANOSIM, *R*>0.7; *P*=0.1%; **Table 2**) and CT42Pp (ANOSIM, *R*>0.7; *P*=0.1%; **Table 2**; except for NP42 to which was separable: ANOSIM, *R*>0.5; *P*=0.1%; **Table 2**]. Both, silver contamination and *P. pruinosus* presence, contributed to explain the complete variation on soil bacterial communities across the samples (29.1%) [14.3% for silver contamination (PCO2 axis) and 14.8% for *P. pruinosus* presence (PCO1 axis) (**Figure 2B**)], but silver contamination showed to be the variable causing soil bacterial communities branching (**Figure 2A**) revealing that the additional presence of the *P. pruinosus* enhanced the structural changes on the soil bacterial communities. This means that the structure of the soil bacterial communities was mainly affected by the silver contamination and then by

the presence of *P. pruinosus* (**Figure 2A**) i.e. all soil samples contaminated with silver grouped (cluster 2 and 3) separately from the control (cluster 1) and all samples where *P. pruinosus* was absent (cluster 2) were separated from those where the isopod was included (cluster 3) (ANOSIM, Ag42, NP42, Ag42Pp and NP42Pp when compared to CT42: $R$=1; $P$=0.1%; **Table 2**). Only exposure to silver cation in the presence of *P. pruinosus* (Ag42Pp) revealed a significantly lower diversity ($H'$=3.39 ± 0.13; one-way ANOVA, Ag42Pp *vs*. CT42; $P$=0.047<0.05, α=0.05; **Table 3**) and richness ($S$=39 ± 2; one-way ANOVA, Ag42Pp *vs*. CT42; $P$=0.035<0.05, α=0.05; **Table 3**).

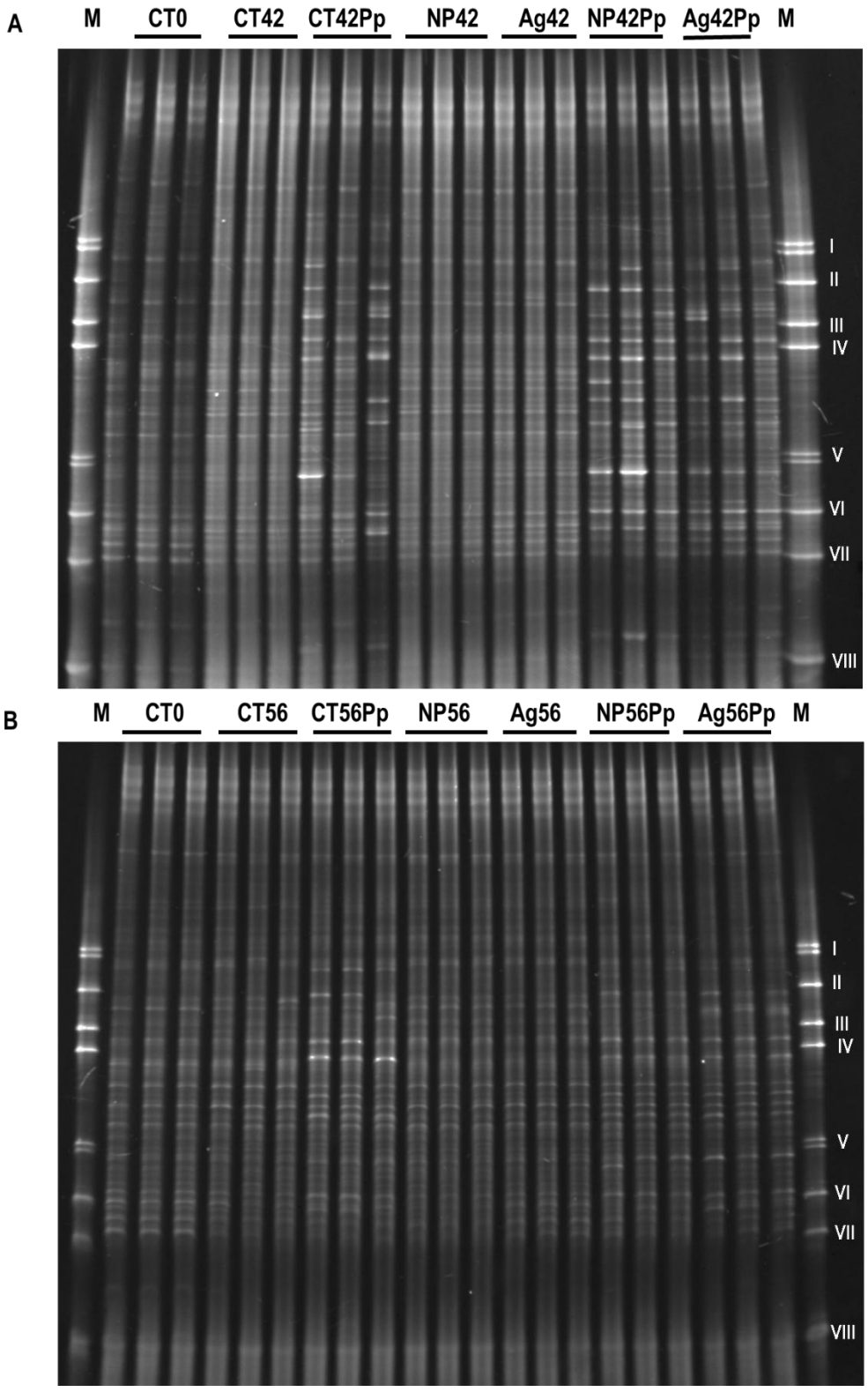



**Figure 1.** PCR-DGGE profile obtained for the soil bacterial community exposed to AgNPs or $Ag^{+}$, during 42 days (A) and 56 days (B). Please see Table 1 for sample identification details. Triplicates of each sample are ordered and grouped. Lane M refers to DGGE marker: I - RAI 70; II - RAN 60; III - RAI 3; IV - RAI 43; V - RAN 18; VI - RAN 12; VII - RAN 140; VIII - RAI 76 (Henriques *et al*., 2004).

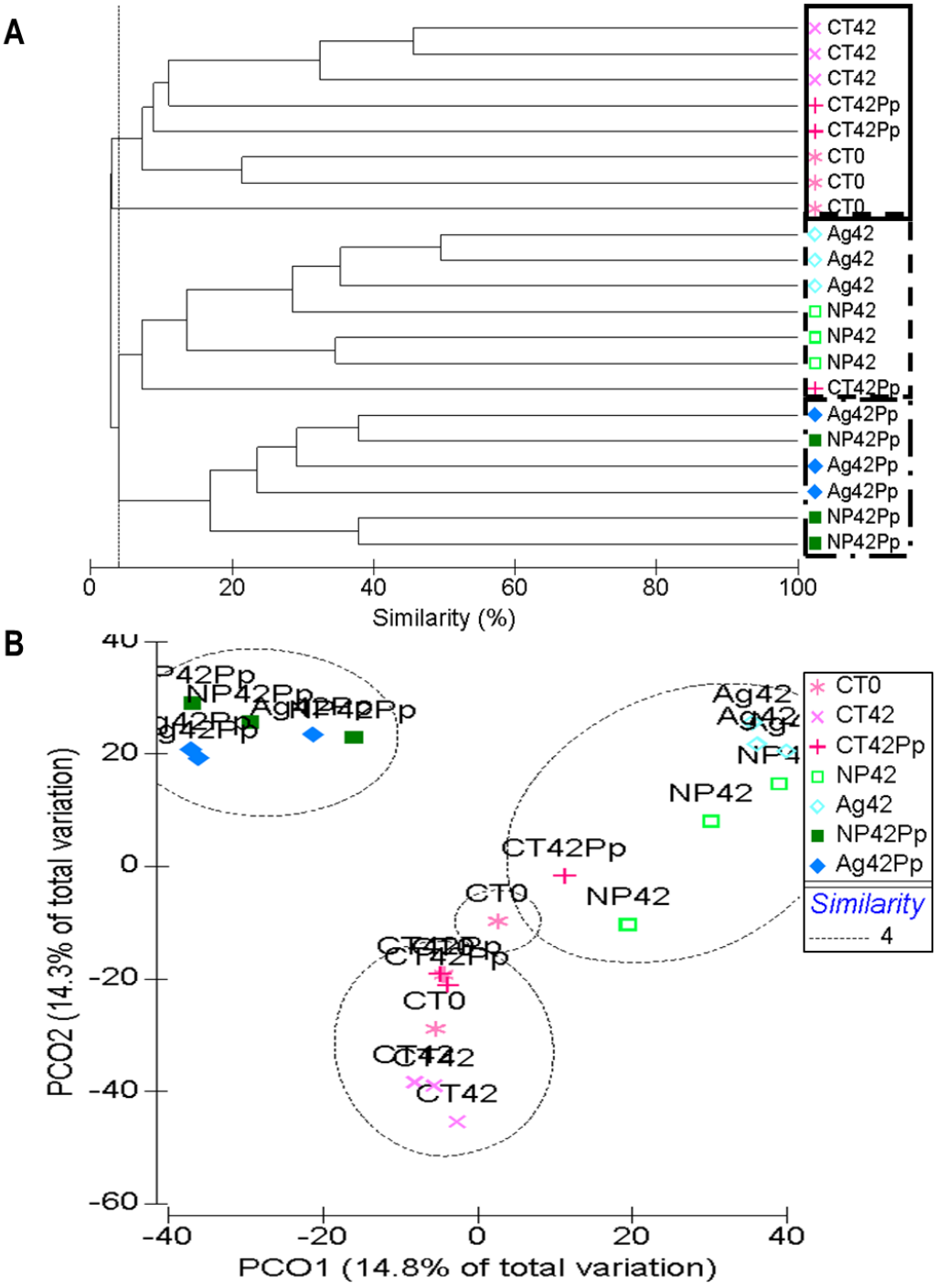



**Figure 2.** Cluster (A) and PCoA analysis (B) of DGGE profiles obtained from the soil bacterial community exposed to AgNPs or $Ag^{+}$ during 42 days. Please see Table 1 for sample identification. For Figure 2A: the cluster mentioned in the text as cluster 1 is delimited by solid line and represents the controls, cluster 2 is delimited by dashed line and represents silver contaminated samples in the absence of *P. pruinosus*; the remaining samples are grouped in cluster 3 which represents silver contaminated samples in the presence of *P. pruinosus*; clusters and PCoA groups were defined at 4% similarity.

**Table 2.** ANOSIM statistical analysis (*R* value; *P*=0.1%) generated from the patterns of bands obtained by DGGE of the soil bacterial community (42 days).

| | Degree of similarity (*R*) | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sample** | **CT0** | **CT42** | **CT42Pp** | **NP42** | **Ag42** | **NP42Pp** | **Ag42Pp** |
| **CT0** | | | | | | | |
| **CT42** | 0.48 | | | | | | |
| **CT42Pp** | 0.22 | 0.63 | | | | | |
| **NP42** | 0.74 | 1 | 0.56 | | | | |
| **Ag42** | 0.82 | 1 | 0.85 | 0.78 | | | |
| **NP42Pp** | 0.82 | 1 | 0.74 | 1 | 1 | | |
| **Ag42Pp** | 0.89 | 1 | 0.82 | 1 | 1 | 0.52 | |

**Table 3.** Richness (*S*) and shannon diversity (*H*') and evenness (*J*') indexes of the soil bacterial community (42 days).

| Soil sample | Richness (*S*) | Shannon diversity (*H'*) | Evenness (*J'*) |
|---|---|---|---|
| **CT0** | 41 ± 1$^{a}$ | 3.45 ± 0.07$^{a}$ | 0.93 ± 0.01$^{a}$ |
| **CT42** | 45 ± 1$^{a}$ | 3.62 ± 0.04$^{b}$ | 0.94 ± 0.02$^{a}$ |
| **CT42Pp** | 44 ± 2$^{a}$ | 3.47 ± 0.15$^{ab}$ | 0.94 ± 0.02$^{a}$ |
| **NP42** | 45 ± 1$^{a}$ | 3.57 ± 0.03$^{b}$ | 0.95 ± 0.01$^{a}$ |
| **Ag42** | 49 ± 2$^{a}$ | 3.67 ± 0.03$^{b}$ | 0.94 ± 0.01$^{a}$ |
| **NP42Pp** | 43 ± 4$^{a}$ | 3.40 ± 0.17$^{b}$ | 0.94 ± 0.01$^{a}$ |
| **Ag42Pp** | 39 ± 2$^{b}$ | 3.39 ± 0.13$^{c}$ | 0.92 ± 0.03$^{a}$ |

Values are means of three replicates ± standard deviation; Different letters within each parameter (richness, diversity and evenness) indicates significant differences towards the respective control according to one-way ANOVA and using *Dunnett's* method.

After 14 additional days of exposure (at day 56) the soil bacterial community naturally changed, even in the absence of *P. pruinosus* (CT56). This time-effect and isopod-effect is depicted from **Figure 3A** where CT0 clustered (cluster 4; **Figure 3A**) separately from CT56 and CT56Pp (cluster 5; **Figure 3A**), which is corroborated by ANOSIM analysis (CT0 *vs.* CT56: *R*=0.63 and CT0 *vs.* CT56Pp: *R*=0.93; *P*=0.1%; **Table 2**). For CT56Pp, a moderate change in the structure of the bacterial community was also observed (ANOSIM, CT56 *vs.* CT56Pp: *R*=0.78; *P*=0.1%; **Table 4**) but was

not sufficient to originate a different cluster and they both grouped in cluster 5 and linked to cluster 6.

Evenness index at 42 days and at 56 days were close to 1 revealing highly uneven soil bacterial communities dominated by few bacterial species. Only in the presence of *P. pruinosus* (CT56Pp), the evenness index decreased and showed statistical differences towards the control (both, CT0 and CT56) (one-way ANOVA, CT56Pp *vs.* CT0, $P$=0.031<0.05 and CT56Pp *vs.* CT56, $P$=0.017<0.05, α=0.05; **Table 5**).

Though it was not possible to observe if the two silver forms (AgNPs or $Ag^+$) caused distinct structural effects of after 42 days, regardless isopod presence or absence (**Figure 2**), time extension of the experiment in 14 days altered this result. Indeed, the soil bacterial communities that were not submitted to *P. pruinosus* were distinctly affected in their structure by AgNPs or by $Ag^+$ [NP56 and Ag56 grouped in different clusters, cluster 5 and 6, respectively (**Figure 3A**); and, NP56 *vs.* Ag56 ANOSIM analysis revealed $R$=0.85; $P$=0.1%; **Table 4**]. Moreover, though NP56 grouped together with CT56 and CT56Pp (cluster 5), the structural modifications induced by the variable time were distinct from those caused by AgNPs contamination (ANOSIM, NP56 *vs.* CT56: $R$=1; NP56 *vs.* CT56Pp: $R$=0.78; $P$=0.1%; **Table 4**).

After 56 days in the presence of *P. pruinosus*, the silver cation revealed no effect on the structure of the soil bacterial communities [Ag56Pp and CT0 grouped in cluster 4 (**Figure 3A**) and Ag56Pp *vs.* CT0 ANOSIM analysis revealed $R$=0.22; $P$=0.1%; **Table 4**]. In opposition, contamination with AgNPs along with isopod organisms (cluster 6), induced structural changes on the soil bacterial communities (ANOSIM, NP56Pp *vs.* Ag56: $R$=0.70; NP56Pp *vs.* NP56: $R$=1; $P$=0.1%; **Table 4**).

Overall, the total variation within the soil bacterial community (23.7%; **Figure 3B**) was explained by the time of exposure, the form of the contaminant (AgNPs or $Ag^+$) and the presence of *P. pruinosus*. Initial branching was caused by time of exposure and then each silver form grouped separately. Moreover, the presence of *P. pruinosus* along with the contamination (AgNPs or $Ag^+$) revealed possible antagonistic effects because these samples clustered apart (Ag56Pp grouped with CT0 in cluster 4 while NP56Pp was in cluster 6). For all exposures (Ag56, NP56, Ag56Pp and NP56Pp), the soil bacterial community evenness significantly decreased in comparison to the control (one-way ANOVA, Ag56 *vs.* CT56, $P$=0.042<0.05; NP56 *vs.* CT56, $P$=0.027<0.05, Ag56Pp *vs.* CT56, $P$=0.028<0.05 and NP56Pp *vs.* CT56, $P$=0.031<0.05, α=0.05; **Table 5**).

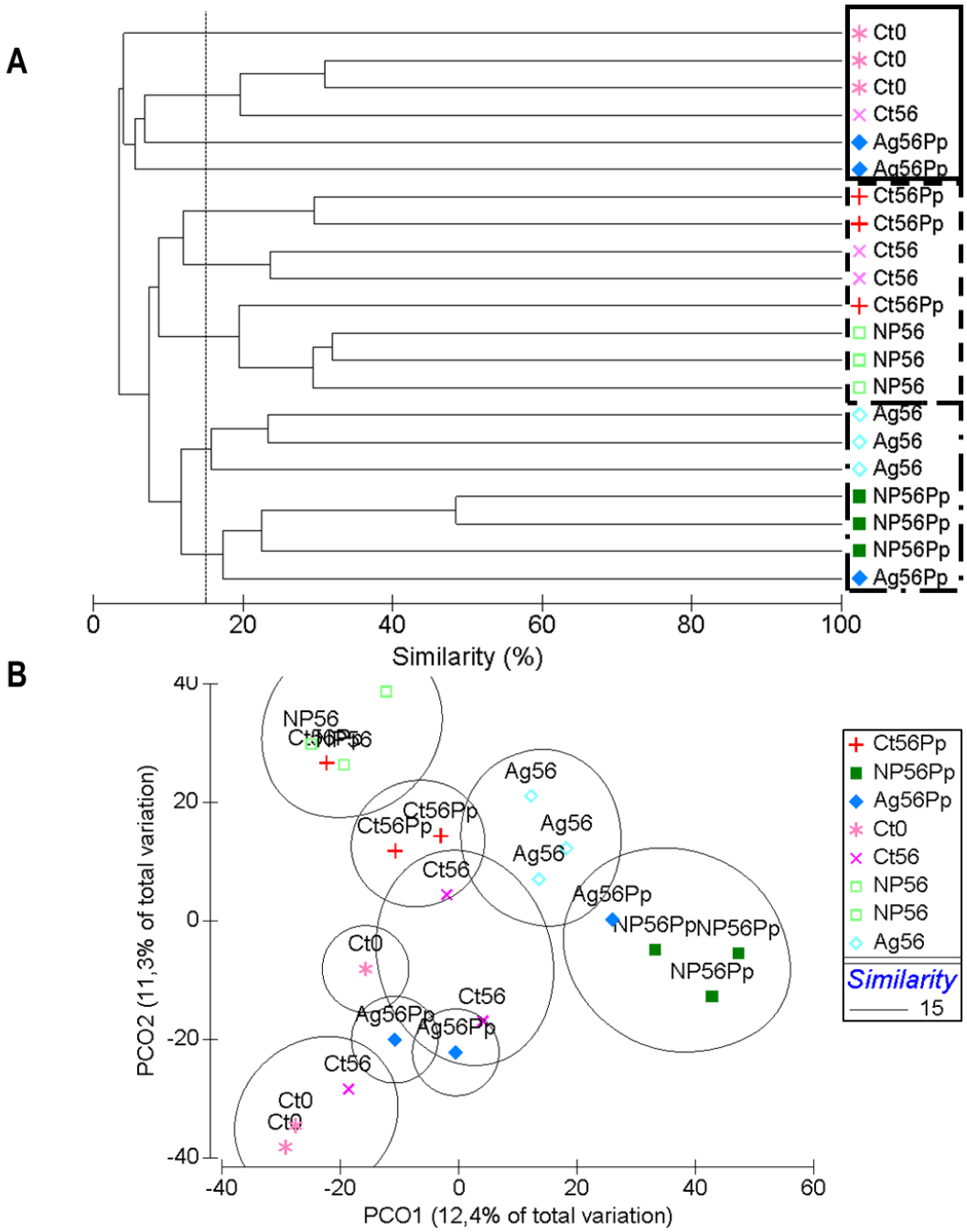



**Figure 3.** Cluster (A) and PCoA analysis (B) of DGGE profiles obtained from the soil bacterial community exposed to AgNPs or $Ag^{+}$ during 56 days. Please see Table 1 for sample identification. For Figure 3A: the cluster mentioned in the text as cluster 4 is delimited by solid line, the cluster 5 is delimited by dashed line and the remaining samples are grouped in cluster 6; clusters in the dendrogram were qualitatively defined for better description in the text; groups in PCoA were defined at 15% similarity.

**Table 4.** ANOSIM statistical analysis (*R* value; *P*=0.1%) generated from the patterns of bands obtained by DGGE of the soil bacterial community (56 days).

| | Degree of similarity (*R*)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | **CT0** | **CT56** | **CT56Pp** | **NP56** | **Ag56** | **NP56Pp** | **Ag56Pp** |
| **CT0** | | | | | | | |
| **CT56** | 0.63 | | | | | | |
| **CT56Pp** | 0.93 | 0.78 | | | | | |
| **NP56** | 0.85 | 1 | 0.78 | | | | |
| **Ag56** | 0.93 | 0.93 | 0.74 | 0.85 | | | |
| **NP56Pp** | 1 | 0.96 | 1 | 1 | 0.70 | | |
| **Ag56Pp** | 0.22 | 0.41 | 0.41 | 0.26 | 0.15 | 0.26 | |

**Table 5**. Richness (*S*) and shannon diversity (*H*') and evenness (*J*') indexes of the soil bacterial community (56 days).

| Soil sample | Richness (*S*) | Shannon diversity (*H'*) | Evenness (*J'*) |
|---|---|---|---|
| **CT0** | 39 ± 2[a] | 3.40 ± 0.06[a] | 0.93 ± 0.01[a] |
| **CT56** | 42 ± 1[a] | 3.14 ± 0.60[a] | 0.93 ± 0.01[a] |
| **CT56Pp** | 42 ± 2[a] | 3.37 ± 0.06[a] | 0.90 ± 0.01[b] |
| **NP56** | 41± 0[a] | 3.38 ± 0.04[a] | 0.91 ± 0.01[b] |
| **Ag56** | 42 ± 1[a] | 3.46 ± 0.05[a] | 0.91 ± 0.01[b] |
| **NP56Pp** | 45 ± 2[a] | 3.46 ± 0.08[a] | 0.91 ± 0.01[b] |
| **Ag56Pp** | 41 ± 1[a] | 3.34 ± 0.10[a] | 0.90 ± 0.01[b] |

Values are means of three replicates ± standard deviation; Different letters within each parameter (richness, diversity and evenness) indicates significant differences towards the respective control according to one-way ANOVA using *Dunnett's* method.

## 4. Discussion

With this study we wanted to know if AgNPs or $Ag^{+}$ affected soil bacterial communities and how this effects changed along the time and if *P. pruinosus* was included.

Indeed, the PCR-DGGE approach confirmed that depending on the time of exposure, distinct results were achieved. After 42 days, silver contamination (regardless of the silver form) and then the presence of *P. pruinosus* were determinant factors deciding the structural changes. On the other hand, after 14 additional days (at day 56), time and isopod presence gained relevance in defining the structure of the soil bacterial

communities. Also, the silver forms showed distinct impacts on the structure of the soil bacterial community exposed for 56 days. Moreover, *P. pruinosus* showed to distinctly impact the soil bacterial community depending on the silver form: $Ag^+$ did not cause structural changes in the soil bacterial communities if the isopods were present. Additionally, the 14 additional days were decisive to observe a decrease in the bacterial evenness as a result of silver contamination. Only the non-exposed communities retrieved at 56 days revealed changes in their structure due to time and *P. pruinosus* presence.

When designing the experiment we wanted to analyze two times of exposure lower than those previously tested and that would provide an idea of how the community response was fluctuating considering the surrounding factors (temperature, humidity, pH, among others) as well as the additional pressure caused by the silver contamination and possible influence of *P. pruinosus*. Because bacteria respond rapidly to environmental changes as well as to stress, we were expecting that soil bacterial community would alter at the first 42 days. Yet this was not observed, and only at 56 days the community changed as a result of time and *P. pruinosus* presence. Because we monitored the temperature, humidity and photoperiod we do not know if any other environmental variable (e.g. pH) could be affecting our experiment. During this period, the isopod might have promoted soil aeration (due to its activity: walking, burying) (El-Wakeil, 2015) which indirectly influenced different bacteria ultimately resulting in a structural change of the community.

In our work, silver contamination (regardless of the form) impacted the structure of soil bacterial communities at 42 days but only after 56 days it was possible to observe that the $Ag^+$ caused distinct effects from those caused by AgNPs in the structure of bacterial community. The distinct effects observed on the soil bacterial communities due to exposure to AgNPs or $Ag^+$ might result from different chemical transformations within the soil that each silver form suffers e.g. sorption, aggregation to soil particles or dissolution in pore water (Klitzke *et al*., 2014; Dwivedi *et al*., 2014). Silver cation caused more distinct structural changes than AgNPs. Another explanation for distinct effects between silver forms is that AgNPs can release lower amounts of silver cations in a prolonged way while the silver cations from the bulk material are released in higher amounts and faster (Settimio *et al*., 2015).

The presence of *P. pruinosus* impacted differently the soil bacterial communities depending on the time of exposure. While at 42 days *P. pruinosus* only impacted the

structure when the silver was present, for 56 days *P. pruinosus* showed to impact both bacterial communities from non-exposed and silver-exposed soil with distinct effects regarding silver forms. However, for 56 days, the structure of the bacterial communities exposed to $Ag^+$ *P. pruinosus* were more similar to the control while for AgNPs the structural changes were more similar to those that occurred for the communities exposed only to the silver.

These results might be explained by occurrence of several processes, acting simultaneously and varying in importance throughout the study.

Even in a healthy soil, bacterial communities are constantly adapting to environmental stress (for instance, temperature, pH as well as the presence of other organisms as *P. pruinosus*). These communities adjust naturally not affecting the structure of the bacterial community (CT0≈CT42≈CT42Pp). Yet, if naturally-occurring stress is prolonged, these effects might result in different community balances. That is why, for the controls, the effects of time on bacteria-isopod interaction were only observed for 56 days (CT0≠CT56≠CT56Pp). Nonetheless, in a context of already altered bacterial communities due to for instance silver contamination, this *P. pruinosus* effect might gain an additional relevance. And that is why the bacterial communities exposed to both forms of silver were grouped according to the presence of *P. pruinosus* (Ag42Pp; NP42Pp). For instance, because silver affected the bacterial communities, preferences of *P. pruinosus* to ingest some bacteria rather than others (Ihnen and Zimmer 2008), might had cause a more pronounced impact on these affected bacterial communities.

On the other hand, it is well known that *P. pruinosus* can ingest and sequestrate several metals [e.g. Cd (Calhôa *et al*., 2006), $Cu^+$ (Golobič *et al*., 2012), $Zn^+$ (Tourinho *et al*., 2013)], besides $Ag^+$ (Tkalec *et al*., 2011) in granules of the hepatopancreas cells thus altering $Ag^+$ bioavailability in soil. Thus, the presence of *P. pruinosus* will decrease the silver cations bioavailable in the soil. Tought the isopod sequestrates within its body silver cations released from both silver forms reducing their bioavailability (Santiago-Martin *et al*., 2015), we cannot exclude the possibility of this contaminant being available for higher consumers (biomagnification).

Because silver cations are more rapidly released from $AgNO_3$ than from AgNPs, the accumulation rate by *P. pruinosus* of $AgNO_3$ is higher for prolonged periods. Thus, the bioavailability of $AgNO_3$ is reduced in soil consequently reducing the pressure of this contaminant. This justifies the grouping of $Ag^+$ exposed bacterial communities with

the control (Ag56Pp≈CT0). This suggests a positive response by bacterial communities to the sequestration of $Ag^+$ by the isopod. In opposite, it is unlikely that a similar pattern occurred to the AgNPs. Indeed, it seems that the pressure of this contaminant was maintained and enhanced by *P. pruinosus*, resulting in a more pronounced structural change (as previously justified).

Contrary to 42 days exposure, distinct structural effects were observed as a result of the two silver forms. This can also be explained by the different released rate of the silver cations from each silver form.

Another interesting result was that all silver exposures at 56 days resulted in a decrease in evenness but still with values near 1. This means that we were in the presence of highly uneven communities dominated by few bacterial species. This scenario is not favourable in a context of environmental stress, because the community is less capable to adapt. After contamination, dominance was reduced suggesting that the dominant groups were not able to adapt to the silver contamination yet this might have a positive outcome as more even bacterial communities might have higher probability to persist in a scenario of multiple contamination.

*P. pruinosus* was the right choice and proved to be suitable to evaluate the effect of AgNPs on the soil bacterial communities. Furthermore, we want to highlight that the presence of the isopod in our experiment [(number of isopods used in accordance to Paolleti & Hassall (1999)] enabled us to study the soil bacterial communities under a more realistic scenario where they naturally interact with the isopod, thus, single tests with bacterial communities might minimize our power to extrapolate the laboratory results (Levard *et al*., 2012). Nonetheless, other organisms must be tested in order to predict impact of AgNPs on a closer to reality scenario (Levard *et al*., 2012; Fajardo *et al*., 2014). Though a clear response was evident for the treatments applied, in future experiments, perhaps composed samples should be considered in order to increase similarity among triplicates. It is also suggested increasing the sampling efforts. Future works should be made in order to clarify the interactive effects of contaminants with *P. pruinosus*. Other molecular analyses such as pyrosequencing would complement this approach and give a better understanding of the effects of these contaminants on bacterial communities.

## 5. Conclusions

This study shows evidence that the response of soil bacterial community to silver contamination is dependent on duration of exposure, the silver form and the presence of *P. pruinosus*. Despite a lack of clear shifts on richness and diversity of soil bacterial communities, evenness and structural changes were evident at 56 days.

We want to highlight that *P. pruinosus* might be useful in minimizing the effects of silver cation at exposures close to 2 months; yet, even in the presence of this isopod, the AgNPs might be a risk for the bacterial communities of the soil compartment.

PCR-DGGE approach might be useful to provide further information about the impacts on the soil bacterial communities when evaluating the environmental hazards of AgNPs.

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# CHAPTER III

SUSCEPTIBILITY OF SOIL BACTERIAL COMMUNITIES TO SILVER (NANOPARTICLE AND CATION)

# SUSCEPTIBILITY OF SOIL BACTERIAL COMMUNITIES TO SILVER (NANOPARTICLE AND CATION)


## Abstract

Silver nanoparticles (AgNPs) are broadly used in several products due to AgNPs' antibacterial properties. However, AgNPs are persistent in the environment therefore soil contamination might hamper natural soil bacterial communities, which plays a fundamental ecological role. Nonetheless, the knowledge about the susceptibility of soil bacterial communities to AgNPs is limited. Thus, this work used an adaptation of the disc diffusion method to assess the susceptibility of soil bacterial communities to AgNPs or silver cation ($Ag^+$) also evaluating the susceptibility responses for different amounts, silver forms joint effect and after previous exposure to silver.

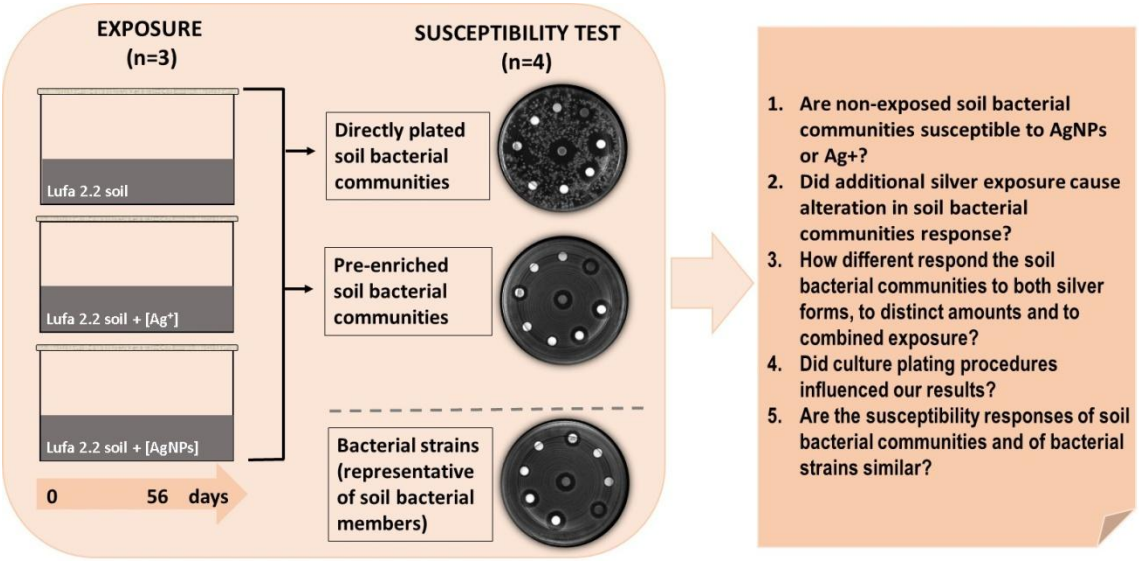
The bacterial communities of LUFA 2.2 soil were exposed to AgNPs or $Ag^+$ (at 5 µg of Ag/Kg of soil) during 56 days. Then, the bacterial communities retrieved from soil were directly plated (dBC) or pre-enriched (pBC) in MHA and their susceptibility was evaluated (using an adaptation of the disc diffusion method). The susceptibility of soil bacterial members (bacterial strains) was also evaluated for comparison purposes. Discs were embedded with each silver form (AgNPs or $Ag^+$), distinct amounts of silver (0.1 µg, 1 µg or 10 µg) or the combination of both silver forms.

All soil bacterial communities (dBC and pBC) as well as tested bacterial strains were susceptible to both silver forms. Overall, our outcomes demonstrated that susceptibility decreased as follows: $Ag^+$ > AgNPs (regarding silver form) and 10 µg > 1 µg > 0.1 µg (regarding silver amount). Also, joint susceptibility response (Combi) was similar to the inibihition on growth caused by adding half of the inhibition caused by each of the highest amounts of each silver form [Combi ≈ ½(10 µg of $Ag^+$) + ½(10 µg of AgNPs)].

This study shows evidence that bacterial communities are susceptible to silver contamination and that, even in lower amounts, a previous exposure to silver might change bacterial susceptibility response.


**Keywords:** silver cation, silver nanoparticle, susceptibility, soil bacterial community, disc diffusion method.

# Graphical abstract



# 1. Introduction

Nowadays, silver nanoparticles (AgNPs) are included in several products: medical devices (Tran *et al*., 2015), textiles, laundry additives, cosmetics, paintings and others (Rai, 2009; Foldbjerg & Autrup 2013). During the life cycle of nano-products and due to inappropriate waste treatment, AgNPs are inevitable released into the environment (Gottschalk *et al*., 2009; Som *et al*., 2010; Rana & Kalaichelvan, 2013), particularly into soil, probably one of the environmental compartments most affected by this contamination (Tourinho *et al*., 2012).

The quality of soil is dependent on microbial balance (Sharma, 2010). Because of this, the antibacterial properties of AgNPs as well as prevalence of AgNPs in the terrestrial environment arouse concerns about the possibility of soil bacterial communities being affected by AgNPs. It is well recognized that the effects of AgNPs are dependent on the nanoparticle characteristics (size, shape and surface area, among others) (Tourinho *et al*., 2015) as well as on the time of exposure (Sierra *et al*., 2015) and concentration (Bondarenko *et al*., 2013) but it is also dependent on the characteristics of the bacterial isolate or bacterial community that it might affect.

The minimum inhibitory concentrations of several antimicrobial agents were described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) but regarding AgNPs there is no available information (Vasileiadis *et al*., 2015). Consequently, several studies have investigated the bacterial susceptibility to AgNPs but focused on bacterial isolates [e.g. *Bacillus subtilis* (Anthony *et al*., 2014), *Staphylococcus aureus* (Tran *et al*., 2015) and *Pseudomonas aeruginosa* (Saeb *et al*., 2014)]. Bacterial groups representative of soil have not yet been used, particularly due to difficult on growing these soil members on culture media. Using soil bacterial representatives is important because we might, in some degree, compare strain and community responses as well as foresee possible impact on the biogeochemical cycles or other function that they might be associated with. *Pseudomonas putida*, for instance, thrives in the rhizosphere providing plant protection and participating in bioremediation of xenobiotic compounds (Gupta *et al*., 2015). *Bacillus* spp. is also common in soil interfering with plant growth and regulating the phosphorus and nitrogen cycles (Hayat *et al*., 2010). *Arthrobacter* spp. is also involved in nitrogen cycling and in soil bioremediation (Westerberg *et al*., 2000).

The bacterial susceptibility is influenced by the several features intrinsic to the bacterial groups with which AgNPs might interact (Klaine *et al*., 2008). Considering, for instance, the distinct composition of the cell membrane of Gram-positive and Gram-negative bacteria, we can hypothesize that these bacteria might respond differently to the presence of silver. Indeed, other studies showed that Gram-positive were more affected and/or susceptible to AgNPs having higher toxicity potential than Gram-negative because of Gram-negative enhanced impenetrability (Losasso *et al*., 2014). Still, some Gram-negative bacteria were reported to be affected by AgNPs namely: *Acinetobacter* (Niakan *et al*., 2013), *Escherichia* (Li *et al*., 2010), *Pseudomonas* (Niakan *et al*., 2013) and *Salmonella* (Losasso *et al*., 2014). Some of the possible mechanisms of silver action that might explain the bacterial susceptibility include the alteration of the permeability of the cell membrane and the production of reactive oxygen species responsible, for instance, for the damage of membranes and inactivation of key enzymes (Klaine *et al*., 2008).

Despite these studies on bacterial isolates, a better understanding is needed about the possible bacterial community responses to this silver contamination (Vasileiadis *et al*., 2015). Furthermore, as a community, the responses of bacterial members might adjust to the community needs as a consequence of external pressures (either environmental changes or *e.g.* the presence of pollutants) showing tolerance/resilience, resistance or functional redundancy (Sharma, 2010).

Considering these knowledge gaps, this study aimed to investigate the bacterial community susceptibility to silver [in different forms (AgNPs or $Ag^+$) and amounts, when the two silver forms act together (combined effect) and if a previous exposure to silver contamination occurred] also comparing it with bacterial members representative of soil. A culture-dependent method, the disc diffusion method, was used to evaluate the inhibitory effect or susceptibility to AgNPs because it is simple, versatile, inexpensive, requires low-tech equipment and the respective outcomes are reliable and reproducible (Matuschek *et al*., 2013).

## 2. Material and methods

### 2.1. Experimental design and sample preparation

Soil contamination with 5 µg/Kg of AgNPs (AMEPOX, 3-8 nm, 1000 mg/L) or $Ag^+$ (in the form of $AgNO_3$; Sigma-Aldrich; CAS 7761-88-8; Germany; 99.0% pure) was done

as previously described (**CHAPTER II**). The experiment was made in triplicate during 56 days (**Table 1**).

**Table 1**. Sample identification of soil experimental setup (n=3).

| Sample ID | Soil treatment | Collection (days) |
|---|---|---|
| **CT0** | Non-treated | 0 |
| **CT56** | Non-treated | 56 |
| **NP56** | AgNPs | |
| **Ag56** | $Ag^+$ | |

In the table: AgNPs – Silver nanoparticles; $Ag^+$– Silver cations supplied as $AgNO_3$.

At day 56, 5 g of soil of each replicate (**Table 1**) was collected and transferred into sterile phosphate buffer solution (PBS, 0.12 M) (1:3, soil:PBS) for posterior homogenization for 30 minutes at 250 rpm, using a shaker (Edmund Bühler GmbH, Germany). Supernatants were then collected into glycerol at 45% (2:1, supernatant:glycerol), fast frozen (nitrogen liquid) and stored at –80ºC for further use.

## 2.2. Disc diffusion method

The disc diffusion method was used to analyze the susceptibility of the cultivable soil bacterial community to silver. The protocol was adapted from (Matuschek *et al*., 2013; Saeb *et al*., 2014) and will be briefly described.

The first step consisted of using Müller-Hinton agar (MHA, **ANNEX 3**) plates for bacterial growth. Thus, the bacterial suspensions previously conserved in glycerol were either directly swabbed (100 µL) in MHA plates (herein after referred as dBC – directly plated soil bacterial communities) or pre-enriched in Tryptic Soy Broth (TSB, **ANNEX 3**) (over-night, 250 rpm, 26ºC) and then swabbed (100 µL) in MHA plates (herein after referred as pBC – pre-enriched soil bacterial communities). Furthermore, in order to compare the susceptibility responses of the soil bacterial community with that of bacterial groups, the following bacterial strains were also included: *Bacillus sphaericus* ATCC 29726, *Pseudomonas putida* NCIMB 10432 and *Arthrobacter arilaitensis* GCNP1_I.

These bacterial strains were chosen because they are well known and assumed to be abundant, or to perform important ecological functions, in soils (Fajardo *et al*., 2014; Janssen, 2006). *Escherichia coli* ATCC 25922 served as quality control of the method (Matuschek *et al*., 2013). All strains were pre-cultured in TSB and growth exponential phase was confirmed by spectrophotometry before being swabbed (100 µL) in MHA plates.

The second step consisted in preparing the sterile filter paper discs. Each disc (6 mm) was loaded with 10 µL of the respective silver amount to be tested: 10 µg of AgNPs or $Ag^+$ (representing the silver nanoparticle and silver cation stock solutions, each at 1 mg/mL), 1 µg or 0.1 µg of each silver form (these amounts represent two serial dilutions of each silver stock solution) and, finally, a mixture of 5 µg of AgNPs with 5 µg of $Ag^+$ (added as equal volumes of each silver stock solution). Loaded discs were then placed in MHA plates (previously swabbed) (**Table 2**). The negative control consisted of PBS for dBC and pBC while TSB was used for plates containing bacterial strains (**Table 2**). The positive controls included the antibiotic ticarcillin-clavulanic acid (TIM) which was used for most assays and chosen due to broad spectrum and the antibiotic imipenem (IMI) only used for *Pseudomonas* spp. because this bacterial strain was not affected by TIM (**Table 2**).

Susceptibility tests were made under aseptic conditions in quadruplicate and incubation occurred for 24 h at 25ºC (Saravanan *et al*., 2011) (**ANNEX 5**). At the end of incubation, and for each disc, the diameter of the zone of growth inhibition (ZoI) was measured to estimate silver inhibitory effects and bacterial susceptibility.

**Table 2.** Identification of the discs used in the susceptibility testing (n=4). (See also **ANNEX 5**).

| Disc ID | Disc content | Loaded amount (µg) |
|---|---|---|
| **$NP_0$** | AgNPs | 10 |
| **$NP_{-1}$** | AgNPs | 1 |
| **$NP_{-2}$** | AgNPs | 0.1 |
| **$Ag_0$** | $Ag^+$ | 10 |
| **$Ag_{-1}$** | $Ag^+$ | 1 |
| **$Ag_{-2}$** | $Ag^+$ | 0.1 |
| **Combi** | AgNPs + $Ag^+$ | 5 (of each) |
| **PBS** | PSB or TSB | N/A |
| **Antibiotic** | TIM or IMI | 85 |

In the table: AgNPs – Silver nanoparticles; $Ag^+$ – Silver cations supplied as $AgNO_3$; Combi – represents a mixture of equals amounts of AgNPs and $Ag^+$; PBS – Phosphate Buffer Solution; TSB – Tryptic Soy Broth; TIM – Antibiotic ticarcillin-clavulanic acid; IMI – Antibiotic imipenem; N/A – not applicable. Final volume within each disc was 10 µL.

## 2.3. Statistical analysis

Data resulting from susceptibility testing, representing the diameters of ZoI, were expressed as mean ± standard deviation (of the four replicates).

Statistical differences were assessed using SigmaPlot v12.0 software. Statistical comparisons were only performed among data that corresponded to the same amount of silver loaded in the discs.

Within each soil treatment (CT0 or CT56 or NP56 or Ag56), the same amount of silver was used to statistically compare susceptibility between the silver forms (AgNPs and $Ag^{+}$) using the one-way analysis of variance (one-way ANOVA, *Dunnett's* test) assuming variances at $p<0.05$.

Between soil treatments (CT0 *vs.* CT56; CT56 *vs.* Ag56; CT56 *vs.* NP56; NP56 *vs.* Ag56), the same amount of silver was used to statistically compare susceptibility not only between previous exposure to silver but also to compare between the silver forms (AgNPs and $Ag^{+}$) (e.g. 10 µg Ag56 *vs.* 10 µg NP56 among NP56 and Ag56 towards CT56) using the two-way analysis of variance (two-way ANOVA) assuming variances at $p<0.05$.

A similar statistical analysis was done for the bacterial strains susceptibility testing.

# 3. Results

## 3.1. Non-exposed soil microbial communities

Except for 0.1 µg of AgNPs and 1 µg of AgNPs at the beginning of the experiment (CT0), all exposures caused growth inhibition of the non-exposed bacterial communities (controls) showing that soil bacterial communities were susceptible to a first silver contamination via discs. Regardless of the plating technique [dBC (**Figure 1A**) or pBC (**Figure 1B**)], the response of the non-exposed bacterial community was similar. Nonetheless, it was possible to observe that distinct bacterial communities were being tested in the dBC and pBC plates as depicted from the distinct uniformity and abundance of the bacterial cultures in the plate as well as the distinct aspect of colonies (**Figure S1**). This explains higher standard deviation values for dBC than for pBC (**Figure 1A**). Furthermore, except for 1 µg of AgNPs, the susceptibility response of the soil bacterial

communities did not changed along the experiment with non-contaminated soil at the beginning (CT0) and end (CT56) not showing significant differences (**Figure 1**). Therefore, the non-contaminated soil that was collected after 56 days (CT56) was considered for the subsequent analysis (showed in 3.2).

Within each silver form treatment, an amount-dependent response was observed meaning that higher amounts of AgNPs or $Ag^+$ resulted in higher growth inhibition. Overall, for both dBC and pBC, the susceptibility to each silver form decreased as follows: 10 µg > 1 µg > 0.1 µg. Comparison of the inhibition zones induced by AgNPs or $Ag^+$ revealed that (for the same silver amount) silver cation was more effective in inhibiting both the dBC and pBC. Also, silver cation was effective for a broader range of silver amount (from 0.1 -10 µg while AgNPs was only for 1-10 µg) (**Figure 1**).

Moreover, for both dBC and pBC, inhibition zones induced by equal amounts of AgNPs and $Ag^+$ (Combi, **Table 2**) revealed to be similar to the inibihition zones caused by adding half of the inhibition caused by each of the highest amounts of each silver form [Combi ≈ ½(10 µg of $Ag^+$) + ½(10 µg of AgNPs)] (**Figure 1**).

The susceptibility test was validated by the positive and negative controls. As expected, no inhibition was observed for the negative control. Regarding positive control, the inhibition zone was closer to 15 mm, for both dBC and pBC (**Figure 1**).

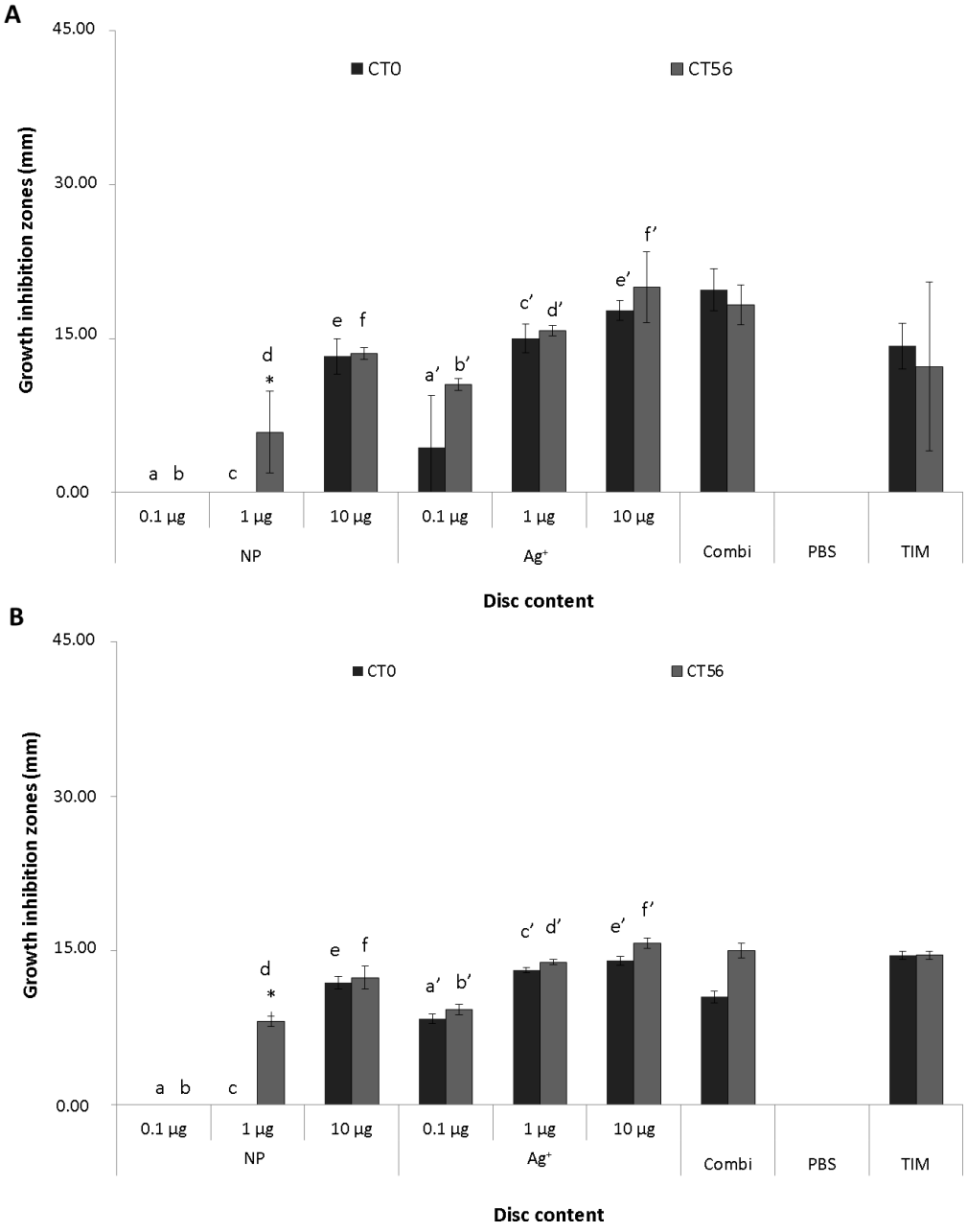



**Figure 1**. Susceptibility of soil bacterial communities (CT0 and CT56) not exposed to silver: **(A)** dBC (directly plated bacterial communities) and **(B)** pBC (pre-enriched bacterial communities). Please see **Table 1** and **2** for sample identification. Data represent diameters mean ± standard deviation (n=4). (*) Statistical differences between soil treatments (CT0 *vs.* CT56), for the same silver amount, according to one-way ANOVA (*Dunnett's* test) for $p<0.05$. Different letters (*e.g.* a and a') corresponds to significant differences, for the same silver amount, within soil treatments (*e.g.* within CT0 or within CT56) and between silver forms (AgNPs *vs.* $Ag^+$) $p<0.05$ (two-way ANOVA).

### 3.2. Soil microbial communities previously exposed to silver

To evaluate if the susceptibility response of the bacterial communities was maintained or altered if a previous exposure via soil to silver forms occurred (NP56 or Ag56), these communities were also submitted to the susceptibility testing and compared to non-exposed bacterial communities (CT56).

Contrary to what was observed to the non-exposed communities (CT0 and CT56, **Figure 1**), the communities previously exposed to silver forms (NP56 and Ag56) revealed distinct susceptibility responses depending on the plating techniques used (dBC and pBC).

For the dBC, only the exposure to 0.1 µg of NP induced an increase of susceptibility in the bacterial community previously exposed to silver cation (Ag56), (**Figure 2A**) while bacterial communities previously exposed to AgNPs (NP56), showed, regardless of the amount, a decrease in the inhibition zones if the second exposure was to $Ag^+$ (0.1, 1 and 10 µg) (**Figure 2A**).

On the other hand, for the pBC, the susceptibility responses were maintained after previous exposures to silver, except for 1 µg of AgNPs for which no inhibition was observed revealing that the bacterial communities might have become tolerant to this amount of AgNPs (**Figure 2B**).

Overall, and similarly to what was observed for non-exposed soil bacterial communities (CT0 and CT56, **Figure 1**), for both dBC and pBC, the susceptibility response after previous exposure to each silver form decreased as follows: 10 µg > 1 µg > 0.1 µg (**Figure 2**). Regardless of previous exposure, silver cation was still more effective than AgNPs (comparing the same silver amount) in inhibiting both the dBC and pBC, as depicted from **Figure 2**.

Regardless of previous exposure, combined contamination of the discs with AgNPs and $Ag^+$ (Combi, **Table 2**) continued to give similar growth inibihition to the one caused by adding half of the inhibition caused by each of the highest amounts of each silver form [Combi ≈ ½(10 µg of $Ag^+$) + ½(10 µg of AgNPs)] (**Figure 2**).

The susceptibility test was validated by the positive and negative controls that showed inhibition zone closer to 15 mm (for both dBC and pBC) or no inhibition, respectively (**Figure 2**).

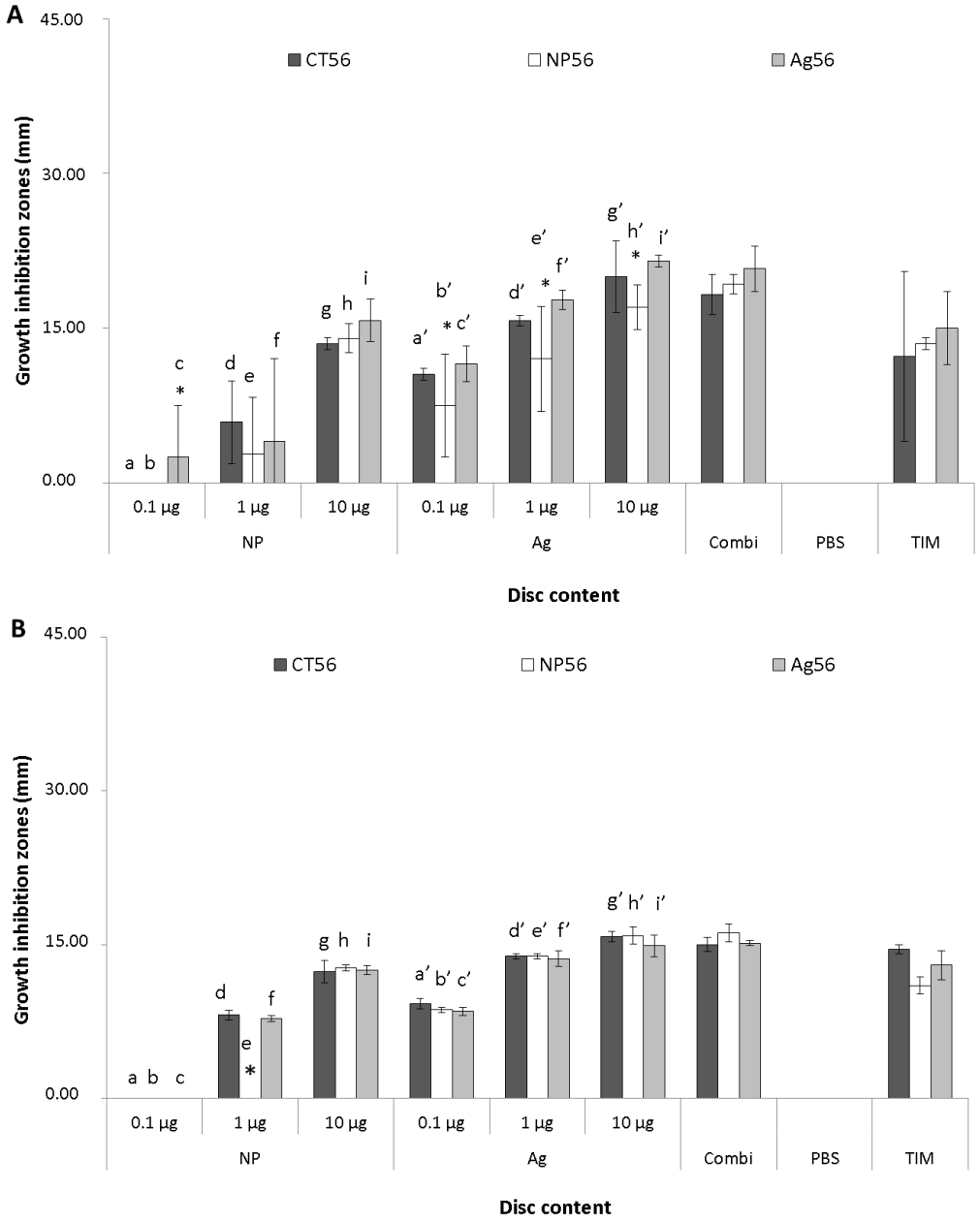



**Figure 2**. Susceptibility of soil bacterial communities previously exposed to silver forms (NP56 or Ag56): **(A)** dBC (directly plated bacterial communities) and **(B)** pBC (pre-enriched bacterial communities). Data relative to non-exposed soil bacterial communities from the end of the experiment (CT56 for dBC and CT56 for pBC) was maintained for comparison purposes. Please see **Table 1** and **2** for sample identification. Data represent diameters mean ± standard deviation (n=4). (*) Statistical differences between soil treatments (CT56 *vs.* NP56 or CT56 *vs.* Ag56), for the same silver amount, according to one-way ANOVA (*Dunnett's* test) for $p<0.05$. Different letters (*e.g.* a and a') correspond to significant differences, for the same silver amount, within soil treatments (*e.g.* within NP56 or within Ag56) and between silver forms (AgNPs *vs.* $Ag^+$) $p<0.05$ (two-way ANOVA).

## 3.3. Bacterial groups representative of soil bacterial community

In order to analyze if the susceptibility of the retrieved soil bacterial communities was similar to the susceptibility of well-known bacterial groups, representatives of soil, three strains were considered: *Bacillus sphaericus* ATCC 29726, *Arthrobacter arilaitensis* GCNP1_I and *Pseudomonas putida* NCIMB 10432.

Only the highest tested AgNPs amount (10 µg of AgNPs) was capable of inhibiting the growth of *B. sphaericus* [ZoI(10 µg of AgNPs)$_{B.\ sphaericus}$ = 13 mm]. *A. arilaitensis* and *P. putida* were only susceptible to the two highest amounts of AgNPs (10 and 1 µg) with *P. putida* being the less susceptible of both (**Figure 4**). Of all bacterial strains, *P. putida* was the less susceptible for the two highest $Ag^+$ amounts (10 and 1 µg) but for 0.1 µg of $Ag^+$ this bacterial strain was the only affected [ZoI(0.1 µg of $Ag^+$)$_{P.\ putida}$= 9 mm] (**Figure 4**).

Overall, the bacterial strains showed decrease susceptibility to each silver form as follows: 10 µg > 1 µg > 0.1 µg (**Figure 4**). Regardless of the bacterial strain, silver cation was more effective in inhibiting the bacterial growth than AgNPs (comparing the same silver amount), as shown in **Figure 4**.

Combined contamination with AgNPs and $Ag^+$ (Combi, **Table 2**) induced similar growth inibihition to the one caused by adding half of the inhibition caused by each of the highest amounts of each silver form [Combi ≈ ½(10 µg of $Ag^+$) + ½(10 µg of AgNPs)] (**Figure 4**).

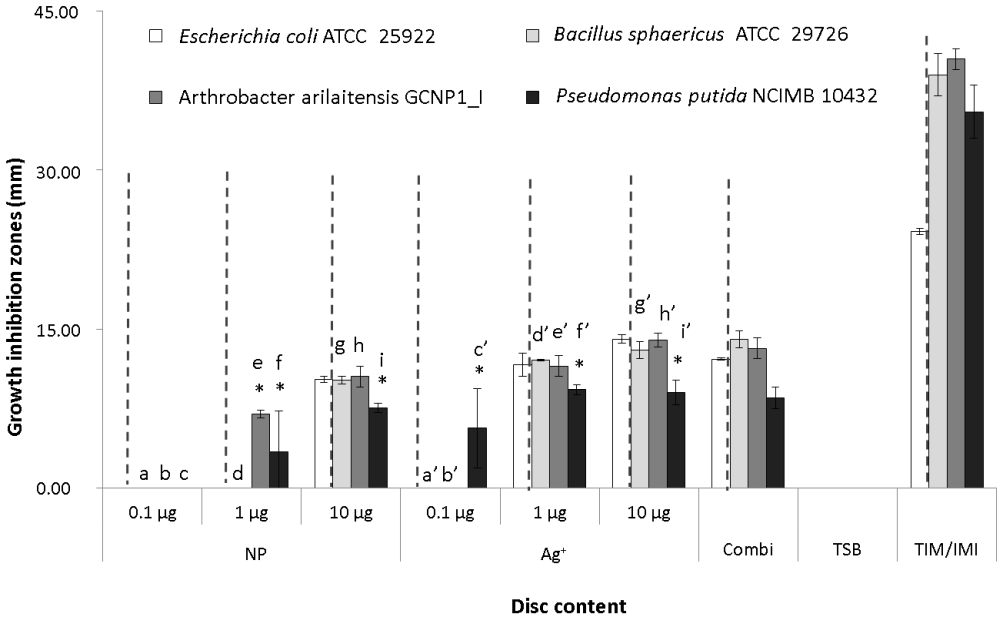



**Figure 3**. Susceptibility of bacterial strains to silver exposure. In the figure: *Escherichia coli* ATCC 25922 was only used as control and is separate from the tested bacterial strains using a dashed line; Antibiotic imipenem (IMI) was only used for *Pseudomonas putida*. Please see **Table 2** for sample identification. Data represent diameters mean ± standard deviation (n=4). (*) Statistical differences among bacterial strains (*Bacillus sphaericus* ATCC 29726 *vs. Arthrobacter arilaitensis* GCNP1_I *vs. Pseudomonas putida* NCIMB 10432), for the same silver amount, according to one-way ANOVA (*Dunnett's* test) for $p<0.05$. Different letters (*e.g.* a and a') corresponds to significant differences, for the same silver amount, within each bacterial strain (*e.g.* within *Bacillus sphaericus* ATCC 29726 or within *Arthrobacter arilaitensis* GCNP1_I or within *Pseudomonas putida* NCIMB 10432) and between silver forms (AgNPs *vs.* $Ag^+$), $p<0.05$ (two-way ANOVA).

## 4. Discussion

To gain a better understanding of the impact of AgNPs on soil bacterial communities, both the non-exposed and the silver exposed soil bacterial communities were investigated. Doing so, we wanted to understand if non-exposed soil microbial communities (CT0 and CT56) showed susceptibility to the AgNPs or to $Ag^+$ and if previously exposed soil microbial communities (NP56 and Ag56) maintained or altered their response after an additional silver exposure via diffusion from discs. In addition, we wanted to evaluate how distinctly the soil microbial community responded to both silver forms and to the three chosen silver amounts. We were also interested in understanding if our culture procedure could influence our results [thus we used both direct plating (dBC) and plating after recover (pBC)] and finally, if the susceptibility response of the microbial community was similar to that of bacterial groups representative of soil.

Overall, we observed that: (1) non-exposed bacterial communities showed susceptibility to both silver forms and duration of the experiment did not changed their susceptibility response (CT0≈CT56); (2) previous exposure to silver altered some of the susceptibility responses; (3) for the same silver amount, $Ag^+$ was more effective in inhibiting growth than AgNPs ($Ag^+$ > AgNPs) while equal amounts of both silver forms (Combi) revealed to induce similar inibihition to the one caused by adding half of the inhibition caused by each of the highest amounts of each silver form [Combi ≈ ½(10 µg of $Ag^+$) + ½(10 µg of AgNPs)]; (4) the susceptibility to each silver form decreased as follows: 10 µg > 1 µg > 0.1 µg; (5) distinct plating techniques (dBC or pBC) only showed different susceptibility responses for bacterial communities that were previously submitted to silver contamination (NP56 and Ag56); (6) comparing bacterial communities to bacterial strains, the same pattern of susceptibility regarding silver amount (10 µg > 1 µg > 0.1 µg), silver form ($Ag^+$ > AgNPs) and joint response of both silver forms [Combi ≈ ½(10 µg of $Ag^+$)+ ½(10 µg of AgNPs)] was observed.

It is well recognized that effects of silver contamination is dependent on the characteristics of: (1) the contaminant (Tourinho *et al*., 2015), (2) the bacterial target, (3) the surrounding media [if soil: organic matter content, pH, type of soil, pore water (Bondarenko *et al*., 2013)] and (4) time (Sierra *et al*., 2015) and (5) amount of exposure (Bondarenko *et al*., 2013).

Despite absence of silver contamination on soil (CT0 or CT56), we observed that the bacterial communities were susceptible to silver.

Herein we analyzed the bacterial susceptibility to engineered AgNPs but the same method has been used to test green AgNPs (green synthesis). Some studies showed that the silver cation was less toxic than AgNPs (Anthony *et al*., 2014; Arokiyaraj *et al*., 2014), another study revealed the opposite (Swain *et al*., 2014) while one other reported similar bacterial susceptibility pattern for both silver forms (Bose & Chatterjee, 2015). Although these studies considered nanoparticles with distinct characteristics, their controversial conclusions regarding toxicity comparison between silver forms induced us to expect that any of those outcomes could be obtained from the bacterial communities (dBC and pBC) herein tested. In fact, in our work, higher susceptibility to silver cation than to AgNPs was observed. The antibacterial activity of AgNPs is due to their dissolved fraction i.e. the silver cations that AgNPs releases in a slow but prolonged way (Engelke *et al*., 2014) when

compared to $AgNO_3$ (Settimio *et al*., 2015). On the other hand, $Ag^+$ (provided as $AgNO_3$) has higher biological reactivity (meaning that contact with the microorganisms is improved) being less persistent in the surrounding environment (Choi *et al*., 2008; Losasso *et al*., 2014). Thus, during incubation time (24 h), the $AgNO_3$ released silver cations faster than AgNPs, consequently interacting quickly with the surrounding bacterial communities and therefore inhibiting their growth. The same pattern was observed for the bacterial strains. Similarly, Dorobantu *et al*. (2015) showed that silver cations (also provided as $AgNO_3$) were more effective than capped AgNPs in inhibition the bacterial growth of different strains (*Pseudomonas aeruginosa* (ATCC 27317); *Staphylococcus aureus* (ATCC 25923) and *Saccharomyces cerevisiae*).

Regarding the amount of silver, our study is in line with what was previously reported by El. Rafie *et al*. (2014) and Zarhan *et al*. (2014), both showing that not only the amount of silver was an important factor for the bacterial susceptibility but also that susceptibility increases with higher amounts of silver.

Susceptibility testing already contemplated some combined exposure. Studies about the combined effect of AgNPs and ultraviolet radiation (UVA) (Zhao *et al*., 2013) and of AgNPs and antibiotics (Thomas *et al*., 2014) were already addressed. For both studies the antimicrobial power was enhance due to this interactions. However, little is known about the combined effect of AgNPs and $Ag^+$ (provided as $AgNO_3$) because most susceptibility tests usually consist of analysing individually each silver form. Nonetheless, analysing the combined effects of both silver forms is of upmost importance because in natural environment both silver forms occur and interact possibly inducing distinct consequences in the microbiota from those that might occur in consequence of single exposure. If we take into account the synergetic effects observed in those investigations (Zhao *et al*., 2013; Thomas *et al*., 2014) we were expecting that a similar result would occur for combined effect between $Ag^+$ and AgNPs. Indeed, our work showed that addition effect between $Ag^+$ and AgNPs occurred because the sum of half of the inhibition growth given by 10 µg of each silver form corresponded to the total inhibition obtained for the combined exposure [Combi ≈ ½(10 µg of $Ag^+$) + ½(10 µg of AgNPs)].

Variation of the susceptibility of the soil bacterial community might be explained due to capability to respond and/or recover (or not) in their performance and composition after contact with a disturbance, which in this case was the $Ag^+$ or the AgNPs. However,

the degree to which the community is susceptible is dependent on the initial composition of the bacterial community inducing a response of resistance, resilience or tolerance and functional redundancy (Allison & Martiny, 2008; Sharma, 2010). It was previously reported that pre-enrichment with TSB can underestimate the diversity of bacteria (Jackson *et al*., 1998). Indeed, some microorganisms might be more capable to growth in detriment of others under TSB culture conditions thus resulting in a more homogeneous culture in the plate. Thus, in our study, by using distinct plating techniques, we analyzed distinct microorganisms retrieved form soil. Because we were analyzing communities with different composition, it was expected distinct results between dBC and pBC (Allison & Martiny, 2008). Comparing non-exposed soil bacterial communities (CT0 and CT56) with silver pre-exposed soil bacterial communities (NP56 and Ag56) we noticed more pronounced differences between dBC and pBC for the communities that had previously suffered silver contamination (i.e. NP56 and Ag56). This might be because, for CT0 and CT56, differences in the community were only due to plating methods while for NP56 and Ag56 were also due to the initial variation of the soil community (altered by silver). Indeed, once in the soil AgNPs can chemically transform by releasing silver cations and further undergo sorption, aggregation and accumulation processes (Klitzke *et al*., 2014; Dwivedi *et al*., 2014) becoming differently bioavailable. Though chemical stress might result in less adapted bacteria (Camargo *et al*., 2005) thus, in more susceptible bacteria, we observed that previous exposure to silver induced distinct susceptibility responses depending on the form and amount of silver. For instance, in the case of dBC, only when the first contamination was due to silver cation, bacteria turn less susceptible to all amounts of $Ag^+$ but when first exposure was done by AgNPs, bacteria turn more susceptible to 0.1 µg of AgNPs. On the contrary, in the case of pBC, only contamination with the 1 µg of AgNPs showed not to cause growth inhibition.

When comparing susceptibility responses between the soil bacterial communities and bacterial strains representing soil bacterial members, consensus in the outcomes were regarding silver amount (10 µg > 1 µg > 0.1 µg), silver form ($Ag^+$ > AgNPs) and joint response of both silver forms [Combi ≈ ½(10 µg of $Ag^+$) + ½(10 µg of AgNPs)]. However, interestingly, the values of growth inhibition were higher for the bacterial strains than for the dBC and pBC. This might be explained due to the higher diversity of the soil bacterial communities which gather distinct bacterial members capable of tolerating or being

susceptible to silver while for the bacterial strains the response is the result of a single group (either susceptible or tolerant).

Bacteria have several possible mechanisms against AgNPs (Choi *et al*., 2008). Bacterial susceptibility might depend on the membrane composition (Anthony *et al*., 2014). So, it is expected that Gram-negative might be less susceptible than Gram-positive bacteria (Negi *et al.,* 2013). Nonetheless, in our work, this was not always the case and was not consistent along all silver amounts. *A. arilaitensis* and *B. sphaericus*, which are Gram-positive, showed similar responses for all silver exposures, except for 1 µg of AgNPs where *B. sphaericus* was not susceptible. On the other hand *P. putida*, a Gram-negative strain, resulted in a distinct response: this strain was always less susceptible than the Gram-positive bacteria, except for 1 µg of AgNPs and 0.1 µg of $Ag^+$. Interesting information was reported by Gambino *et al.* (2015) showing that *B. subtilis* were activated by low AgNPs concentrations by promoting activities as inorganic phosphate solubilisation. So, a similar approach might be being used by *B. sphaericus.* It was described that *P. putida* uses efflux pumps to expel the silver cations from the cell (Yang *et al*., 2012). However, efflux pumps activity can be hampered by production of reactive oxygen species due to silver presence. This disruption occurs by interaction of the bacteria with $Ag^+$ and consequently, the bacteria lose its membrane integrity leading to cell lysis (Abdel-Aziz *et al*., 2013). Therefore, it is understandable that *P. putida* revealed susceptibility for such lower $Ag^+$ amounts as 0.1 µg.

Cultivable methods only allow analyzing viable cells screening a minor percentage (1%) of the bacterial community that effectively occur in soils (Sharma, 2010). Nonetheless, this method proved to be valuable to detect the effect of anthropogenic contaminants as AgNPs and $Ag^+$.

Similar studies have reported silver effects on bacteria for higher amounts than those herein tested (Fajardo *et al*., 2014). Though these works are valuable, their outcomes are difficult to be extrapolated into real scenario. Also, to our knowledge, analysis of the susceptibility of bacterial communities previous exposed to silver was never tested before. Furthermore, our approach is innovative because it included susceptibility analysis at the community level retrieved from soil and not only bacterial strains. These premises were used in the attempt to provide a scenario as close as possible to the reality.

We wanted to highlight that it is important to assess the impact of AgNPs in soil bacterial communities, considering their crucial role for soil ecosystem balance and health.

## 5. Conclusions

Overall, $Ag^+$ induced higher bacterial susceptibility response than AgNPs. Another bacterial susceptibility pattern was that the highest the silver amount, the highest the susceptibility (10 µg > 1 µg > 0.1 µg). Also, joint susceptibility response of both silver forms revealed to correspond to addition of suscetibilities of half of the single exposure to the highest amounts of each silver form [Combi ≈ ½(10 µg of $Ag^+$) + ½(10 µg of AgNPs)].

Our results reinforces that soil bacterial communities are susceptible to silver contamination and that, bacterial communities previously exposed to silver might change their susceptibility towards silver even if low amounts of silver are present.

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# 8. Supplementary data

## 8.1. Figures

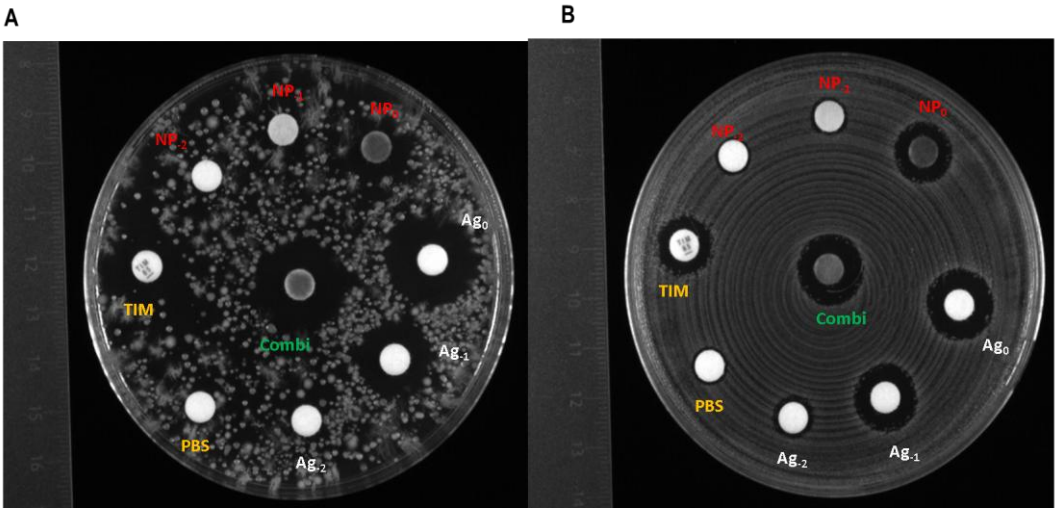


**Figure S1**. Plates of cultured bacterial communities not exposed to silver (CT56) after testing response to silver forms and amounts: **(A)** dBC (directly plated bacterial communities) and **(B)** pBC (pre-enriched bacterial communities). Please see **Table 1** and **2** for sample identification. See also **ANNEX 5**.

# CHAPTER IV
FINAL DISCUSSION AND CONCLUSIONS

# 1. Final discussion and conclusions

The overall goal of this master thesis was to investigate if the soil bacterial community was affected/changed as a result of AgNPs exposure. This was justified because AgNPs are non-biodegradable and have antimicrobial activity being constantly produced and embedded in daily products as well as continuously released into the soil where bacterial communities play an essential ecological role in biogeochemical cycling (VandeVoort *et al*., 2014). The main aim of this thesis was achieved by using: PCR-DGGE (culture-independent) and disc diffusion (culture-dependent) methods.

Both methodologies used the same environmental sample, yet their results cannot be compared to each other (Lapanje *et al*., 2012) but rather seen as complementary methods done to obtain a more robust outcome. Indeed, PCR-DGGE showed the impact of silver contamination regarding the structure, diversity, richness and evenness of the soil bacterial community while the disc diffusion method revealed the effect of silver contamination by means of growth inhibition. Though both methodologies cannot fully describe the soil bacterial communities, the small part that each method represents is very important. In the PCR-DGGE, silver effects were analyzed in both the viable and not viable cells, while in the disc diffusion method only the viable bacterial members retrieved from the soil were considered. The PCR-DGGE gives higher resolution in terms of bacterial diversity yet the interpretation of results requires attention due to the drawbacks of this technique (Vaz-Moreira *et al*., 2011). Indeed, natural-occurring microorganisms might be efficiently detected by culture-independent methods which may gain advantage during the DNA extraction and PCR amplification stages. On the other hand, natural-occurring microorganisms are sometimes fastidious in their nutritional requirements being difficult to represent through culture methodologies (Shameli *et al*., 2012; Matuschek *et al*., 2013). Moreover, time of incubation plus preparation of media and material can be time consuming in culture dependent methods. There are already some studies using culture-dependent and -independent methods because these combination can more efficiently show the overall effects of the AgNPs on soil bacterial community (Fajardo *et al*., 2014; Carbone *et al*., 2014).

Though PCR-DGGE is used since 1993, it is still used as an important tool to evaluate the effects/changes on bacterial communities (**CHAPTER II**). By using PCR-DGGE, we demonstrated that the silver forms (AgNPs and $Ag^{+}$), time (56 days) and the presence of *P. pruinosus* were relevant to alter the structure of soil bacterial community.

*P. pruinosus* showed to be probably useful in minimizing the effects of silver cation for exposures close to 2 months; yet, even in the presence of this isopod, the AgNPs might still be a risk for the soil bacterial communities.

As the maximum effect obtained in structure of bacterial community occurred after 56 days, the susceptibility tests (**CHAPTER III**) were performed for this time. But this was also because we wanted to evaluate the effects of contaminants on the cultivable bacterial community from soil that suffered the highest exposure in terms of time. Also, soil exposure occurred at 25ºC which is the annual average temperatures in Portugal. By using this method we demonstrated that the silver form, the amount and the combination of both silver forms affected the susceptibility of the soil bacterial communities. All bacteria analyzed (dBC, pBC and bacterial strains) were more susceptible to $Ag^+$ than to AgNPs, probably because $Ag^+$ release from bulk material ($AgNO_3$) was more rapid/efficient in a short period of time than AgNPs, thus interacting quicker with the microorganisms (Settimio *et al*., 2015). Also, an amount-dependent effect was demonstrated as well as an addition effect. The bacterial communities previously exposed to silver were evaluated considering the uncontrolled release of AgNPs into the environment, and consequently that silver contamination migh occur in different periods of time and amounts (Zhao *et al*., 2013). Results were dependent on the distinct bacterial community being analyzed (resulting from not only the effects of silver but also on the distinct plating techiques) as well as on distinct susceptibilities regarding the silver form and its amount. Although the bacterial communities being analyzed by the two methods were different, overall patterns can be identified: bacteria are affected by silver forms, particularly by showing altered community structure and susceptibility and these effects are stronger for $Ag^+$ than for AgNPs. Thus, this thesis strengthens how valuable soil bacterial communities are to evaluate the soil quality as a good indicator because they proved to be sensible to silver contamination by both methodologies. Indeed, the determination of effects on bacteria should be an integrative part of the environmental risk assessments of contaminants in soil, as stated by Kuperman, *et al*. (2014).

The present thesis considered a soil exposure of 5 µg Ag/Kg (100 x PEC for AgNPs), indicated as a worst case scenario by the NanoFATE project. Predicting the environmental concentrations of AgNPs is very difficult considering that the AgNPs production volumes and release are continuously increasing (Rana & Kalaichelvan, 2013). To give an idea, in the last years investigators have modulated several PEC

values (depending on the scenario assumed): 4.26 µg/Kg (Boxall *et al*., 2007), 0.02 µg/Kg (in a realistic scenario) or 0.1 µg/Kg (in a high-emission scenario) (Mueller & Nowack 2008) and 0.227 µg/Kg per year (Gottschalk *et al*., 2009). In other investigations, higher concentrations than ours were tested, yet the possibility of extrapolation of these results in a soil environmental risk assessment perspective is limited. Yet, lower concentrations than ours are imperative to be tested as we obtained most of the susceptibility changes for lower amounts (1 and 0.1 µg). Different responses of soil bacterial communities depending on previous exposure to silver exposure might be explained by the capability of bacteria to adapt and use silver (Mertens *et al*. 2007) or not (thus activating their mechanisms of defense: integrity of membrane, efflux pumps, among others). Furthermore, individual bacterial response is also influenced by communication cell-to-cell, e. g. quorum-sensing (Gambino *et al*., 2015), leading to a community response against contaminants (positive, neutral or negative) (Dinesh *et al*., 2012). To better understand all these possible bacterial responses, of individual groups and as a community, additional analysis is need including a better knowledge of the bacteria interactions with the AgNPs, the bacterial responses at gene expression level, chemical changes along silver exposure as well as analysis at the ecosystem level (by including other biota besides *P. pruinosus*) (Fajardo *et al*., 2014). Furthermore, we advise that these studies include higher sampling effort because of low repeatability.

Because the toxicity of silver forms, their mobility and bioavailability is influenced by physicochemical properties of soil (Hund-Rinke & Schlich, 2015), the choice of the soil to be tested is a relevant aspect. An investigation comparing five soils indicated that silver nanomaterials toxicity was higher for acidic soils than for alkaline ones and higher for those rich in clay content rather than in sand (Hund-Rinke & Schlich, 2015). Other soils were addressed, as in the work of Chunjaturas *et al*. (2014) that evaluated two agricultural soils from Thailand (clay soil and sandy loam soil) and in the investigation of Fajardo *et al*., (2014) that used LUFA 2.2 and LUFA 2.4. All these studies also supported that choosing the soil is of relevance for evaluating the effects of a soil contaminant as silver on bacterial communities. In the last decades, the OECD soil has been also used in toxicological tests, but this soil has the disadvantage of being artificial (van Gestel *et al*., 2012). On the other hand, soils from natural environments are not a good choice, because these soils might be contaminated with different chemicals. To use soils collected from the environment without this additional contamination it must be ensured that it has not been used for agriculture in at least 30

years (Henriques *et al*., 2015). Furthermore, the use of soils collected from the environment would require analysis of its properties, e.g. pH, organic matter, among others. Therefore, in this thesis, the LUFA 2.2 was a good choice because it is a natural standard soil (loamy sand according to texture) has been broadly used in hazard assessment of soil contaminants (Bastos *et al*., 2014) thus representing a good ecological approximation for risk evaluation and allowing further comparison studies. Another advantages of using this soil is that it does not require time to analyze the soil properties because they are previously analyzed (**ANNEX 1**).

Besides the work described in **CHAPTER III** which was done considering 25ºC, additional studies were done. For instance, the susceptibility assays were also performed at 37ºC including the analysis for dBC, pBC and bacterial strains (**ANNEX 6** and **ANNEX 7**). The temperature for the optimal growth of the bacterial strain for quality control in this assay (*Escherichia coli* 25922) was considered as being 37ºC (Anthony *et al*., 2014). On the other hand, the annual average of the environmental temperatures in Portugal is near 25ºC. If the first approach is more suitable for the susceptibility tests including the bacterial strains, the last would provide us a scenario closer to reality. Therefore, although we evaluated the effect of the two temperatures (25ºC or 37ºC) in the susceptibility tests, following the same experimental design, we only included in the article the results obtained at 25ºC, justified because this temperature represents a more realistic scenario in Portugal (**CHAPTER III)**. Besides, for the highest tested temperature (37ºC) regardless of the previous exposure treatment, the susceptibility responses maintained in comparison to the control at the end of the experiment. Furthermore, the susceptibility patterns observed for 25ºC and 37ºC were similar [$Ag^+$ > AgNPs; 10 µg > 1 µg > 0.1 µg; Combi ≈ ½(10 µg of $Ag^+$) + ½(10 µg of AgNPs)]. This aspect is interesting because, although the bacterial communities tend to be highly responsive to changes in temperature (Kuperman *et al*., 2014), very little information exists about the effects of temperature in the interaction of AgNPs with soil bacterial community. Results obtained by Xu *et al*. (2012) explained that higher temperature, as 37ºC, can influence the attachment and transport of $Ag^+$ into the plasma membrane. Due to higher permeability of the bacterial membrane, the transport of AgNPs might also be facilitated. Thus, the production of ROS can be stimulated due to the autoxidation of NADH dehydrogenase II in the respiratory chain, which is also accelerated by higher temperatures (Xu *et al*., 2012). Thus, Xu *et al*. (2012) showed that higher temperatures resulted in higher bacterial mortality when in the contact with AgNPs. Still considering

temperature as a variable, another study evaluated different temperatures [winter-summer (10-15ºC)] in arctic soil exposed to AgNPs revealing that the addition of AgNPs into soil during winter to summer transition was capable to interfere with normal and temporal microbial community changes decreasing the fertility of the soil (Kumar *et al*., 2014). Therefore, this aspect regarding temperature and silver bacterial effects deserves to be further explored.

A lot of work remains to be done in the scope of nanotechnology, specifically to assess the environmental impact of AgNPs in soil microbiota. For instance, experiments to evaluate of the effects of these contaminants on bacterial community of isopod gut and feces because this organism ingests silver forms possibly changing the bacterial community inside the isopod, and thus those that are spread in soil through the pellets (feces). From another perspective, and to add to what was mentioned before, using others tools, as pyrosequencing, can also be useful to provide additional/complementary information about the composition of bacterial communities.

The potential harm that the AgNPs represents to the soil bacterial communities appears to be of low impact, compared to silver cation, for the methodologies herein used. However, nowadays, nanotechnology is still a growing industry meaning that risk assessment is needeed. Thus, it is urgent to fulfill a gap concerning AgNPs' risk assessment: the uniformity and standardization of methodologies to assess AgNPs effects on bacterial communities. Though this thesis presented two complementary alternatives to assess the effects of AgNPs on soil bacterial communities, other methodologies should be further explored to understand how valuable they might be comparing to those studied in this thesis.

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# ANNEXES

# ANNEX 1. Properties of LUFA 2.2 soil

**Table A1.1** – Main properties of LUFA 2.2 soil.

| LUFA 2.2 soil | Mean ± STD DEV† |
|---|---|
| Organic carbon (% C) | 1.61 ± 0.2 |
| Nitrogen (% N) | 0.17 ± 0.02 |
| pH - value (0.01 M $CaCl_2$) (%) | 5.5 ± 0.10 |
| Cation exchange capacity (meq /100 g) (%)* | 10.0 ± 0.70 |
| Clay (%) | 7.3 ± 1.2 |
| Silt (%) | 13.8 ± 2.7 |
| Sand (%) | 78.9 ± 0.70 |
| WHC (%)# | 40.0 ± 3.0 |

† Mean values of different batch analyses ± standard deviation (all values refer to dry matter);*meg/100g: milliequivalent of hydrogen per 100 g of dry soil; # WHC: water holding capacity.

# ANNEX 2. Electrophoresis of the PCR products obtained prior to DGGE.

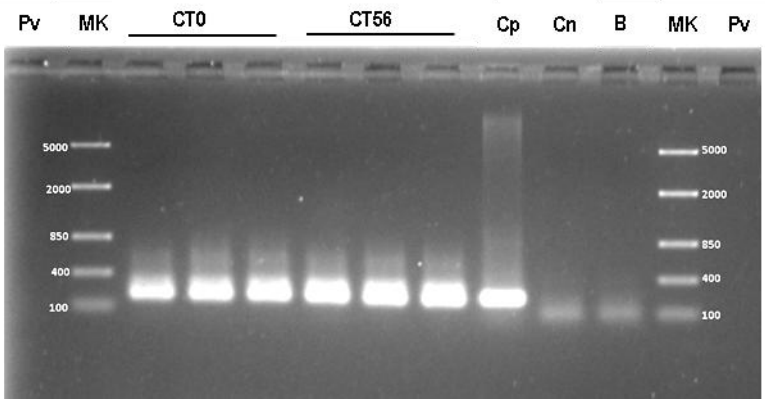


**Figure A2.1.** Electrophoresis in agarose gels (1.5% in TAE 1x; 120V; 40 min) of the PCR products obtained from controls samples (not exposed soil) at 0 (CT0) and 56 days (CT56). Triplicates are grouped. In the figure: Cp - Positive control (consists of all reagents used during sample processing but contains a DNA that you know it will amplify instead of sample); Cn - Negative control (consists of all reagents used during sample processing but contains water instead of sample; it can also be a DNA that you know it will not amplify); B - Reagent blank (consists of all reagents used during sample processing but contains no sample) control; Pv - Empty well; MK - Thermo Scientific™ FastRuler Middle Range DNA Ladder (#SM1113) [composed of the following DNA fragments (in base pairs): 5000, 2000, 850, 400 and 100].

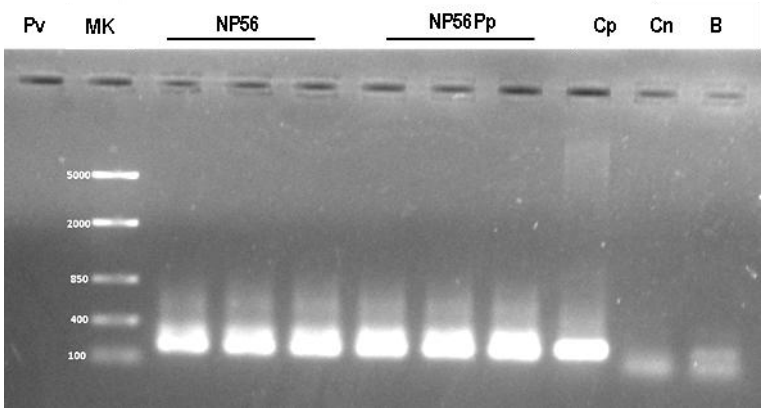


**Figure A2.2.** Electrophoresis in agarose gels (1.5% in TAE 1x; 120V; 40 min) of the PCR products obtained from soil samples after exposure to AgNPs during 56 days in the absence (NP56) and presence (NP56Pp) of *P. pruinosus*. Triplicates are grouped. In the figure: Cp - Positive control; Cn - Negative control; B - Reagent blank control; Pv - Empty well; MK - Thermo Scientific™ FastRuler Middle Range DNA Ladder (#SM1113) [composed of the following DNA fragments (in base pairs): 5000, 2000, 850, 400 and 100].

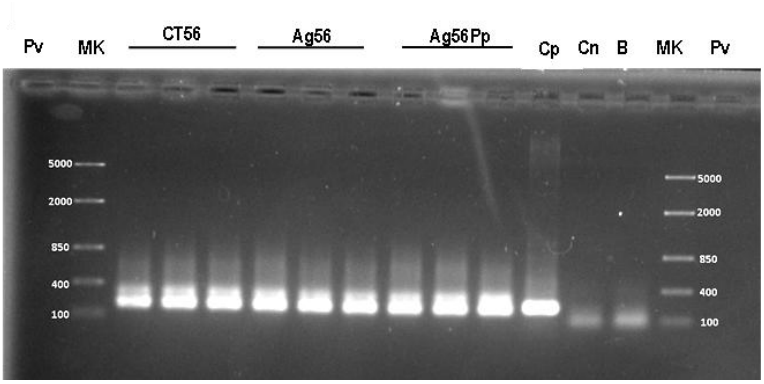


**Figure A2.3.** Electrophoresis in agarose gels (1.5% in TAE 1x; 120V; 40 min) of the PCR products obtained from soil samples after exposure to $Ag^{+}$ during 56 days in the absence (Ag56) and presence (Ag56Pp) of *P. pruinosus*. Triplicates are grouped. In the figure: Cp - Positive control; Cn - Negative control; B - Reagent blank control; Pv - Empty well; MK - Thermo Scientific™ FastRuler Middle Range DNA Ladder (#SM1113) [composed of the following DNA fragments (in base pairs): 5000, 2000, 850, 400 and 100].

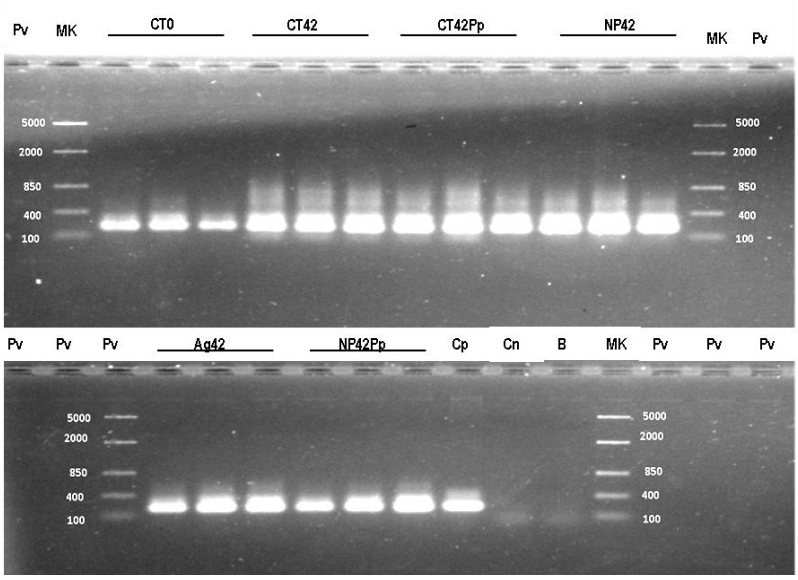


**Figure A2.4.** Electrophoresis in agarose gels (1.5% in TAE 1x; 120V; 40 min) of the PCR products obtained from non-contaminated soil samples at the beginning (CT0) and at 42 days in the absence (CT42) and presence (CT42Pp) of *P. pruinosus* as well as contaminated soil samples (with AgNPs in the absence (NP56) and presence (NP56Pp) of *P. pruinosus* or to $Ag^{+}$ in the absence (Ag56) and presence (Ag56Pp) of the isopod). Triplicates are grouped. In the figure: Cp - Positive control; Cn - Negative control; B - Reagent blank control; Pv - Empty well; MK - Thermo Scientific™ FastRuler Middle Range DNA Ladder (#SM1113) [composed of the following DNA fragments (in base pairs): 5000, 2000, 850, 400 and 100].

# ANNEX 3. Culture media used in the susceptibility tests: preparation

Medium was prepared by adding 30 g of Tryptic Soy Broth (TSB) (MERCK, Germany) in 1 L of distilled water, according to manufacturer instructions.

For Mueller Hinton Agar (MHA) (MERCK, Germany), 38 g of powder was dissolved in 1 L of distilled water. Both mediums were sterilized at in 121ºC for 15 min and stored at 4ºC.

**Table A3.1.** Composition on the medium TSB and MHA (MERK, Germany).

| **Medium** | **TSB** | **MHA** |
|---|---|---|
| **Composition** | Peptone from casein 17.0 g/L<br>Peptone from soy meal 3.0 g/L<br>Sodium chloride 5.0 g/L<br>Di-potassium hydrogen phosphate 2.5 g/L<br>D(+)glucose monohydrate 2.5 g/L | Infusion from meat 2.0 g/L<br>Casein hydrolysate 1.75 g/L<br>Starch 0.15 g/L<br>Agar - Agar 1.7 g/L |
| **pH** (25 °C) | 7.3 ± 0.2 | 7.4 ± 0.2 |

# ANNEX 4. Susceptibility of bacterial strains to antibiotics

**Table A4.1**- Susceptibility patterns to the two antibiotics Imipenem and Ticarcillin - Clavulanic Acid [Adapted from: CLSI document M100-S23 (M02-A11): "Disc diffusion supplemental tables"].

| | | **Organisms for quality control in susceptibility testing** [zone of growth inhibition in diameters (mm)] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Antibiotic** | **Potency** | ***E. coli* ATCC® 25922** | ***S.aureus* ATCC® 25923** | ***P.aerug.* ATCC® 27853** | ***E. coli* ATCC® 35218** | ***H. influ.* ATCC® 49247** | ***H. influ.* ATCC® 49766** | ***N. gon.* ATCC® 49226** | ***S.pneumo.* ATCC® 49619 p** |
| **Imipenem** | 10 µg | **26-32** | — | 20-28 | — | 21-29 | — | — | — |
| **Ticarcillin - Clavulanic Acid** | 75/10 µg | **24-30** | 29-37 | 20-28 | 21-25 | — | — | — | — |

**Note:** The quantity of antibiotic used in our experiment was of 85µg per disc and the organism used for quality control in our susceptibility testing was *E. coli* ATCC 25922 (indicated in bold).

# ANNEX 5. Images of the resulting plates from the susceptibility test at 25ºC.

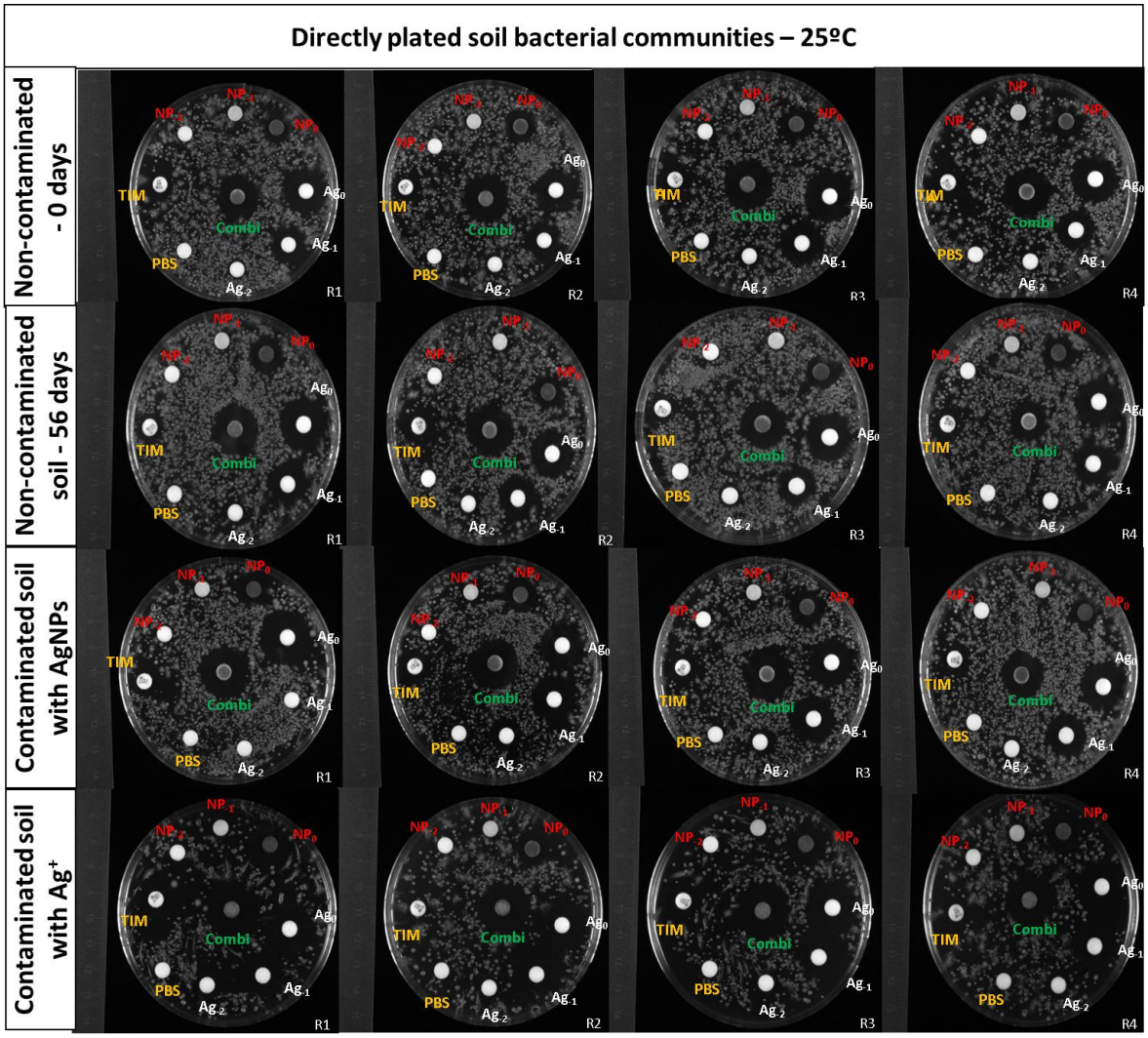


**Figure A5.1.** Susceptibility assays of the soil bacterial communities [directly plated soil bacterial communities: dBC; non-exposed (at 0 and 56 days) and exposed to AgNPs or $Ag^{+}$] by disc diffusion method at 25ºC. Please see **Table 2** (**Chapter III**) for disc identification details. Quadruplicate assay (R1, R2, R3 and R4).

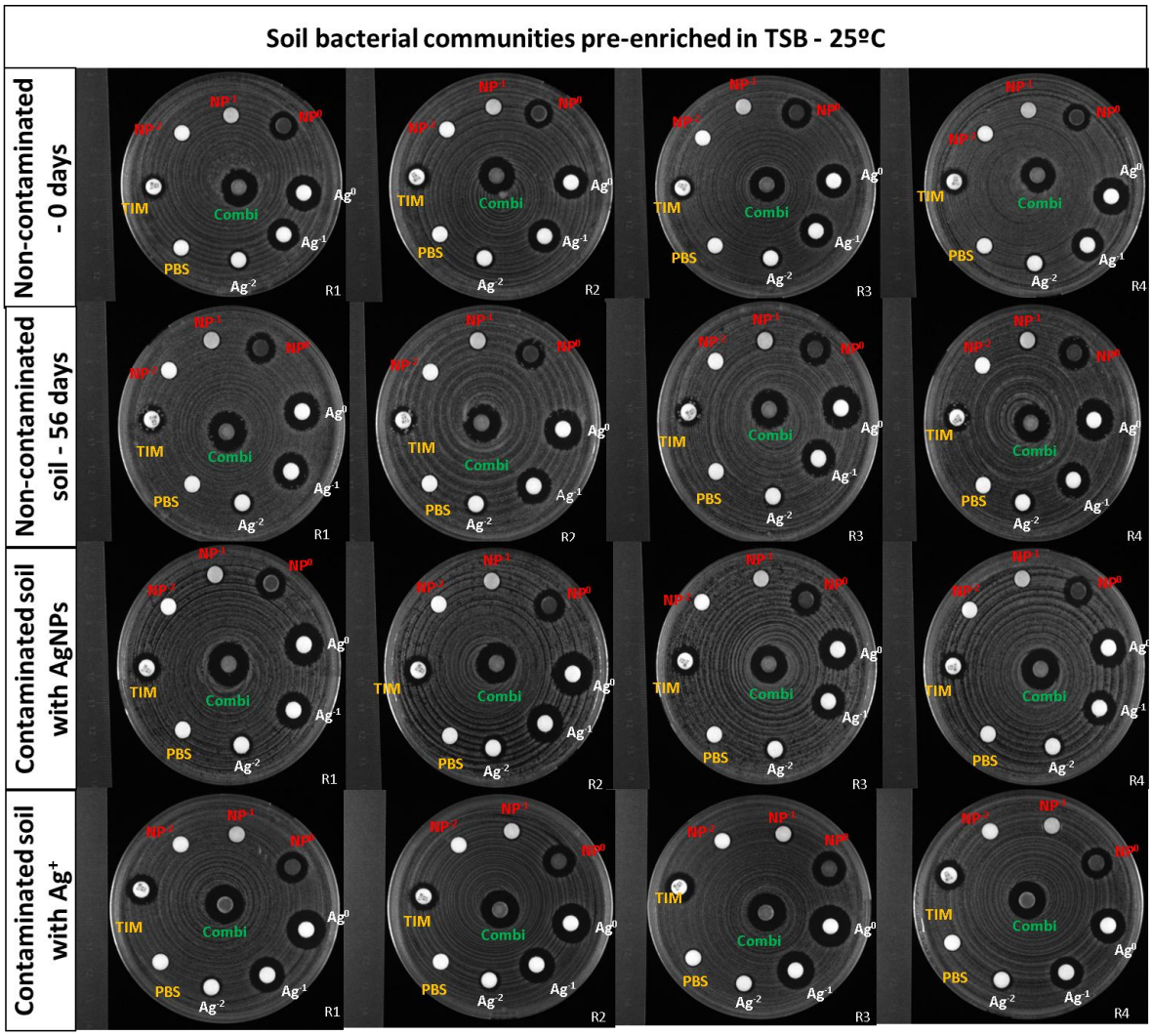


**Figure A5.2.** Susceptibility assays of the soil bacterial community (after recover to TSB) to AgNPs or $Ag^{+}$ exposures, by disc diffusion method at 25ºC. Please see **Table 2** (**Chapter III**) for sample identification details. Quadruplicate assay (R1, R2, R3 and R4).

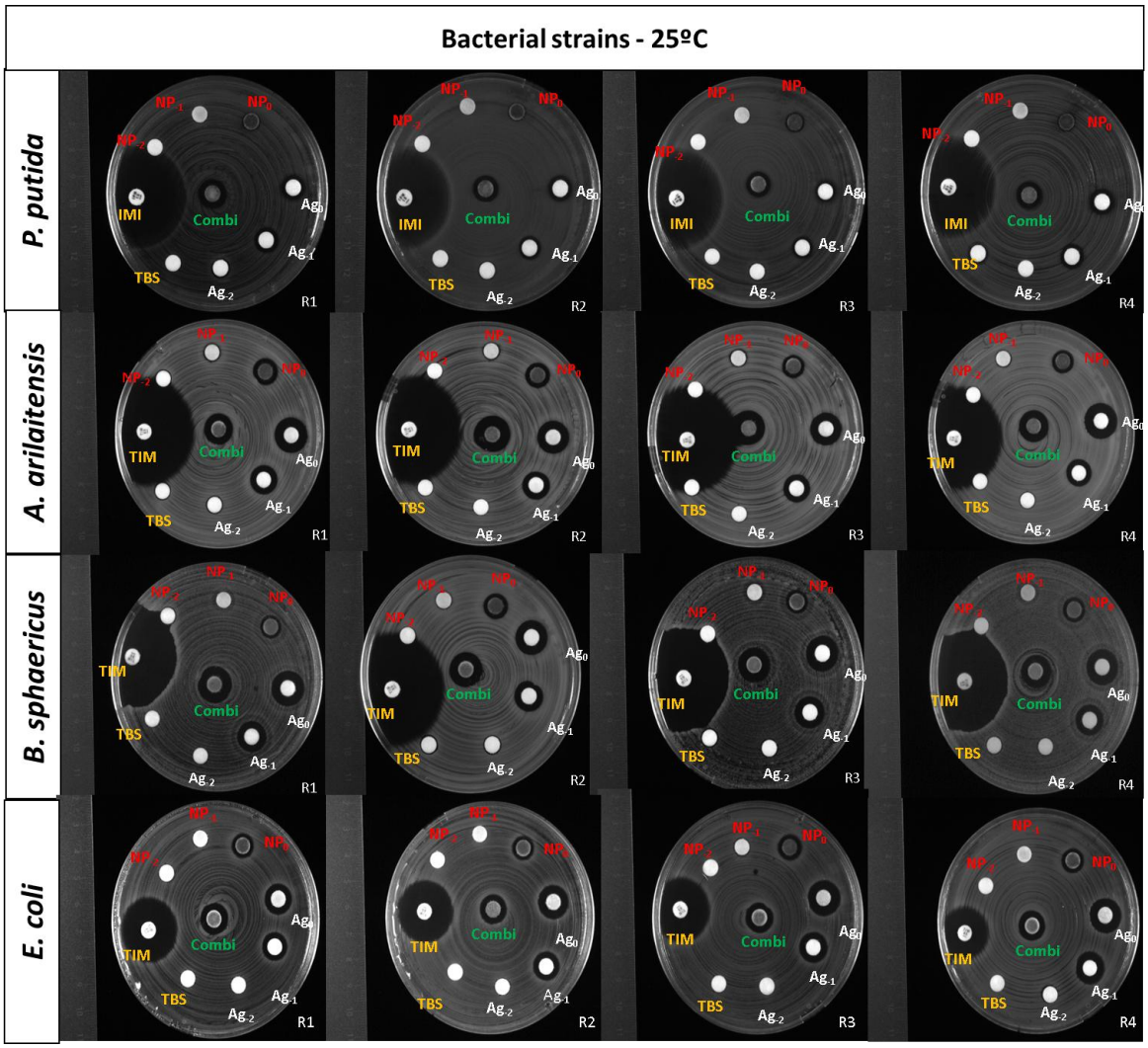


**Figure A5.3.** Susceptibility assays of the bacterial strains (*P. putida; A. arilaitensis; B. sphaericus* and *E. coli*) to AgNPs or $Ag^{+}$ exposures, by disc diffusion method at 25ºC. Please see **Table 2** (**Chapter III**) for sample identification details. Quadruplicate assay (R1, R2, R3 and R4).

## ANNEX 6. Susceptibility tests at 37ºC

Here we present the preliminary results for the susceptibility tests conducted at 37ºC following the same experimental design as the one used at 25ºC (**Chapter III**; **Table 1** and **2** for sample identification).

Except for 1 µg of AgNPs (where the susceptibility decreased after 56 days), the susceptibility response did not change as a function of time: no significant differences ($p<0.05$) were observed between the bacterial communities retrieved from the non-contaminated soil at 0 day or at 56 days. Thus, for further statistical analysis only the non-contaminated soil bacterial community results (control) collected after 56 days (CT56) was considered (**Figure A6.1**).

The susceptibility response, screened at 37ºC, of non-exposed soil bacterial communities (CT0 and CT56) decreased as following: 10 µg > 1 µg > 0.1 µg (higher susceptibility for higher silver amounts) (**Figure A6.1**).

For the same silver amount, the growth inhibition zones were higher for $Ag^+$ treatment than for AgNPs ($Ag^+$ > AgNPs) and susceptibility was noticed for lower silver amounts (for instance, 0.1 µg of $Ag^+$ induced susceptibility while 0.1 µg of AgNPs did not induced susceptibility) (**Figure A6.1**).

Combined contamination of the discs with AgNPs and $Ag^+$ (Combi) give similar susceptibility response to the one caused by adding half of the inhibition caused by each of the highest amounts of each silver form [Combi ≈ ½(10 µg of $Ag^+$) + ½(10 µg of AgNPs)] (**Figure A6.1**).

All these observations are similar to those observed for the susceptibility tests at 25ºC (**Chapter III**).

The inhibition decreases when lower amounts are present (10 µg > 1 µg > 0.1 µg), regardless of the contaminants (either for AgNPs or $Ag^+$) (**Figure A6.1**).

The inhibition zones were higher for $Ag^+$ treatment. So this contaminant might be more toxic than AgNPs (**Figure A6.1**).

**Previous exposure with $Ag^+$ treatment:** The susceptibility of pBC was maintained (**Figure A6.1**).

**Previous exposures with AgNPs treatment**: For a silver amount at 1 µg, the dBC non-contaminated was affected not showing inhibition zone, but after AgNPs exposure the dBC did not recover (**Figure A6.1**).

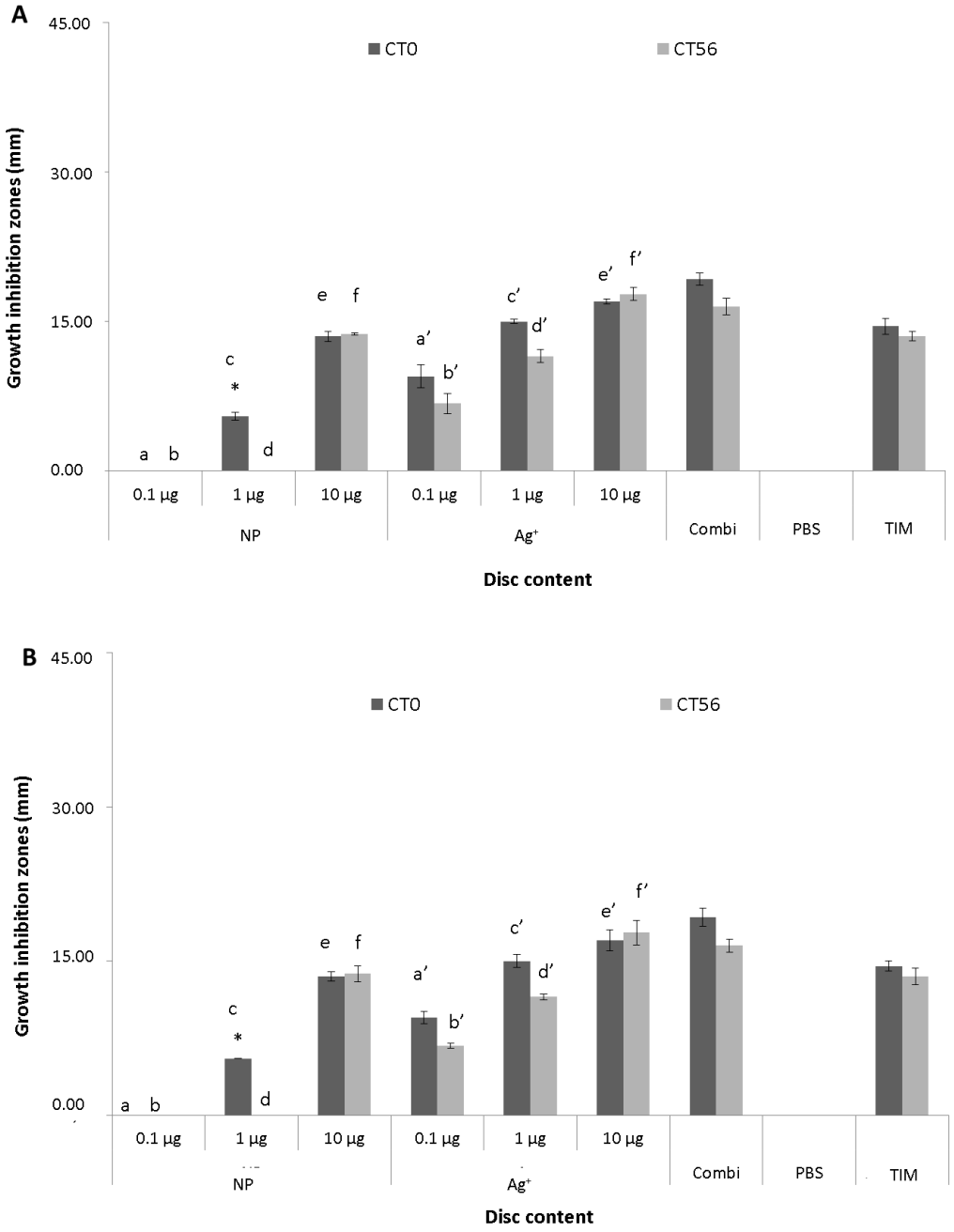


**Figure A6.1.** Susceptibility of soil bacterial communities previously not exposed to silver forms (CT0 and CT56) at 37ºC: **(A)** dBC (directly plated bacterial communities) and **(B)** pBC (pre-enriched bacterial communities). Please see **Table 1** and **2 (Chapter III)** for sample identification. Data represent diameters mean ± standard deviation (n=4). (*) Statistical differences between soil treatments (CT0 *vs.* CT56), for the same silver amount, according to one-way ANOVA (*Dunnett's* test) for $p<0.05$. Different letters (*e.g.* a and a') corresponds to significant differences, for the same silver amount, within soil treatments (*e.g.* within CT0 or within CT56) and between silver forms (AgNPs *vs.* $Ag^+$), $p<0.05$ (two-way ANOVA).

The susceptibility response of the dBC (directly plated bacterial communities) and pBC (pre-enriched bacterial communities) at 37ºC (**Figure A6.2**) revealed that:

- Comparing bacterial communities previously exposed to silver (NP56 and Ag56) with non-exposed soil bacterial communities (CT56), all exposures maintained their

susceptibility (for both dBC and pBC) except when pBC was previously exposed to AgNPs (NP56) suffered exposure of 1 µg of AgNPs becoming no susceptible to AgNPs.

- The growth inhibition, and consequently the susceptibility, increases according to increasing silver amounts (10 µg > 1 µg > 0.1 µg), regardless of the silver form (AgNPs or $Ag^+$) and previous exposure (NP56 or Ag56).

- For the same silver amount, the growth inhibition zones were higher for $Ag^+$ treatment than for AgNPs ($Ag^+$ > AgNPs) and susceptibility was noticed for lower silver amounts (for instance, 0.1 of $Ag^+$ induced susceptibility while 0.1 µg of AgNPs did not induced susceptibility).

- Regardless of previous exposure, combined contamination of the discs with AgNPs and $Ag^+$ (Combi) continued to give similar inibihition growth to the one caused by adding half of the inhibition caused by each of the highest amounts of each silver form [Combi ≈ ½(10 µg of $Ag^+$) + ½(10 µg of AgNPs)].

- Standard deviation regarding growth inhibition was higher for dBC than for pBC.

These observations were not similar comparing to the susceptibility tests at 25ºC (**Chapter III**).

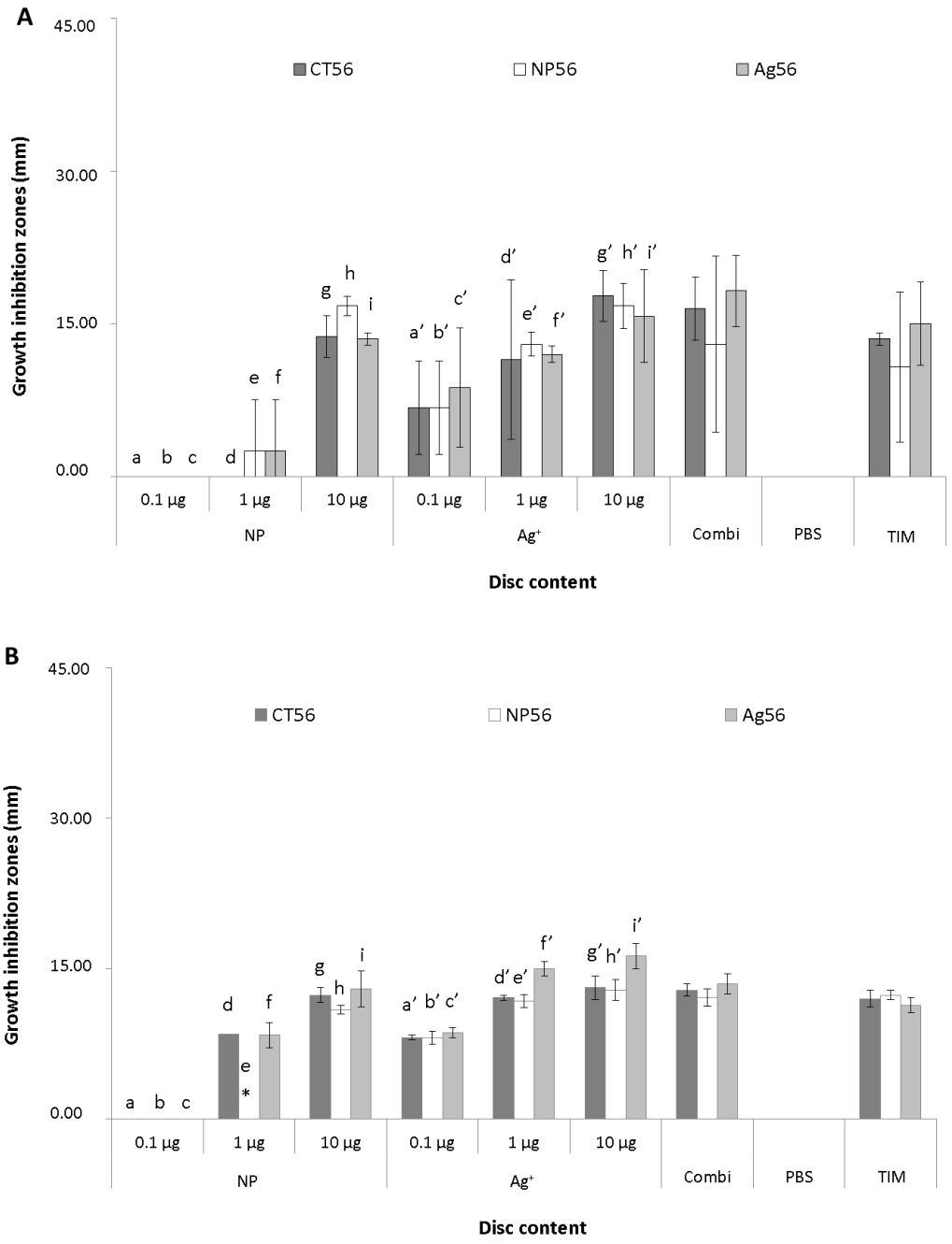


**Figure A6.2** Susceptibility of soil bacterial communities previously exposed to silver forms (NP56 or Ag56) at 37ºC: **(A)** dBC (directly plated bacterial communities) and **(B)** pBC (pre-enriched bacterial communities). Data relative to non-exposed soil bacterial communities from the end of the experiment (CT56 for dBC and CT56 for pBC) was maintained for comparison purposes. Please see **Table 1** and **2 (Chapter III)** for sample identification. Data represent diameters mean ± standard deviation (n=4). (*) Statistical differences between soil treatments (CT56 *vs.* NP56 or CT56 *vs.* Ag56), for the same silver amount, according to one-way ANOVA (*Dunnett's* test) for $p<0.05$. Different letters (*e.g.* a and a') corresponds to significant differences, for the same silver amount, within soil treatments (*e.g.* within NP56 or within Ag56) and between silver forms (AgNPs *vs.* $Ag^+$), $p<0.05$ (two-way ANOVA).

In case of susceptibility of the soil bacterial communities and of well-known soil bacterial groups were similar; three representative strains (*Bacillus sphaericus* ATCC 29726, *Arthrobacter arilaitensis* GCNP1_I and *Pseudomonas putida* NCIMB 10432) were chosen.

Only the highest AgNPs amount (10 µg of AgNPs) was capable of inhibiting the growth of *B. sphaericus* [ZoI(10 µg of AgNPs)$_{B.\ sphaericus}$ = 13.5 mm]. *A. arilaitensis* and *P. putida* were only susceptible to the two highest amounts of AgNPs (10 and 1 µg) with *P. putida* being the less susceptible of both (**Figure A6.3**). Of all bacterial strains have a similar susceptibility pattern for the two highest $Ag^+$ amounts (10 and 1 µg) but for 0.1 µg of $Ag^+$ the *P. putida* was the only affected [ZoI(.01 µg of $Ag^+$)$_{P.\ putida}$= 11 mm] (**Figure A6.3**).

Overall, the bacterial strains showed decrease susceptibility to each silver form as follows: 10 µg > 1 µg > 0.1 µg. Regardless of the bacterial strain, silver cation was more effective in inhibiting growth than AgNPs (comparing the same silver amount), as shown in **Figure A6.3**.

Combined contamination with AgNPs and $Ag^+$ (Combi, **Chapter III**; **Table 1** and **2** for sample identification) induced similar growth inibihition to the one caused by adding half of the inhibition caused by each of the highest amounts of each silver form [Combi ≈ ½(10 µg of $Ag^+$) + ½(10 µg of AgNPs)] (**Figure A6.3**).

The susceptibility test was validated by the by *E. coli* results [ZoI(TIM)$_{E.coli}$ = 25.7 mm, which is within the established range 24 - 30 mm (**ANNEX 4**).

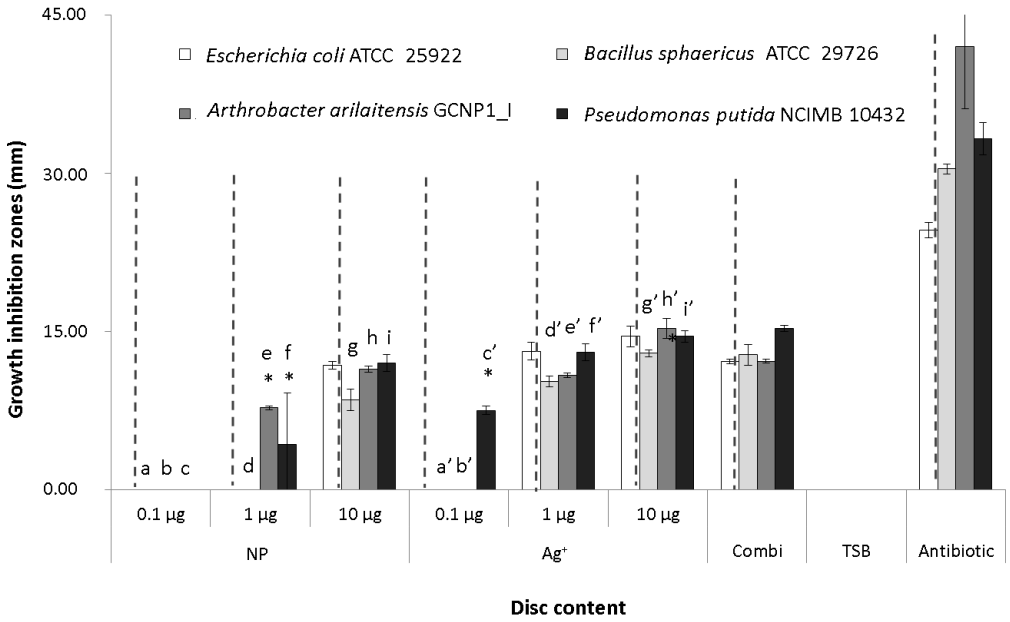


**Figure A6.3.** Susceptibility of bacterial strains to silver exposure at 37ºC. In the figure: *Escherichia coli* ATCC 25922 was only used as control and is separate from the tested bacterial strains using a dashed line; Antibiotic imipenem (IMI) was only used for *Pseudomonas putida*. Please see **Table 2 (Chapter III)** for sample identification. Data represent diameters mean ± standard deviation (n=4). (*) Statistical differences among bacterial strains (*Bacillus sphaericus* ATCC 29726 *vs. Arthrobacter arilaitensis* GCNP1_I *vs. Pseudomonas putida* NCIMB 10432), for the same silver amount, according to one-way ANOVA (*Dunnett's* test) for $p<0.05$. Different letters (*e.g.* a and a') corresponds to significant differences, for the same silver amount, within each bacterial strain (*e.g.* within *Bacillus sphaericus* ATCC 29726 or within *Arthrobacter arilaitensis* GCNP1_I or within *Pseudomonas putida* NCIMB 10432) and between silver forms (AgNPs *vs.* $Ag^+$), $p<0.05$ (two-way ANOVA).

# ANNEX 7. Images of the resulting plates from the susceptibility test at 37ºC.

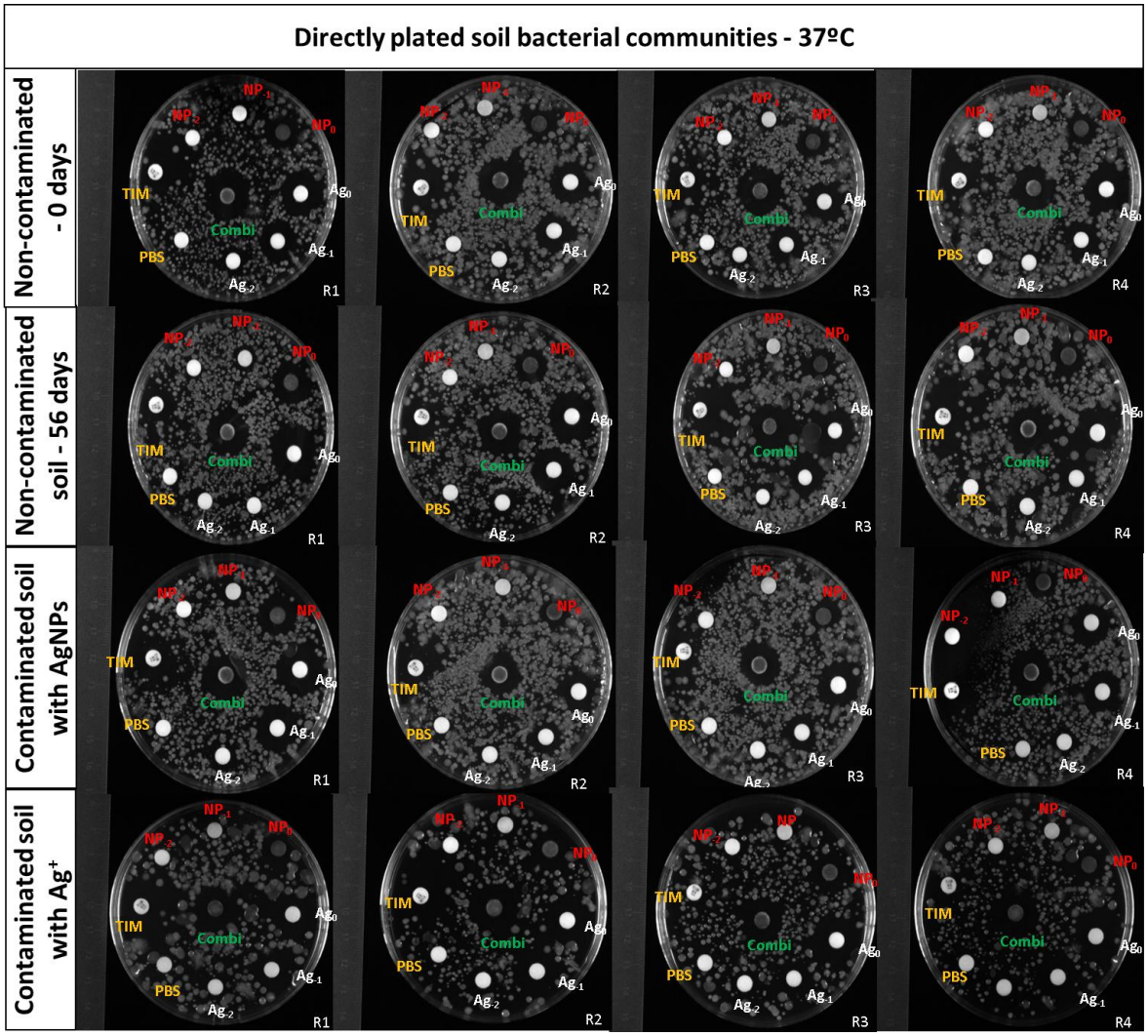


**Figure A7.1.** Susceptibility assays of the soil bacterial community to AgNPs or $Ag^{+}$ exposures, by disc diffusion method at 37ºC. Please see **Table 2 (Chapter III)** for sample identification details. Quadruplicate assay (R1, R2, R3 and R4).

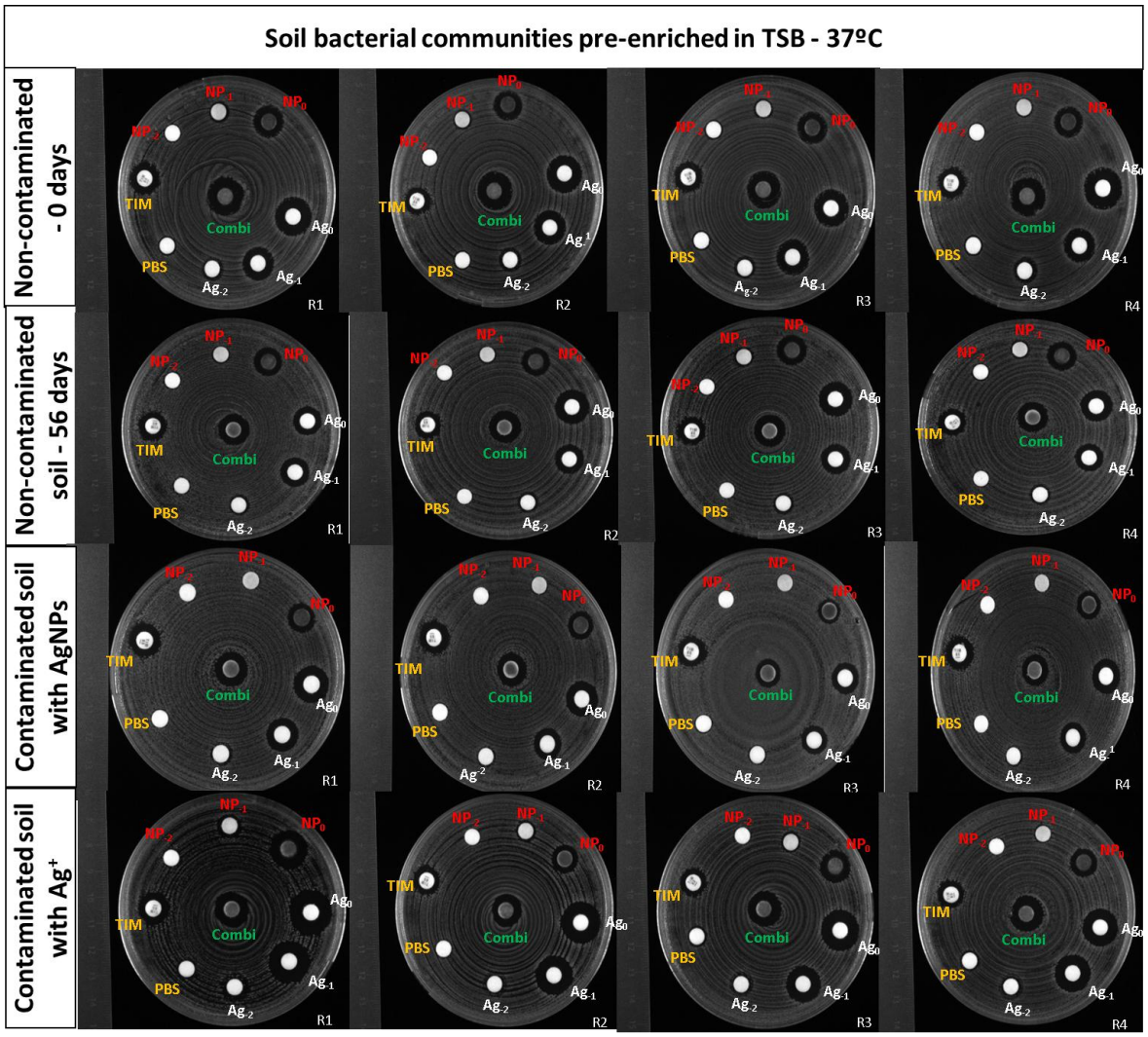


**Figure A7.2.** Susceptibility assays of the soil bacterial community (after recover to TSB) to AgNPs or $Ag^{+}$ exposures, by disc diffusion method at 37ºC. Please see **Table 2** (**Chapter III**) for sample identification details. Quadruplicate assay (R1, R2, R3 and R4).

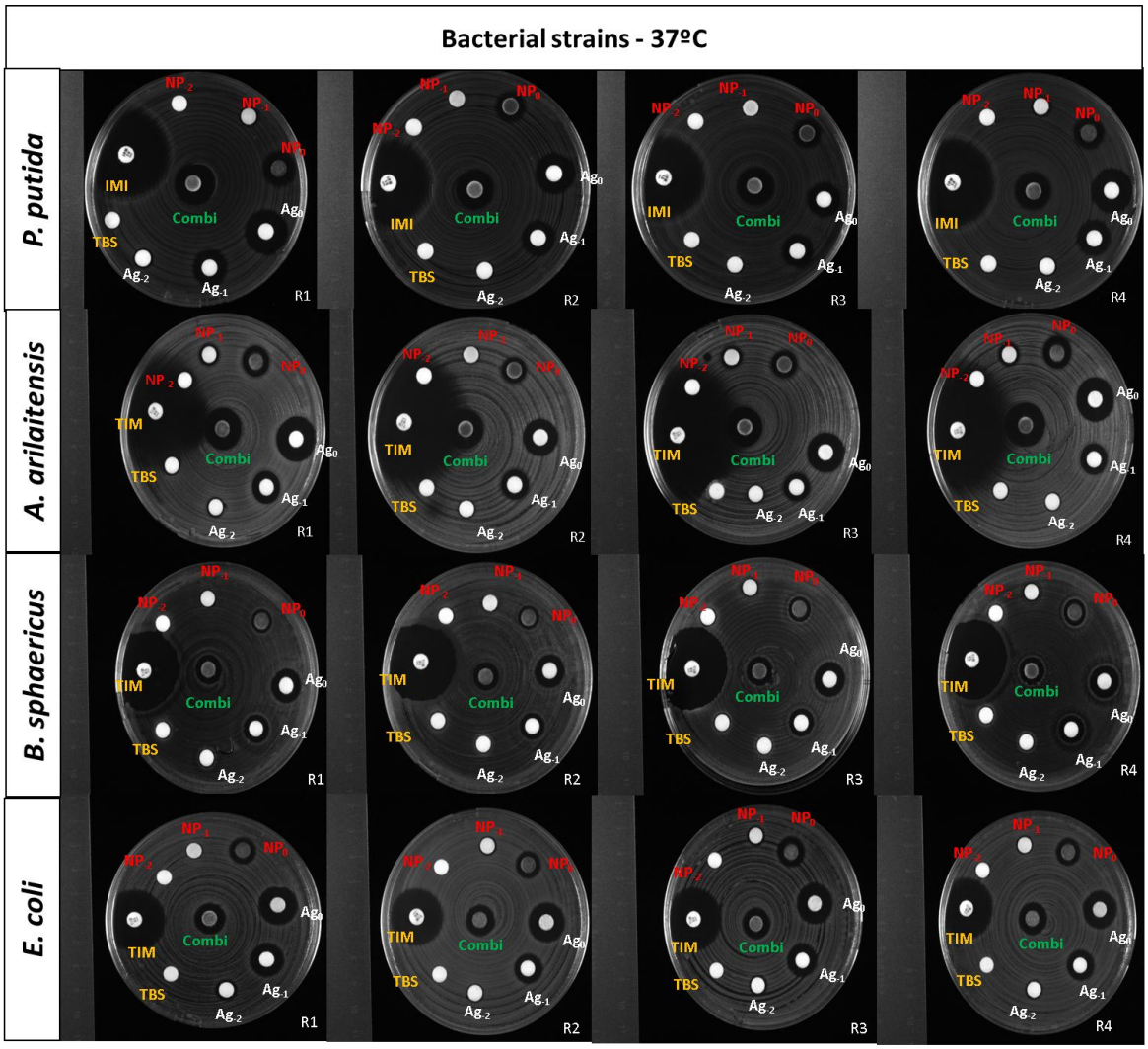


**Figure A7.3.** Susceptibility assays of the bacterial strains (*P. putida; A. arilaitensis; B. sphaericus* and *E. coli*) to AgNPs or $Ag^{+}$ exposures, by disc diffusion method at 37ºC. Please see **Table 2** (**Chapter III**) for sample identification details. Quadruplicate assay (R1, R2, R3 and R4).