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**Interactions of nanoenabled agrochemicals with soil microbiome**

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**Abstract**
Soil is a dynamic, physically, spatially and temporally heterogeneous but well-organized, three-dimensional porous matrix mixing mineral and organic matter and living organisms. Among them, soil microbiota constitute a reservoir in which plants select a specific microbiome, contributing to their growth and their health. Microbes in soil also contribute to many ecosystemic services in agrosystems, as the recycling of major nutrients in the soil ecosystem (carbon, nitrogen, phosphorus, sulfur…).
Nanoagrochemicals are active substances based on nanotechnologies and nanoformulations to improve the characteristics and properties of active molecules as pesticides for agronomy purposes, e.g., biocides, herbicides but also nutrients. Nanotechnologies have burst into agronomy with a potential for innovation in order to improve the efficiency of pesticides, nutrients, their delivery and thus contribute to the reduction of inputs in agriculture. However, the impact of these nanopesticides on the soil microbiota as non-target organism remains underestimated up to now.
The chapter review the approaches and trends in the evaluation of nanopesticides implications on soil microbiota, focusing on copper- and silver-based nanoparticles as pesticides or on formulation or nanocarriers of conventional pesticides. By confronting the current knowledge and comparing methodologies, the potential and the pitfalls to overcome are discussed, together with future directions.

**Keywords: Nanoagrochemicals, non-target organisms, soil microbiota, metallic nanoparticles, polymer-based pesticide nanoformulations**

## I. Introduction

Agrochemicals, also known as phytopharmaceuticals products or pesticides, are substances used in agriculture to increase crop yield and to control pests, such as plant pathogens (fungi and bacteria), herbs and nematodes. Nanoagrochemicals or nanopesticides are active substances based on nanotechnologies and nanoformulation to improve their characteristics and properties. Nanoenabled agrochemicals encompasses nanofertilizers, nanopesticides, soil enhancer and more recently nanosensors (Parisi *et al.* 2015; Fraceto *et al.* 2016; Baker *et al.* 2017; Adisa *et al.* 2019). Unlike nanomaterials, which are defined as materials with at least one dimension between 1 and 100 nm, nanopesticides encompasses a range of heterogeneous products in terms of particle size: most of nanopesticides exceed the 100 nm size threshold. However, the nanoscale dimension usually provides particles with new chemical and physical properties, and is source of innovation in agricultural sector. The outcome of nanotechnologies applied to pesticides are smart objects, endowed with increased efficacy, due to the reduction of losses and controlled delivery of the active ingredient, together with potential reduction of doses (Kah *et al.* 2018).

This reduction in the quantities of pesticides, used to increase agricultural productivity, could be particularly welcome in a paradoxical context that confronts the injunction for a more sustainable agriculture to preserve the earth's resources, feeding an increasing world population expected to reach from 7.7 billion to 9.7 billion in 2050 (https://population.un.org/wpp/), and fluctuating yields due to global warming and climatic events (drought, flooding heatwave events, etc.).

Pesticides and nanopesticides, sprayed on plants and soils or used as seed coating, can interact with the soil ecosystem, with potential consequences for the soil microbiomes, the soil fertility and ecosystemic services.
Soil microbiota, encompasses a community of microorganisms, bacteria, archaea, fungi, viruses and protists, associated to this environment. Soil microbiota plays a fundamental role in the cycle of elements, especially carbon but also nitrogen, phosphorus, sulfur and other elements, the recycling of organic matter, the degradation of pollutants and the soil formation, by water and microbial alteration of rocks. Hence, soil organisms are key drivers for relevant ecosystem services in agricultural landscapes, such as nutrient cycling, soil structure, pest control and biodiversity.
But more importantly for agriculture, the soil is a reservoir of microorganisms, in which the plant selects a specific microbiome, which contributes to the growth of the plant and its health. Thus, via the selected microbiome, the plant acclimates more quickly to stress, whether abiotic (drought, flooding, chemical toxics) or biotic (plant pathogens). The role of the plant microbiome is often compared to that of the intestinal microbiome for humans (Schlaeppi *et al.* 2014). Soil microbiome is considered as the second genome of the plant and the agricultural potential of the soil. Some microbiomes associated to soils can be suppressors of plant pathogens and naturally help controlling plant diseases.
Thus, understanding the interactions of nanopesticides with soil and plant microbiomes is essential in order to develop smart nanoagrochemicals that associate efficiency and eco-compatibility, in order to preserve the microbial diversity of the soil.

Before jumping into the nanoworld of pesticides, we would like to highlight the fact that the impact on soil microbiome as non-target organisms of regular (non-nano) pesticides is not so well described, even if they are currently used on agroecosystems at a rate of billions of tons. Pesticide risk assessment on soil microorganisms, is certainly sidelined when considering the effects on non-target organisms. In Europe, as far as environmental risk assessment is concerned on non-target soil microorganisms, obtaining a marketing authorization from EFSA (European Food Safety Authority ) only requires to evaluate the effect of the active substance on nitrogen mineralization (OCDE 216 2000; Thiour-Mauprivez *et al.* 2019). However, the European Food Safety Authority (EFSA) Panel on Plant Protection Products and their Residues recently proposed specific protection goals and testing strategy (*e.g.,* functional assays based on soil respiration, exoenzyme activity and potential ammonium oxidation, PAO, test), which takes into account the relevant exposure routes for in-soil organisms and the potential direct and indirect effects.

Many pesticides are systemic in plant and may act on a target that both can be found in plants and in microorganisms, as it for herbicides (Thiour-Mauprivez *et al.* 2019). Pesticides that control biotic plant disease, can indiscriminately affect microorganisms pathogenic or beneficial to the soil ecosystem and to the plant. Regular use of organophosphates or pesticides reduces the microbial community and soil fertility though pesticides are not always toxic for microbial communities (Lo 2010). Some effects can be transient, *e.g.* the modulation of soil enzymatic activities by biopesticides (Shao & Zhang 2017). Pesticides can be both a felicity or a curse to soil microbial community (Karpouzas *et al.* 2016). Indeed, some pesticides are used as source energy for microbes and can challenge and select some specific and competitive microbial communities. However, whether these selected microbes are friendly or not is a main concern. As example, glyphosate, one of the most used herbicide in the world, enhances the resistance to chloramphenicol and kanamycin in *E. coli* and *S. typhimurium* (Kurenbach *et al.*, 2017). Thus, crossed-resistances to herbicide and antibiotics could be a major concern, as exposure of bacteria to non-antibiotic chemicals such as herbicides could promote the resistance to antibiotics (Rangasamy *et al.* 2018; Van Bruggen *et al.* 2018).
Thus understanding the impact of pesticides and nanopesticides on non-target organisms and the resilience of the soil ecosystem is an evidence and an open question, and the approaches are still debated.

This chapter analyses the interactions and impacts of nanopesticides on soil microbial communities. It is not an exhaustive review but rather an illustration of the knowledge in the field, the gaps and future prospects.

Before getting into the details of microbial nanopesticides interactions and their impacts on soil life, it is necessary to understand: i) the complexity of soil and plant-soil-microbial system, indeed, the nanoform of pesticides may alter their fate and diffusion in the soil matrix, and ii) the main methods to characterize impacts on soil activity and soil microbiota.

## II. Soil-Plant-Microbiota : a complex system

- **Soil is complex and heterogeneous matrix**

Soil is biomaterial and the support for microbial communities that form the foundation of trophic food webs, supporting terrestrial life. A fertile soil contains up to $10^{12}$ bacteria and 25 km of fungi. However, as cells cluster together, only about a tiny fraction of the soil surface area ($10^{-6}$ %) is covered by soil microbes (Young *et al.* 2008).
Soil is a dynamic, physically, spatially and temporally heterogeneous but well-organized, three-dimensional porous matrix made from mineral and organic matter, different physical matter states (solids, liquids and gases) and living organisms. There is a complex feedback between the chemistry of the matter and the biology of microorganisms living in soil habitat (Figure 6.1). At a local scale, soil is a 3-dimensional hierarchical network based on aggregates and on pores that are periodically connected during wetting events. Aggregates are the functional unit of a soil ecosystem (Wilpiszeski *et al.* 2019). Organo-mineral associations drive the formation of clusters (2-20 microm) through electrostatic and hydrophobic interactions between clays and organic matter, especially extracellular polymeric substances (Santaella *et al.* 2008) forming hutches for bacteria and fungi (Totsche *et al.* 2018; Watteau & Villemin 2018). The formation of stable clusters is stimulated at the interface between the plant root

and the soil, the rhizosphere, as plant exudates and desquamated cells promote hot spots of bacteria (Watteau & Villemin 2018). These clusters assemble into microaggregates (<250 microm) cementing mineral agents (oxides, hydroxides, and oxyhydroxides of iron, manganese, aluminum, silicon, aluminosilicates, and carbonates  and entangling organic matter (Totsche *et al.* 2018). Temporary binding through hyphae from fungi or actinomycetes, roots, proteins and extracellular polymeric substances gathers microaggregates into macroggregates (> 250 mm) and pores. This architecture creates a variable flow of water and nutrients that can be accessed by soil organisms (Wilpiszeski *et al.* 2019). Proteins with enzymatic activities can be everywhere, inside cells, inside or at the surface of microaggregates, and macroaggregates and even in pores during a waterlogging event. Soil and especially clays, organic matter and minerals can sorb chemical compounds circulating in the pore water solution (the so-called cation exchange capacity) and interact with microrganisms.
The microstructure of soil aggregates directly impacts soil communities and functional diversity. The diffusion gas, water and nutrients is modulated according to the diameter of pore spaces from 10 to 30 μm in inter-aggregates to 1 to 2 μm within intra-aggregates. Soil microstructure offers micro-niche for microorganisms. As example, nitrogen cycle relies on communities inhabiting distinct portions or the soil structure. Nitrifiers are most abundant and active in 2- to 20-μm microaggregates, while nitrogen-fixing bacteria were most abundant in the <2 microm clay fraction (references in Wilpiszeski *et al.* (2019).

Recently, Driouich *et al.* 2019 described a new structure, the Root Extracellular Trap (RET), expected to set in soil the interactions and relations between plant and rhizosphere microorganisms. At the tip of the root, cap-derived cells (AC-DCs, Driouich *et al.* 2019) are released in the rhizosphere as single cells (border cells, Hawes *et al.* , 2000) or files of cells still attached together (border-like cells, Vicré *et al.* 2005). These two types of cells are implicated in the root defense (Hawes *et al.* 2012, 2016; Plancot *et al.* 2013). At the scale of a root system, root cap-derived cells and their secretions form a cloudy network of 'sticky' mucilage between the soil and the roots, composed of cells and defense-related compounds released into the surrounding soil environment and, consist mainly of glycan-containing molecules (*i.e.*, proteoglycans and polysaccharides), antimicrobial compounds including proteins, peptides and secondary metabolites, histones and extracellular DNA (Hawes *et al.* 2016; Ropitaux *et al.* 2019; Driouich *et al.* 2019), regulating interactions and relations of the plant with rhizosphere microorganisms.

All these architectural structure in the soil controls the interactions between plants, microbes, and also pollutants. This is why understanding the interactions between the soil matrix and nanopesticides will be so important.

- **Microbiome *vs* microbiota**

There is some confusion and quite a controversy in the use of these two words, supported by the semantic analysis of the word stem as « microbi-ome » or « micro-biome » (Lederberg & Mccray 2001). According to the author of that word, « microbiome » refers to "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease" (Lederberg & Mccray 2001). However, this definition overlaps with that of the microbiota, quite equivalent to the microflora in the gut, defined as the microbial communities that inhabit our gastrointestinal tract. In the dynamic trend of -omes and omics, microbiomes could tend to define a population of microorganisms and their genetic potential while microbiota defines the collection of microbes. The composition of a microbial community as described by high throughput sequencing approaches (see the next paragraph) refers to a microbiome, while a fecal microorganism transplantation refers to a microbiota.

- **How to analyze the impacts of nanopesticides on soil microbiota**
    - Microbiome analysis

Microbiome analysis relies on metagenomics and more generally omics (transcriptomics, proteomics and metabolomics), which allows microorganisms to be studied in their environment without the need for a culture step. Microbial communities can be characterized by their composition (who is there), abundances (how many of them?), their activities, *e.g.*, RNA, proteins and metabolites (what are they doing).

One approach to characterize microbiome is amplicon sequencing or "DNA metabarcoding". DNA Metabarcoding is based on high throughput sequencing of amplicons of taxonomic markers, such as ribosomal RNA genes (16S rRNA for bacteria and archaea, 18s RNA for eucaryota) or Internal Transcribed Spacer (ITS, for fungi), as universal barcode sequences of the microorganism identity (Caporaso *et al.* 2011; Shokralla *et al.* 2012). 16S rRNA and 18S rRNA genes code ribosomal RNA, a non-coding RNA (not translated to protein) that is part of the small subunit of the ribosome, responsible for the translation of messenger RNAs (mRNAs) into proteins. These genes are not submitted to lateral gene transfer, and contained conserved and variable regions termed V1 to V9.
This allows to analyze the composition and the abundance of taxa, which are groups of closely related organisms, using a sequence similarity criterion. A deeper investigation of microbiomes can be reached by metagenomics, which analyzes the whole set of genes present, leading to the composition, but also to the whole set of functions potentially displayed by the microbiome.

How to interpret changes in the abundance of specific taxa, drifts in microbial community profiles or potential alteration in microbial functions? Hugerth & Andersson (2017) provide a comprehensive analysis of how sequencing data are obtained and processed for microbial community analysis.
Next-generation-sequencing data are usually interpreted in terms of alpha- and betadiversity. Alphadiversity will refer to the diversity within a single type of sample based on replicates (Whittaker 1960). This diversity is characterized by an estimation of the richness (number of sequence, Chao 1 estimator) or as richness and evenness (e.g., the Shannon diversity index). Evenness corresponds to the regularity of the presence of a taxon in a community. Apart from the fact that it is difficult to correctly estimate alphadiversity, the interpretation of this data is hampered by the preconceived idea that higher diversity is better. The temptation to conclude to drama is great when comparing the richness of a control sample to that of a treatment. Shade (2017) advises to consider these data as a starting point for further inquiry of ecological mechanisms rather than an 'answer' to community outcomes.

Betadiversity measures the extent to which two samples are different. For this purpose, different tools based on metrics, allow to measure the distance between microbiomes, based on OTU (operational taxonomic unit) abundance and/or on phylogenetic distance. Changes in defined taxa, and shifts in community profiles can be detected. However, it is difficult to interpret the meaning and consequences of these changes on soil functioning. The role and importance of taxa in an ecosystem is not always related to their abundance. As example, rare microorganisms with an abundance less than 0.1%, could act as a reservoir to rapidly respond to environmental changes and contribute to community stability (Shade *et al.* 2014). Moreover, inferring functional role of a microbial community basedon 16S rRNA partial gene sequence is unsatisfactory.

Beyond diversity patterns, interaction networks of ecological or functional associations between taxa are essential drivers of ecological community structure and dynamics. Keystone microbes are those whose interactions cascade through the community (Berry & Widder 2014). Some highly connected keystone taxa can be good predictors of whole-community compositional change under environmental disturbance (Herren & McMahon 2018).

#### o Microbial Enzymatic activities

The interaction between soil and pesticides may result in altered biochemical processes driven by microorganisms. Soil contain many enzymes, as free, immobilized and extracellular or intracellular entities. Soil enzyme activities are soil quality indicators, playing many roles in nutrient element cycling and organic matter decomposition (García-Ruiz *et al.* 2008; Karaca *et al.* 2011). Thus, soil enzyme activities are good biological responses to analyze the soil response to a stress such as pesticides.

Soil enzymes have a crucial role in element cycling such as C cycle (glycosyl hydrolases, oxidases, and peroxidases), N cycle (proteases, peptidases, urease, and chitinase), P cycle (phosphatases) and S cycle (arylsulfatase). Dehydrogenase are intracellular enzymes found in all living organisms that are involve in energy transfer in microbial metabolic reactions and biological oxidation of soil organic matter. They are widely used as an indicator of overall soil microbial activity (Wolińska *et al.* 2015).

## III. Impact of nanopesticides on non-target soil microorganims and microbiomes

Most of nanopesticides are systemic and are intended to be active inside the plant. However as nanopesticides are disseminated in the environment, soil microbiota and microfauna and plants become non-target organisms, and exposed to the impacts of these bioactive molecules.

Regarding nanopesticides impact, the standpoint of non-target organisms is still not already set in the literature. A Web of Science (WOS) bibliometric analysis (october 2019) of (nanopesticide* AND non-target) yields 23 references.

As non-target organisms, plant or microbes are not viewed with the same importance. The search for keywords nanopesticide* AND soil* AND microb* in WOS (october 2019) returned 12 references while (nanopesticide* AND plant*) yielded 106 references. The importance of soil microorganisms for ecosystem functioning remains greatly underestimated.

The chapter will focus on microbiome and microbiota as non-target organisms of nanopesticides.

**III. Impact of nanopesticides on soil microbiomes and microbial communities**

Different types of pesticides have been formulated as nanopesticides, including nanoformulations of conventional pesticides or nanomaterials as pesticides, many of them being metallic and metal oxide nanoparticles.

- **Nanopesticides based on metal and metal oxide nanoparticles**
  - Copper

The impacts of metal and metal oxide nanoparticles, on the microbiome of the soil and rhizosphere, has been widely studied, mainly with the envision of environmental pollution effects (Anjum *et al.* 2013; Simonin & Richaume 2015; Tian *et al.* 2019; Rajput *et al.* 2020). Among the most investigated in toxicity studies, nanoparticles based on $TiO_2$, Ag, ZnO, Cu and Fe rule the ranking.

Currently, two types of nanomaterials have resulted in nanoenabled commercial agrochemicals, available on the market: copper nanoparticles as fungicides to control diseases on fruit tree, vegetables and crops, and colloidal silver to treat fungal pathogens on seeds, tubers and vegetative plants (He *et al.* 2019).

We will focus on reports of the impacts of Cu- and Ag-based nanomaterials on soil microbial communities, especially those for which the doses tested were compatible with applications in agriculture, as nanofertilizers or nanopesticides.

Copper is both an essential nutrient for living organisms as plants and microorganisms, and a renowned biocide since ages. Some copper-based pesticides are currently authorized in organic farming as fungicides and bactericides on grapes, trees and fruits. Initially used as lime neutralized copper sulfate in the Bordeaux mixture to cure grapes infected with downy mildew (Millardet *et al.* 1933), copper-based pesticides can exist as copper hydroxide, cuprous oxide, copper oxychloride, copper ammonium carbonate, and copper octanoate. Indeed, as the solubility of copper sulfate favors phytotoxicity and decreases the persistence on the plant/tree leaves and fruits, and fungicide activity, less soluble forms known as fixed-coppers have been developed (*e.g.*, copper hydroxide, copper oxychloride, basic copper sulfate cuprous oxide, etc.). These fixed-coppers are particles whose size determines coverage and adherence to plant leaves, and release of copper ions. Initially marketed as micronized particles, copper nanosized particles have rapidly been developed and commercialized to improve the coverage of the plant fruits or leaves, and to control the release of Cu ions. Currently, at least two nanosized copper formulations are available : Kocide® 3000 (DuPont) and NANOCU (Bio Nano Technology) (He *et al.* 2019).

(Simonin *et al.* 2018a) assessed the impact of nanosized bare CuO (~50 nm, specific surface area 23 $m^2g^{-1}$, 0.1, 1, and 100 $mg.kg^{-1}$ dry soil) *vs* Cu ions ($CuSO_4$) in five agricultural soils with contrasting properties (pH between 6.4 and 8.21), to take into account soil biological complexity and physico-chemical diversity. Soil moisture was adjusted to the water holding capacity specific to each soil, and soil microcosms were incubated in the dark at 28°C, over 90 days. At the highest concentration (100 $mg.kg^{-1}$ dry soil), in the five soils tested, CuONPs cause significant reductions that worsen over time, on soil microbial activities involved in carbon and nitrogen cycles, respiration, nitrification, and denitrification. Lowest doses show limited effects, mostly at 90 days, with decreases of respiration in the sandy-loam soil from 1 $mg.kg^{-1}$, and in denitrification at 1 $mg.kg^{-1}$ in the loamy soil. Globally, denitrification is the

most sensitive microbial activity to CuONPs in most soil types, while soil respiration and nitrification are mainly impacted in coarse soils. CuONPs and ionic Cu show distinct impact on soil microbial activities, likely explained by the low dissolution of CuONPs, less than 2% in soil solution, over time. Thus at low and agricultural-relevant concentrations, CuONPs have limited effects on soil microbial activities involved in carbon and N cycles. Occasionally, coarse soil texture with low organic matter or clays contents, are more likely to be affected.
In this type of soil (loamy soil with low clay content), potentially more sensitive, enhanced with CuONPs (1 and 100 $mg.kg^{-1}$), Simonin *et al.* (2018a) grew winter wheat (*Triticum aestivum*) over 50 days in climatic chambers. The plant exudates stimulates heterotrophic microbial activities as microbial respiration and denitrification. However, this does not counterbalance or even worsen (*e.g.*, 1 $mg.kg^{-1}$ CuONPs for microbial respiration) the effects of CuONPs on these enzymatic activities. Thus the plant influences the microbial response to CuONPs exposure but does not mitigate the negative effects of CuONPs.
VandeVoort & Arai (2012) confirmed the toxicity of Cu-based NPs to nitrifiers and the very different behaviour between CuONPs and $Cu^{2+}$ ions in terms of $Cu^{2+}$ release, adsorption and impact on nitrification in batch nitrification kinetic experiments.

Asadishad *et al.* (2018) investigated the impact of nanosized CuO and Cu ions on soil enzyme activity and microbial community composition of a biosolid-amended agricultural soil, over 30 days. Surface soil (pH 6.7) was sampled from an agricultural site at the Macdonald campus of McGill University (most likely sandy loams, loamy sands or clay soils based on Collaborative Geographic Information Systems, Authors' note) amended with a biosolid from a waste water treatment plant, was enhanced with bare CuONPs (40 nm) at 1, 10, and 100 mg total $CuNPs.kg^{-1}$ soil. In soil solution, CuONPs dissolution occurs within the first 2 h (70%) and remains stable up to 30 days, likely because of soil dissolved organic matter binding to reactive sites on the NP surface.
The activities of five soil extracellular microbial enzymes involved in C, N and P nutrient cycling were measured in the soil amended with biosolids and exposed to bare CuONPs or Cu ions at 2 h, and 30 days after treatment with the NPs suspensions or ionic solutions. After some transient inhibitory at 2h, no significant enzyme inhibition is observed for the soil-biosolids slurry exposed to CuONPs after 30 days. CuONPs and $Cu^{2+}$ show similar effects on soil enzyme activities at short term but CuONPs tends to stimulate some enzyme activity at longer exposure time, suggesting a specific nanoeffect. Over 70% of the CuONPs was dissolved at 2 h, and this dissolution increased to 77% in 30 days suggesting that most of the CuONPs ended up as $Cu^{2+}$ or Cu organic complexes explaining their similar trends for some of the enzymes. The initial decrease in enzyme activity observed at 2 h may be linked to the antimicrobial activity of $Cu^{2+}$ and CuONPs. Nonetheless, these data shows that the activity of the five extracellular soil enzymes generally recovers after 30 days of exposure to CuONPs.

Kocide® 3000 (Dupont) is fungicide/bactericide based on copper hydroxide, approved by the US EPA for citrus, conifers, field crops, small fruits, tree crops, vegetables, vines and some other fruits. Kocide® 3000 contains micronized particles made from nanosheets of $Cu(OH)_2$ embedded in a carbon-based matrix that promptly dissociates in water (Adeleye *et al.* 2014).

Simonin *et al.* (2018b) designed outdoor terrestrial mesocosms with a sandy-clay-loam soil (57.7% sand, 20.5% clay, 21.9% silt, 4% organic matter, pH = 5.8) seeded with seven forage crops composed of forbs, graminoids, and legumes as representatives of the three main plant functional groups. To assess the environmental impacts of sequential applications under low-input or conventional farming scenarios, the nanopesticide was applied alongside three different mineral fertilization levels (Ambient, Low, and High). The foliage of forage was sprayed with the Kocide® 3000 suspension (6.68 $mg.L^{-1}$ in water, 30 $mg.m^{-2}$, at Day 0, 75, and 155, and 15 days before each subsequent plant harvest). The mean particle size was 38.7 ± 8.2 nm (TEM) and an average hydrodynamic diameter of 120 ± 30 nm in the dosing water with a secondary peak with particles size greater than 700 nm (Simonin *et al.* 2018c). The authors monitored enzymatic activities involved in C, N, P and S cycling, soil $N_2$ fixation rates (conversion of molecular $N_2$ in the air to ammonia or nitrogenous compounds available to the plant) and mycorrhizal colonization of plant roots, over a year. The authors report no detrimental effects on the forage biomass and mycorrhizal association with plant roots. However, they evidence a dual, beneficial or negative, interactive effects between nanopesticide and fertilization treatments on extracellular microbial enzymatic activities. In the Ambient fertilization, Kocide® 3000 applications transiently inhibited enzyme activities at short term (15 days) and decreased P and C cycling at long term (6 months after the last Kocide® 3000 applications), while positive effects on plant biomass and enzyme activities occurred in the High fertilization treatment. In Ambient fertilization, the authors hypothesize that at short term, nutrient limitation combined to the copper biocide activity could decrease the ability of microbial community to cope with the stress. At long term, the decrease of enzymatic activities could be related to responses to Kocide® 3000 driven by seasonal effects and low water availability.

At long term, Kocide® 3000 treatment stimulated or unaffected enzyme activities in the Ambient and High fertilizations. This could arise from the adaptation of the microbial community to Cu, with the selection of Cu-tolerant species, and the depletion of resources in soil, with a nutritional effect of Kocide® 3000 and contained micronutrients.

The authors conclude on limited or positive effects of repeated Kocide® 3000 applications on forage production and soil microbial processes in conventional farming with high fertilization rates, but they warn about detrimental effects on microbially mediated soil processes involved in C and P cycling and on forage production in the context of lower-intensity fertilization (e.g., organic farming). This study of the impact of Cu-based nanopesticide on the microbial compartment is certainly the most complete, examining the impact of sequential applications over a growing season in an outdoor mesocosm. However, it would be interesting to verify the last conclusions in soils under organic farm, using fertilizers suited for this mode of cultivation. Here the soil was supplemented with an inorganic fertilizer**,** while in organic farming, fertilizers are usually derived from animal and vegetable matters or agricultural practices.

Zhang *et al.* (2019) applied a commercial $Cu(OH)_2$ nanopesticide formulation, the active ingredient of this formulation, the synthesized $Cu(OH)_2$ nanotubes with comparable morphology to the active ingredient, and $CuSO_4$ to a silty soil (pH 8.17, organic content 3.4%) at 0.5, 5 and 50 $mg.kg^{-1}$, followed by an application of neonicotinoid thiacloprid, an insecticide, after an interval of 21 d. The overall pattern of soil bacterial community composition shows that $Cu(OH)_2$ nanopesticides at 50 $mg.kg^{-1}$ significantly decreased the alpha-diversity of bacteria in soil and drastically altered the community composition. The relative abundance of *Gemmatimonas* decreased by ~30% in soil with $Cu(OH)_2$

nanopesticides 50 mg.kg$^{-1}$ as compared to control. Their relative abundance showed a significant positive correlation ($r=0.89$, $p < 0.05$) with the degradation rate constant of thiacloprid. The $Cu(OH)_2$ nanopesticides reduced nitrile hydratase activity and downregulated thiacloprid-degradative *nth* gene abundance that contributes to the mitigation of thiacloprid degradation. The authors suggest to reconsider the use of nanopesticides based on $Cu(OH)_2$. However, in this study, the authors used a concentration of $Cu(OH)_2$ that is ten-fold the recommended dose of this nanopesticide (5 mg.kg$^{-1}$). Moreover, the Cu applied (50 mg.kg$^{-1}$) was high as compared to the Cu background (4.1 mg.kg$^{-1}$), while in Simonin *et al.* (2018b) the Cu amount applied to the mesocosms (5.43 mg/mesocosm containing 81 kg of soil) was much lower than the background concentration (90.5 mg.kg$^{-1}$). The presence of background Cu in soils may select tolerant communities, which would be less affected by the additional addition of Cu.

Assessing how CuNPs may interact with pollutants and pesticides in soil, Parada *et al.* (2019) incubated CuNPs (40–60 nm) at 0.05 and 0.15% w/w and ATZ (3 mg.kg$^{-1}$) in an Andisol (a soil rich in organic matter) for 30 days. Microbial community profiles assessed by PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) on bacteria, fungi and nitrifying bacteria, remained relatively stable throughout the experiment However, CuNPs at 0.15% w/w caused a significant decrease in ATZ dissipations showing an increase in the persistence of ATZ in soil. This persistence was mostly associated to physical-chemical interaction with soil particles.

Paddy soils are typical soils agricultural soils in China, and are under periodical flood–dry water management, constantly changing redox potential in the soil environment. Shi *et al.* (2018) exposed two paddy soils (organic content 4.1 and 8.01%) to CuONPs (hydrodynamic diameter in water 240.0 nm) and CuO bulk particles (BP, average particles size of 1346 nm) at 10, 100, and 1000 mg.kg$^{-1}$ for CuONPs and 1000 mg.kg$^{-1}$ for CuOBPs. The authors show differentiated behavior between NPs and BPs in paddy soils and a role for the organic matter. Microbial available Cu was higher for CuONPs than for CuOBPs. In the low organic matter soil, CuONPs changed the soil properties by increasing the pH and Eh, accelerated the degradation or mineralization of the organic matter, as well as the Fe reduction process, by increasing the Fe(II) content by 293% after flooding for 60 days. The microbial biomass carbon in both soils was severely inhibited by CuO NPs and to a minor extent by BPs at 100 mg.kg$^{-1}$. The organic matter could partly mitigate the negative effects of CuO NPs.

For a complete review of copper-based nanoparticles implication on terrestrial and aquatic environment, see Rajput *et al.* (2020).

- **Silver**

Silver is known as a biocide since ages. Silver-based nanopesticides show antimicrobial/biocidal properties against a broad of classes of microorganisms, *e.g.*, bacteria, fungi and virus (Durán *et al.* 2016).
Some silver-based nanopesticides are already patented and commercial in the technology of plant protection, the processing of seed material, and the enhancement of plant development. Some examples are WA-CV-WA13B, WA-AT-WB13R, and WA-PR-WB13R (Bio-Plus Co.Ltd.,Pohang, Korea), and Zerebra® agro, Zeroxxee®, Silver leaf, Zeromix®

(AgroKhimProm Group, Russia and Commonwealth of Independent States, Grand Harvest Research Innovation Company). Even if these nanopesticides are claimed to effectively inhibit phytopathogen diseases in a broad set of plants, to strengthen the plant immune system, and to reduce stress reduction (Jung *et al.* 2010; Parada *et al.* 2019), most of the published knowledge on the impact of silver-based nanopesticides on non-target microbes and microbiomes originates from studies on the environmental impact of AgNPs.

Hund-Rinke *et al.* (2019) amended a loamy, acidic sand (73% sand, 22% silt and 5% clay; pH 5.6, low organic matter content, 1.1%). with biosolids and AgNPs (NM-300K dispersed in a mixture of a stabilizing agents, particle size of ~ 15 nm, 99%) to achieve a target concentration of 0.19 to 15 $mg.kg^{-1}$ soil. Soil samples amended with biosolids and AgNPs or standard ionic solutions were kept static in the dark at 22 °C for up to 30 days. The impact of AgNPs wad assessed by soil respiration (Micro-Resp test), exoenzyme activity, potential ammonium oxidation (PAO) test and next-generation sequencing to survey bacterial diversity by sequencing the 16S rRNA gene. The four tests showed similar sensitivity towards the silver nanomaterial, with significant effects at AgNPs concentrations from at least 1.67 $mg.kg^{-1}$. The authors evidenced no differences in the Shannon index or evenness as indicators of alphadiversity. However, next generation sequencing evidenced a different sensitivity of bacterial orders, and shift in the microbial community, with an enrichment of Proteobacteria (*Caulobacterales*, *Burkholderiales*, and *Xanthomonadales*), *Cytophagales* and *Sphingobacteriales*. The adverse impact on some nitrifiers (*Nitrosomonadales*) matched the inhibition of PAO activity.
Examining the long term effect of these AgNPs (140 days) on ammonium oxidizing bacteria (AOB), Schlich *et al.* (2018) incubated AgNPs (NM-300K, diameter~15 nm, and a small proportion at ~95 nm) and $AgNO_3$ added to a sandy loam soil (pH 5.61, 0.93% organic content) at 0.56, 1.67 and 5 $mg.kg^{-1}$ dry matter soil. At 1.67 and 5 mg $mg.kg^{-1}$ AgNPs, they show a relative inhibition of AOB starting from day 14, which increases up to 140 days, while inhibition occurs from day one and increases over time, even at the lowest dose (0.56 $mg.kg^{-1}$) in the case of silver ionic form.

Vitali *et al.* (2019) analyzed the effects of AgNPs on the phyllosphere and rhizosphere associated microbiota of a black poplar tree. Nanopowder, amorphous-carbon-coated with Ag nanoparticles (1 $mg.L^{-1}$,average particle size ~25 nm, specific surface area 23 $m^2$ $g^{-1}$, dispersed in water with a soap surfactant) were chronically supplied at leaf and root level of three-year-old poplar trees (3 m, 15 L pots filled with soil fertilizer mixture) over 10 weeks (4 weeks with single supply followed by 6 weeks with twice supply). The final concentration exposure of plants to AgNPs was 16 mg $L^{-1}$ (volume not indicated) in both leaf and root treatments (surface of the pot estimated to 615 $cm^2$, Authors' note). The soil was protected during foliar exposure, and no fertilizer was added during the time of the experiment. The author used next generation sequencing of the V3 -V4 region of 16S rRNA and the ITS 1 region to analyze the bacterial and fungal microbiomes, respectively. Leaf AgNPs treatment increased bacteria and fungi evenness and determined a significant reduction in both microbial groups, while root AgNPs treatment reduced the bacterial and fungal biodiversity. Bioinformatics functional analysis showed that AgNPs treatment reduced the aerobic and stimulated facultative anaerobic and oxidative stress-tolerant bacteria. However, in this study, the AgNPs treatments mimicked a polluted environment and not an agricultural treatment with Ag nanopesticide. As example, Ag concentration in Zerebra® Agro, a commercial silver-based nanopesticide, is 0.5 $g.L^{-1}$ and the recommended dose for plant treatment is 0.1 $L.t^{-1}$ in seed, and 0.1$L.ha^{-1}$ (50 $mg.ha^{-1}$) for application in vegetation period on agricultural crops from 1 to 3 times, instead of 20 $g.ha^{-1}$ in Vitali *et al.* (2019) study (assuming at least 100 ml were used).

Asadishad *et al.* (2018) investigated the impact of AgNPs (50 nm citrate-coated AgNPs) and their dissolved ions on soil enzyme activity and microbial community composition of a biosolid-amended agricultural soil. Surface soil (~35 cm depth, pH 6.7) was collected at the Macdonald campus of McGill University amended with a biosolid from a municipal wastewater treatment plant (soil/biosolid weight ratio 50/1). AgNPs were added at 1,10, and 100 mg total $AgNP.kg^{-1}$ soil. Dissolution occurred within the first 2 h and remained stable up to 30 days. At short term (2h), AgNs showed no effect at 1 and 10 $mg.kg^{-1}$ extracellular enzymatic activities implicated in P, C and N cycling. At 100 $mg.kg^{-1}$, AgNPs moderately impacted these enzymatic activities as compared to $Ag^+$, likely because only 37% of the AgNPs was dissolved at 2 h. The microbial community of the soil was analyzed by 16S rRNA gene amplicon sequencing after 2h and 30 days of exposure. The relative abundance of the *Gammaproteobacteria* class was significantly higher for $Ag^+$ ions and AgNPs at 100 $mg.kg^{-1}$ soil than in all other treaments. The *Alphaproteobacteria* community responded differently to dissolved Ag and AgNPs, with a decrease in the relative abundance $Ag^+$ 100 $mg.kg^{-1}$ soil.

Also focusing on long term experiments, Grün *et al.* (2018, 2019) incubated at 15 ± 4.5)°C over a period of one year, AgNPs, (BAM-N001 AgPure) with concentrations ranging from 0.01 to 1 mg $AgNPs.kg^{-1}$ soil from an arable field cultivated with wheat. The soil was classified as a loamy soil (pH 7.1 in $CaCl_2$, clay content of 17%–30%, total organic content 2.8%).The toxicity of AgNPs to the microbiota was indicative of the time-dependent reactivity in the complex physicochemical soil system. Over time, AgNPs (0.01 $mg.kg^{-1}$) have short-term (1 day and 1 week) stimulatory effects on *Acidobacteria*, *Actinobacteria* and *Bacteroidetes*. After one month, *Actinobateria* are negatively impacted. The relative abundance of *beta-Proteobacteria* is decreased from the first day of incubation until to the end of the experiment (one year). On the average, for the three concentrations tested, the negative effects were the highest for *beta-Proteobacteria* and *Bacteroidetes*. *Actinobacteria* and *alpha-Proteobacteria* were statistically unaffected by AgNPs treatments after 1-year exposure.
Globally, the author report fluctuations of positive and negative effects over time with a strong toxicity event at 90 days and a decline of silver toxicity on some bacterial phyla at day 28, 180 and 365 at the different concentrations tested. These trends are likely explained by potential transformations such as changes in aggregation and oxidation state, dissolution, sulfidation, sorption of inorganic and organic species that result in a transient pattern of dissolution or stability of AgNPs. In response to these events, the bacterial community showed transient resistance and resilience mechanisms.

Grün *et al.* (2018) show that one year of exposure to 0.01 mg $AgNPs.kg^{-1}$, negatively impacted the microbial soil community involved in nitrogen, with a decrease in the abundance of AOB (*amoA* gene copy numbers), the leucine aminopeptidase activity (N substrate turnover), and the abundance of nitrogen fixing microorganisms (*nifH* gene copy numbers).

Guilger *et al.* (2017) biogenically synthesized silver nanoparticles using the fungus *Trichoderma harzianum.* The AgNPs (spherical nanoparticles size distribution between 20 and 30 nm by scanning electron microscopy, $0.15.10^{12}$ and $0.31.10^{12}$ $NPs.mL^{-1}$) were incubated $0.15.10^{12}$ and $0.31.10^{12}$ $NPs.mL^{-1}$ in an agricultural soil (pH 6.8, 14% organic content) at 25°C for 6 months. The authors quantitatively followed overtime the distribution and abundance of several genes involved in the nitrogen cycle (Figure 6.2): *nifH* (nitrogen fixation), *amoA* (nitrification), *nirK*, *nirS*, and *narG* (first stage of denitrification), and *cmorB* and *nosZ* (second stage of denitrification).
Over time, the authors evidence a sequential modulation of the abundance of bacteria and genes involved in N cycle in the samples exposed to the biogenic AgNPs. During the first 30 days, a higher increase in the abundance of bacteria in the samples exposed to AgNPs than in the control sample is observed, but the distribution of genes stay comparable. Over time, this increase in the abundance of bacteria still happens, which could traduce a stimulation of bacteria involved in N cycle in the samples exposed to biogenic AgNPs. At 90 days, differences do occur in the distribution of genes, with decreases in the bacteria producing nitrate reductases (*narG*) that persists up to 180 days, and reduction nitrogenase reductase enzymes (*nifH*) and oscillations in the proportions of *nifH* and up to 180 days. Bacteria that presented the *cmorB* nitrate reductase genes increased up to 90 days post-exposure and decreased after this period, while the bacteria that presented the nitrous oxide reductase gene (*nosZ*) oscillated in the opposite way, increasing for the first two periods and decreasing for the last two periods (90 and 180 days). The coating of the nanoparticles may have retarded the release of $Ag^{+}$, which could explain possessed a coating, which could have delayed the release of $Ag^{+}$ and explain the latency phase observed in the changes in abundance of bacteria and genes involved in the nitrogen cycle. Thus the impact of the biogenic AgNPs tend to show a stimulation of bacteria involved in N cycle together with some cycles of impact and recovery of the community.

VandeVoort *et al.* (2014) incubated AgNPs (PVP coated 50 nm and 15 nm) at 1, 10 and 100 $mg.L^{-1}$ in a Toccoa soil (AgNPs display near 100% sorption onto Toccoa soil surfaces at all concentrations used for the denitrification experiments). PVP coated 50 nm AgNPs did not show significant differences in NO3 -depletion rate from the control condition at any concentration while the smallest PVP coated 15 nm AgNPs showed the greatest differences from the control condition in the reaction rate and a concentration dependent inhibition. At 1 $mg.L^{-1}$ the depletion rate was not significantly different than that of the control, and it took 68 h to achieve 90% $NO_3$ depletion, while at 10 $mg.L^{-1}$ and 100 $mg.L^{-1}$ it took 111h and 194h respectively. The dissolution of 15 nm AgNPs was an order of magnitude greater than the larger AgNPs and they displayed a better colloidal stability. Phase transformation readily occurred in 15 nm AgNPs as ~ 75% of Ag(0) speciation in pAg15 was changed to $Ag_2S$ and Ag(I) sorbed humic acid during the incubation period. The Ag speciation changed to a much lesser extent 50 nm AgNPs. These results show designing the NPs characteristics and the dose, denitrification can be unaffected by AgNPs.

AgNPs can undergo phase transformation in the aquatic environment and in soil, especially sulfidation (Hashimoto *et al.* 2017). Judy *et al.* (2015) investigated the impact of AgNPs, focusing on different Ag speciation and NPs coating. They exposed a biosolids-amended sandy loam soil (pH 6.8) to 1,10, or 100 mg $Ag_2S$ NPs, polyvinylpyrrolidone (PVP) coated AgNPs and $Ag^+$. The soil mixture was inoculated with a commercial inoculum or an arbuscular mycorrhizal fungi (AMF) , prior to sewing tomato seeds (*Solanum lycopersicum*). The authors monitored the colonization of tomato roots by the fungi), overall microbial community structure in biosolids-amended soil using neutral lipid fatty acids (and phospholipid fatty acids analysis, and ammonium nitrate extractable Ag concentrations. Except for three treatments (100 $mg.kg^{-1}$ for Ag-PVP NPs and $Ag^+$ and 10 $mg.kg^{-1}$ for AgS NPs), mycorrhizal colonization of tomato roots for all Ag treatments at 1 mg $kg^{-1}$ and 10 $mg.kg^{-1}$ was not significantly different compared to the control. The microbial community was affected even at 1 $mg.kg^{-1}$ for Ag-PVP NPs and $Ag^+$, and $Ag_2S$ NPs with an impact on fungi and bacteria, among them Actinomycetes.

The overuse of antibiotics in medical treatment and animal fodder have generated the occurrence and dissemination of antibiotic resistance genes (ARGs) in the environment (Allen *et al.* 2010; Marshall & Levy 2011). The primary mechanism of ARGs dissemination, is horizontal gene transfer (HGT) between cells. At environmentally relevant and sub-lethal concentrations, AgNPs and ionic silver $Ag^+$ can facilitate the conjugative transfer of plasmid-borne ARGs across bacterial genera (Lu *et al.* 2020). Moreover, heavy metal and biocides can also promote the proliferation of ARGs via co-selection (Seiler & Berendonk 2012; Zhu *et al.* 2013; Baker *et al.* 2017). This prompted to investigate the potential ecological risks of environmental levels of AgNPs as an abiotic pressure to co-select antibiotic resistance genes (ARGs) or promote plasmid transfer between bacteria by horizontal transfers. Chen *et al.* (2019) used high throughput quantitative PCR to analyze the effect of AgNPs (100 ppm) on the co-selection pressure of ARGs in the rhizosphere and phyllosphere of 3 months aged *Coriandrum sativum* L growing on a soil (pH 6.69) containing Cr, Cu Zn and Pb, and exposed to (~20 nm and ~50 nm) AgNPs. The exposure to AgNPs did not induce any significant increases in the total abundance of ARGs in either the rhizosphere or phyllosphere. However, the overall pattern of resistome was shifted following AgNPs application, with a significance increase in the relative abundance of efflux pumps genes, which is an important mechanism for co-selection of antibiotic resistance genes by heavy metals.

- Others nanopesticides based on inorganic nanomaterials

Other nanopesticides are envisaged, based on nanomaterials of $TiO_2$, ZnO, $CeO_2$, Si NPs and even carbon nanotubes. For reviews on environmental impacts on microbiota of these NPs see Liné *et al.* (2017) and Tian *et al.* (2019).

- Tentative conclusion on Ag- and Cu-based NPs in agriculture

Altogether these data could tend to underline that Cu- and AgNPs can drastically shift the composition of microbial communities, and alter the activities of extracellular enzymes involved in element cycling. However, except one (Simonin *et al.* 2018b), many of these studies were dedicated to environmental impact of NPs and not to evaluate the impact of Cu- and Ag-based nanopesticides on off-target soil microbiota. At agronomical relevant concentrations and use, Kocide® 3000 ($Cu(OH)_2$) and CuONPs (0.1 $mg.kg^{-1}$, (Simonin *et al.* 2018a; Simonin *et al.* 2018b) showed limited effects on soil microbial activities involved in carbon and nitrogen cycles.

For AgNPs, based on commercial AgNPs nanopesticides as Zerebra Agro® (Patent of the Russian Federation 2419439 as of 27.05.2011), the concentration targeted for agronomical applications is estimated to 0.2 $mg.kg^{-1}$ (assuming a dispersion of AgNPs on a bulk soil density of 1.2 $mg.cm^{-3}$, and a soil depth of 20 cm). At concentration close to this operational concentration, Grün *et al.* (2018, 2019) evidenced some long term impact on Proteobacteria and bacteria involved in N cycle. Note that AgNPs used in this study are AgPure®, which are designed for the antimicrobial functionalization of surfaces and bulk materials. Zerebra Agro® is composed of silver NPs modified with polyhexamethylene biguanidine, a polymer also endowed with biocide properties.

The behavior and fate of Cu- and AgNPs in soil depends on variables inherent to the NPs, *e.g.,* particle size, surface charge, isoelectric point (pH at which the NPs carry not net electrical charge) and extrinsically related to the properties of the complex soil matrix. The shape of nanoparticles is a big player in governing the dissolution, and the interactions with cells. The properties of AgNPs, and NPs in general, can differentially affect the composition and functions of microbial communities depending on the level of exposure (Zhai *et al.* 2016).

Globally, the NPs can experience dissolution, transformation (oxidation and reduction), aggregation with soil colloids, adsorption especially on clays, (for a review, see Anjum *et al.* (2013) and reference inside). Important parameter that control the fate of Ag and Cu-based NPs, are the soil texture, clays are key players in the retention of NPs (Cornelis *et al.* 2014), pH, organic content, divalent cations, etc.. High soil pH value increase the number of negatively charged sites and enhance Ag-sorption, while low pH tend to promote the dissolution of AgNPs. As shown by Schlich & Hund-Rinke (2015) in a variety of soils, AgNPs toxicity towards microbial activities such as substrate-induced respiration and to ammonia oxidizing bacteria declined with increasing clay content and increasing pH. Simonin *et al.* (2018a) also conclude on the same line about occasional impacts of CuONPs at agricultural relevant concentration, on coarse soil texture with low organic matter or clays contents. For the record, acidic soils occupy approximately 30% of the world's ice free land area but only about 4.5% of the acid soil area is used for arable crops (von Uexküll & Mutert 1995). The use of acidic soil can favors the dissolution of Cu- and AgNPs with the release of free ions, that can enhance the short term impact of the nanos. In many studies commented in this chapter, the soils used were acid, and contained low clay contents, which make them worse case studies.

An interesting results from the literature is that the ionic or nano form of the pesticide can show differentiated impacts, likely related to the fraction of ions released (*e.g.,* Asadishad *et al.* 2018). Some authors already pointed that ionic and nanoforms of a metal may show similarities and differences, in the mode of antibacterial activity (Kędziora *et al.* 2018) or in the impact on a microbial community extracted from a soil and exposed in vitro to AgNPs (Zhai *et al.* 2016).

In long-term studies, the toxicity of NPs is kinetic and seems related to dissolution or transformation events in the soil, that lead to transient adjustment and adaptation of the microbial community. As evidenced by VandeVoort *et al.* (2014), tuning the surface properties of NPs could help to control the dissolution and phase changes, and likely to reduce the toxicity towards microbial cells.

As shown by Guilger *et al.* (2017), promising direction probably relies on biogenic nanoparticles, that show minimal impact on human cells, and denitrification, but strong activity toward a set of plant pathogenic fungi.

Among microbial activities that may be affected by NPs, denitrification ranks first. At a microscale level, soil structural organization provides diverse niches that are favorable to bacteria with different needs (aerobic or anaerobic) and lifestyle. Examining the localization on denitrifiers in soils, Lensi *et al.* (1995) showed that the <2 µm fractions contains a moderate density of denitrifiers, with high specific activity while the 20-2 µm fraction contained microaggregates and exhibited the highest microbial biomass C and organic N content and a high density of denitrifiers, with a moderate specific denitrifying activity. The main factors positively influencing denitrification are the absence of oxygen, the availability of nitrate and carbon (source of electrons) (Zumft 1997). Denitrifiers are also sensitive to pH (ŠImek & Cooper 2002), and hydric conditions (Szukics *et al.* 2010). Denitrification is favored at lower soil redox potential values, which in turn is related to soil texture (Kunickis *et al.* 2010). Sandy textured soils generally show redox values too high for denitrification, while clayey textured soils provided lower redox values that were within the range for this biological transformation. VandeVoort & Arai (2012) related negative impacts on denitrification to the silver nanoparticle affinity for soil surfaces and to the physicochemical properties e.g., size, coating, sedimentation rate, solubility, surface charge properties, dispersibility (VandeVoort *et al.* 2014), showing that AgNPs properties could be tuned to avoid impact on denitrification. Hence, the biogenic AgNPs synthetized by green process (Guilger *et al.* 2017) did not show dramatic impact on the nitrogen cycle.
An understanding of the microbiome interactions with NPs at a microscale level could support a better design of the structure and properties of the NPs.

- **Impact of nanopesticides based on nanoformulation of pesticides**

Nanotechnology has the potential to positively impact the agrifood sector, minimizing adverse problems of agricultural practices on environment and human health, improving food security and productivity (Fraceto *et al.* 2016). In this context, nanocapsulated formulations, nanoemulsion, nanogel of conventional pesticides and metal and metal oxide nanoparticles have been designed in order to control the release of the active ingredient, favor adsorption on plant leaves and reduce the dose, protect the active molecule (Fraceto *et al.* 2016; Chhipa 2017). While the impact of metal and metal oxide nanoparticles on the soil microbiota as non-target organism have been addressed in the literature, those of nanoformulation of pesticides still stay poorly documented.

Liu *et al.* (2014) synthesized a new nanopesticide CM-ß-CD-MNPs-Diuron (average diameter of 25 nm by TEM) from an inclusion complex of cyclodextrin-$Fe_3O_4$ magnetic nanoparticles as host and diuron as guest molecules. Their potential toxic effects on soil microbiota was evaluated by microcalorimetry, measure of urease enzyme activity and qPCR. By recording heat flow rate of microbial growth, microcalorimetry provides information on microbial biochemical processes and evaluate the metabolism of microbial biomass in soil. Soil samples (1.00 g) in ampoules were spiked with glucose and ammonium sulfate were exposed to different concentrations of CM-ß-CD-MNPs-Diuron (5.00, 20.0, 80.0, and 150 $mg.g^{-1}$ in dry soil samples) at 28°C. CM-ß-CD-MNPs-Diuron leads to the inhibition of the metabolic activity of microorganism in soil. Urease catalyzes the conversion reaction of urea to carbon dioxide and ammonia, leading to available nitrogen for plants. There was a significant effect ($p<0.05$) of CM-ß-CD-MNPs-Diuron on the urease enzyme activity at 7, 14 and 21 days of incubation. Real-time qPCR and universal probes were used to quantify the impact of different concentration (0.00, 5.00, 20.0, 80.0, and 150 $mg.g^{-1}$) of CM-ß-CD-MNPs-Diuron on population size of the microorganism community in soil for 21 days. The abundance the soil bacterial community decreases when the dose of CM-ß-CD-MNPs-Diuron increases while for *Actinobacteria*, the population does not change significantly at the different doses. Diuron has a negative effect on the microbial population (Prado & Airoldi 2001) and iron-based nanoparticles are toxic to bacterial community of soil due to the generation of reactive oxygen species (He *et al.* 2019; Guilger *et al.* 2017). Altogether, these results show CM-ß-CD-MNPs-Diuron exerts a stress on soil microorganism and that encapsulation of Diuron did not help to decrease the toxicity.

As a counter-example Maruyama *et al.* (2016) encapsulated Imazapic and Imazapyr herbicides in alginate/chitosan and chitosan/tripolyphosphate nanoparticles (an average size of 400 nm). These systemic herbicides are used to control weeds in many crops, and are used as combination to bypass the resistance of plants. An agricultural soil was sampled and exposed to the herbicides using doses equivalent to the application rates employed in the field.
The impact of the formulations on soil was assessed by qPCR of genes involved in nitrogen cycle. Quantification of *nifH*, *nirk*, *nirS*, *narG*, *norB*, and *nosZ*, bacterial genes in the soil samples treated with the nanoparticles for 7 and 30 days showed that the encapsulated herbicides were less toxic, compared to the free Imazapic and Imazapyr compounds.

Essential oils are a promising option for substituting the synthetic pesticides used in agriculture. Neem oil is effective against a wide range of pests, exhibiting a broad spectrum of action due to its systemic and transmembrane activities. However, its use in the field is limited by its short persistence in the environment (Shah *et al.* 2017; Kumar *et al.* 2019). Pascoli *et al.* (2019) formulated neem oil loaded zein nanoparticles as spherical particles of 100-200 nm (atomic force microscopy). Zein is a corn protein. The impact of these NPs on soil microbiota was also assessed by qPCR of specific genes from nitrogen cycle bacteria: *nifH*, *amoA* (encoding ammonia monooxygenase, nitrification enzyme: conversion of ammonia to hydroxylamine), *haO* (encoding hydroxylamine oxidase, nitrification enzyme: oxidation of hydroxylamine to nitrite), *narG*, *nirK* and *nirS*, *cnorB* and *nosZ*. No change in the number of genes which encode nitrogen-fixing enzymes and denitrifying enzymes was detected, suggesting no effect on soil bacteria involved in nitrogen cycle. The encapsulation in zein nanoparticles reduced the genotoxicity of neem oil to *Allia cepa* and nullified the toxicity in *Caenorhabditis elegans*. Thus encapsulation of the herbicides could improve their mode of action and reduced their toxicity.

Heconazole is a pest control and a plant growth regulator. In order to reduce its adverse effects in some plants (Kumar *et al.* 2015) have developed nanoparticles of hexaconazole using polyethyleneglycol-400 (PEG) as the surface stabilizing agent. The nanoparticles show an average size of 100 nm (SEM). The impact of heconazole NPs on non-target soil microbiota was assessed by measure of the quantities of ammonium, nitrite and nitrate-nitrogen as indicators of soil nitrification activity. Ammonia and nitrite oxidizing bacteria are unaffected by heconazole NPs, and commercial formulation of heconazole.

- Different nanovectors of pesticides and different impacts

The different examples discussed above show that in most cases, the nanoformulation of pesticides and herbicides using organic polymers, improved or did not worsen the impact of the active ingredient on non-target soil microbiota. Compared the inorganic metal and metal oxide nanoparticles discussed in the first part, the average size of these pesticides encapsulated in polymers are far higher than those of the inorganic nanoparticles and these organic formulations seem safer toward nitrogen cycle (nitrification and denitrification).

Regarding nitrogen cycle, many studies focus on the abundance of nitrification and denitrification genes, using qPCR. Taking advantage of the diversity revealed by metagenomic in microbial functional groups, Ma *et al.* (2019) reevaluated the coverage of existing DNA primers for denitrification functional genes, using *in silico* approach. They confirm that the existing primers reveal a partial vision of the denitrifiers community. As example, the non-specific coverage of fungi lead to underestimation of the potential importance of fungal denitrifiers.

## IV. Conclusion and future directions

Nanotechnology sounds promising to decrease pesticides impact on non-target soil microorganisms. There is a great potential in modulating the surface of the NPs, to tune their properties, their interaction and fate in soil. Encapsulation of active ingredients in polymers, formulation of biogenic NPs, and designing the properties of NPs to reduce their impact appear as promising opportunities.

From a futuristic perspective, but already under development, smart nanoparticulate vectors of pesticides can be designed in order to deal with controlled and targeted release, taking advantage of environment stimuli responsive nanopesticides (Camara *et al.* 2019). All these smart-devices should rely on green-technologies and biocompatible materials.

An important prerequisite to the development of nanopesticides is to assess their innocuity on soil microorganisms in order to preserve the soil ecosystem, and to control the diffusion of nanopesticides In the soil matrix, to avoid contamination of the water compartment. Soil depth targeted release could be envisioned using as synthetic virus-based model nanopesticides those mobility whose mobility allows them to reach different depths in soil (Chariou *et al.* 2019).

Currently, research is focusing on the search for microbiota that allow plants to defend themselves against abiotic (drought, flooding, etc.) or biotic (plant pathogens) stresses or to improve the growth and yield of field crops. Nanopesticides must fit in this scheme, and allow combined uses of both approach, in preventive (seed treatment, disinfection or stimulation of seedling transplanting) and curative (plant treatment) treatments.

Regarding the impact on microbiome, the methology focuses on diversity revealed by sequencing an amplicon of 16S rRNA, to the impact on the bacterial community present. A broader approach would address the diversity of bacteria, together with those of archaea, fungi, protozoa, etc., allowing to examine how nanopesticides are disrupting the networks of interactions between these communities. A sharper advance could focus on the active communities (complementary DNA) and the expression of genes. Indeed, extracellular DNA can persist in soil, and hide some changes in the community.

Interaction between microorganisms and macroorganisms should be deciphered, especially addressing how nanopesticides present in the soil or systemic in the plant may modify the microbiota recruited in plant roots and shoots, which is currently poorly documented. Some organisms inhabiting soils, such as nematodes, can also modify the impact of nanopesticides on soil microbiota. Recently, Bart *et al.* (2019) evidenced that nematodes can mitigate the toxicity of pesticides on soil microbial enzymatic activities.

Going back to soil, which is the basic matrix for agronomy, the microstructure of soil aggregates directly impacts soil communities and functional diversity, and likely the implications of nanoagrochemicals. To overcome the complexity of soil matrix, microfluidic techniques provide new ways of studying soil microbial ecology by allowing simulation and manipulation of chemical conditions and physical structures at the microscale in soil model habitats (Aleklett *et al.* 2018).

As final conclusion, soil must be considered as a super-organism (Lovelock 1993). In order to design smart solutions for agronomy, the soil ecosystem has to be addressed globally and in interaction with the air and water compartment.

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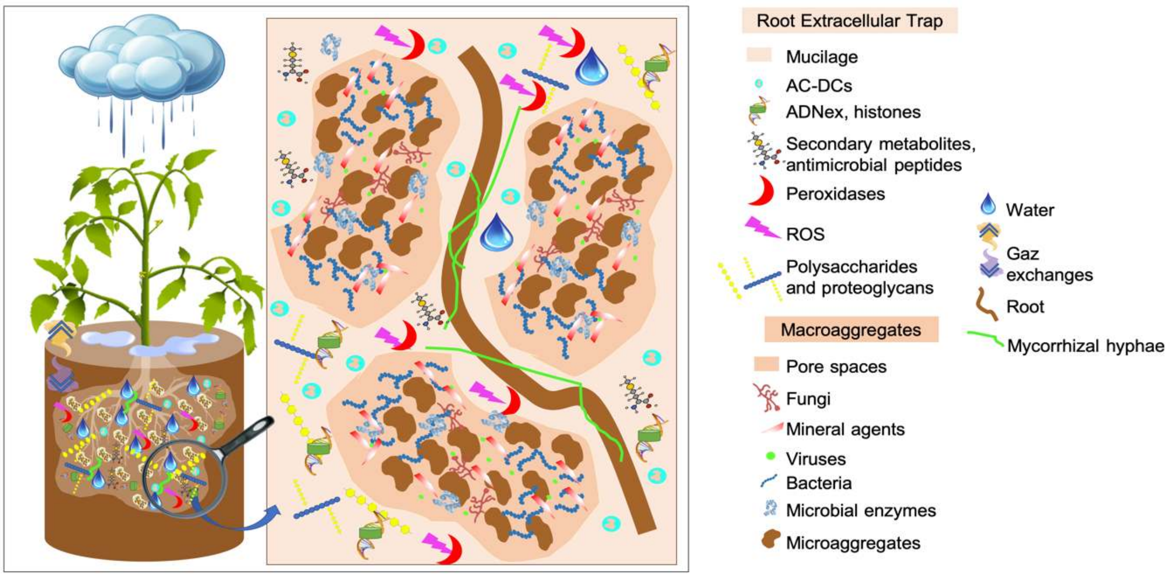


**Figure 1:** Soil matrix, a complex system (adapted from Wilpiszeski *et al.*, 2018 and Driouich *et al.*, 2019). The microstructure of soil aggregates host different soil communities and functional diversity. Pore spaces within microaggregates (1-2 μm) and interaggregates (10 to 30 μm) allow gases, water and nutrients to diffuse. Diffusion gas, water and nutrients is modulated according to the diameter of pore spaces from 10 to 30 μm in interaggregates to 1 to 2 μm within intraaggregates.

At the root tip, a network polysaccharides and proteoglycans embeds cap-derived cells (AC-DCs) and exudates.

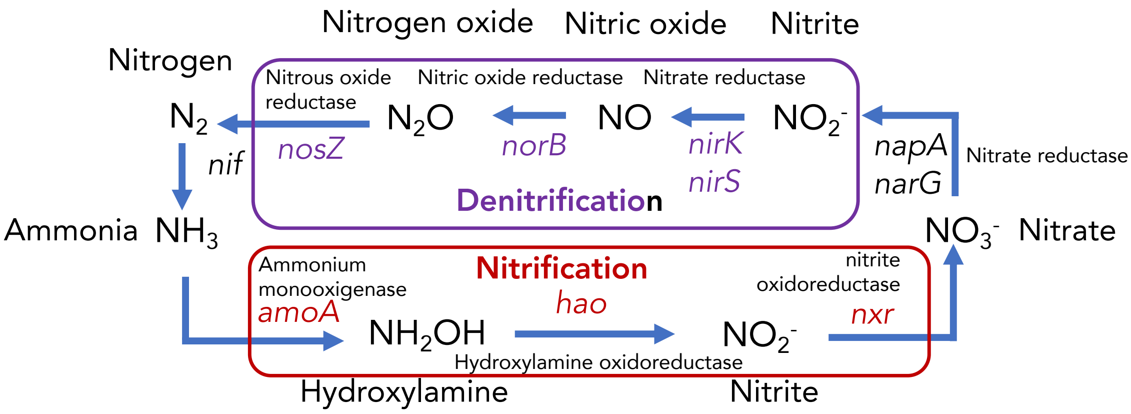


Figure 2 : Nitrogen cycle