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An integrated approach to highlight biological responses of *Pisum sativum* root to nano-TiO_2 exposure in a biosolid-amended agricultural soil

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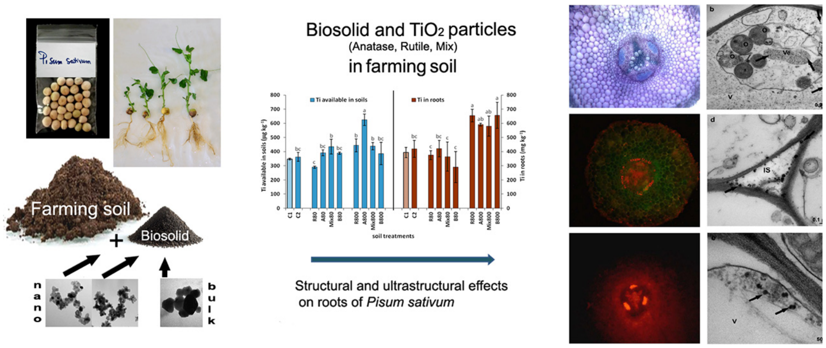
HIGHLIGHTS

- Biosolid spiked to farm soil disturbed pea growth, causing root cellular damages.
- Further damages were recorded spiking TiO_2 particles in the sludge-amended soil.
- Toxicity of nano TiO_2 may depend on the cell ability to compartmentalize t. hem.
- Toxicity of bulk particles was confirmed also in this plant system
- Caution in use of biosolid and nanoparticles is suggested for environmental health.

GRAPHICAL ABSTRACT



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ABSTRACT

This study focused on crop plant response to a simultaneous exposure to biosolid and TiO_2 at micro- and nano-scale, being biosolid one of the major sink of TiO_2 nanoparticles released into the soil environment. We settled an experimental design as much as possible realistic, at microcosm scale, using the crop *Pisum sativum*. This experimental design supported the hypotheses that the presence of biosolid in the farming soil might influence plant growth and metabolism and that, after TiO_2 spiking, the different dimension and crystal forms of TiO_2 might be otherwise bioavailable and differently interacting with the plant system. To test these hypotheses, we have considered different aspects of the response elicited by TiO_2 and biosolid at cellular and organism level, focusing on the root system, with an integrative approach. In our experimental conditions, the presence of biosolid disturbed plant growth of *P. sativum*, causing cellular damages at root level, probably through mechanisms not only oxidative stress-dependent but also involving altered signalling processes. These disturbances could depend on non-humified compounds and/or on the presence of toxic elements and of nanoparticles in the biosolid-amended soil. The addition of TiO_2 particles in the sludge-amended soil, further altered plant growth and induced oxidative and ultrastructural damages. Although non typical dose-effect response was detected, the most responsiveness treatments were found for the anatase crystal form, alone or mixed with rutile. Based on ultrastructural

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observations, we could hypothesise that the toxicity level of TiO_2 nanoparticles may depend on the cell ability to isolate nanoparticles in subcellular compartments, avoiding their interaction with organelles and/or metabolic processes.

The results of the present work suggest reflections on the promising practice of soil amendments and on the use of nanomaterials and their safety for food plants and living organisms.

1. Introduction

Contaminants of emerging concern are increasingly gaining ground in all the ecosystems, due to the unintentional or intentional release into the environment of new molecules/compounds or to a new employment and disposal of complex and potentially polluted matrices (Halden, 2015). In this context, the reuse of sludge from wastewater treatment plants (WWTP) in farming soils is recognized as a cost-effective practice to dispose of a byproduct that can be applied to the soil-plant system as a fertilizer, rich in organic matter and nutrients (Lu et al., 2012; EPA, 1994). Regulations governing the reuse of biosolid (Bs) in farming applications take several broad forms in different countries, but basically they follow a code of good practice, which foresees specific treatments and maturation aimed to guarantee defined chemical, physical and microbiological standards (EEC, 1986; EPA, 1993).

On the other hand, due to the uncertainty of its content not thoroughly tested for safety, Bs can result a possible sink of organic and inorganic unknown priority pollutants as well as of not commonly monitored chemicals, such as nanoparticles (NPs) (Brar et al., 2010; Yang et al., 2014).

The nanotechnology revolution and its challenges has been going on for some time, accompanied however by a series of ethical/safety implications related to the release into the environment of new nanochemical compounds whose effects on ecosystems and living organisms are not yet fully clear and unambiguously interpretable (Maurer-Jones et al., 2013). Besides, NPs behavior is poorly estimated in the different environmental matrices, especially in agricultural soils. In such complex matrices, the bioavailability of the different NPs often is not predictable, due to their tendency to aggregate, to adsorb/precipitate on solid phase, as well as to be coated by organic molecules (Tourinho et al., 2012; Pachapur et al., 2016). In addition, the overall picture of their possible interactions with crop plants and with food chains are not at all clear (Ruffini Castiglione and Cremonini, 2009; Remédios et al., 2012; Rico et al., 2011; Tassi et al., 2017). Given that we cannot afford to miss the opportunity of exploiting nanotechnologies, it is priority and urgent to dispel these uncertainties, that nowadays remain, about the possible harmful effects of these nanomaterials, otherwise transferred in farming soils, on crop plants and food chains.

TiO_2 NPs are among the top five nanomaterials widely used for various applications (Chuankrerkkul and Sangsuk, 2008), ranging from food and personal care products (Weir et al., 2012) to specific medical devices coating (Villatte et al., 2015) and drug delivery systems (Bakhshizadeh et al., 2017), from coating pigments production (El-Sherbiny et al., 2014) to their employment in certain farming sectors and in environmental cleanup technologies (Bhawana and Fulekar, 2012; Liu, 2011). A broad sector of the current body of literature on the environmental impact of NPs is focused on this class of nanomaterials: in recent years, the number of studies on their effects on higher plants is increasing, as well as the different experimental approaches and endpoints considered to evaluate NPs uptake, translocation, accumulation in plant tissues/organs and potential toxicity (Larue et al., 2012; Song et al., 2013; Ruffini Castiglione et al., 2014; Ruffini Castiglione et al., 2016; Amini et al., 2017). The researchers' guidance on these issues is also connected to the general awareness and concern that the most used NPs, including TiO_2, may easily and in a short time reach significant environmental concentrations and enter in the food chains through crop plants, thus affecting the whole living organisms (Rico et al., 2011).

Most of the works published so far on TiO_2 NPs effects on plants of agronomic interest report data obtained in hydroponics, water suspensions or under any other experimental conditions to monitor the short-term effects, testing high concentrations of TiO_2 (Maurer-Jones et al., 2013; Cox et al., 2016), often not realistic, even in the case of accidental pollution (Sun et al., 2014). In this context, there are few studies involving the use of agricultural soils as growth substrates for plants along with the application of treatments (Du et al., 2011; Burke et al., 2014; Gogos et al., 2016).

In this report, we settled an experimental design as much as possible realistic, at microcosm scale, using a biosolid-amended agricultural soil as growth matrix for the crop *Pisum sativum*. We aimed to investigate the effects of TiO_2 in the form of bulk material and in three different nanoparticulate formulations: crystals of anatase, rutile, and a mix of both, all applied at two different concentrations in the range established simulating an environmental contamination, and under long term exposure. This experimental design supports the hypotheses that the presence of Bs in itself may influence plant growth and metabolism and that, after TiO_2 spiking, the different dimension and crystal forms of titanium dioxide might be otherwise bioavailable and differently interacting with the plant system. To test our hypotheses, we have chosen to take into account different aspects of the response elicited by TiO_2 and Bs in tissues/organs, at cellular and organism level, focusing on the root system, with an integrative approach.

2. Materials and methods

2.1. Growth substrates

The farming soil (C1) was collected at CiRAA - Agri-Environmental Research Center 'Enrico Avanzi' from University of Pisa, Italy. The soil was air-dried, sieved (0–2 mm) and homogenized before its analysis and use as growth substrate. C1 soil was characterized by a sandy texture (93.3% of sand, 4.6% of silt and 2.1% of clay) with a pH near the neutrality (7.7), low organic matter content (OM, 1.1%), medium value of cation exchange capacity (CEC, 15.5 $\mathrm{cmol^{(+)}\,kg^{-1}}$) and electrical conductivity (EC) of 0.80 $\mathrm{mS\,cm^{-1}}$.

Bs was obtained from a small WWTP in Pisa (Italy) as a dewatered sludge qualified for its use in an agricultural soil. Bs was further characterized by having a solid residue (at 105 °C) of 18%, pH of 6.9, high OM (57.3%), EC of 11.5 $\mathrm{mS\,cm^{-1}}$ and total concentration of Ti of 699 ± 105 $\mathrm{mg\,kg^{-1}}$ (dw basis). Titanium background found in Bs is in line with that from other studies and model predictions (Josko and Oleszczuk, 2013; Kim et al., 2012; Sun et al., 2014). Heavy metals (As, Cd, Cr, Hg, Ni and Pb), PAH (polycyclic aromatic hydrocarbons) and *Salmonella* spp. content were all below the limit of law reference for its use in farm soils (Italian Legislative Decree 99/92; Commission Regulation (EU) no 1357/2014).

Commercial powder of TiO_2 was bought from US Research Nanomaterials Inc. (Houston, USA) as anatase or rutile NPs (nominal size of 30 nm) and from Sigma-Aldrich (Saint Louis, USA) as bulk particles (>100 nm), all having at least 99.9% of purity (producers' information).