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Effect of cobalt and silver nanoparticles and ions on *Lumbricus rubellus* health and on microbial community of earthworm faeces and soil

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ABSTRACT

The aim of this study was to investigate the impact of silver and cobalt, supplied both as ions and nanoparticles (Ag^+, Co^{2+}, AgNPs, CoNPs) through contaminated food to earthworms (*Lumbricus rubellus*), on their health as well as on microbial community of both soil and earthworm faeces. Earthworms and microbes were exposed to the contaminants in laboratory microcosms with artificial soil. Contaminants were supplied once a week for 5 weeks by spiking them on horse manure. The accumulation of CoNPs and Co^{2+} in earthworm tissues was two and three times greater than AgNPs and Ag^+, respectively. Except for AgNPs, contaminants significantly affected microbial community structure of earthworm faeces by increasing G- bacteria, thus also increasing the bacteria/fungi ratio while decreasing the G+/G- bacteria ratio. Such shift was also reflected on soil microbial community, thus suggesting a close relationship between microbial community of soil and of earthworm faeces. Neither of the Co treatments affected soil microbial basal respiration whereas they increased the microbial biomass specific respiration or metabolic quotient, suggesting some stress induction on soil microorganisms. Earthworm health was strongly affected as revealed by the reduced fluidity of fatty acids extracted from the body tissues. In addition, the histological investigations, after the depuration period, showed positive results about the NPs toxicity. In particular, TUNEL-positive nuclei in epidermis and in peritoneum, suggest the presence of toxicosis.The ESEM-EDS technique revealed the presence of Ca-P spherules (calcification) between mouth and clitellum of earthworms fed with Co^{2+} contaminated food.

1. Introduction

Metallic and ionic Ag has a long history of use as an antimicrobial agent in various industries, and is gradually being replaced by Ag nanoparticles (AgNPs) in agrochemicals, food preservatives and storage containers, textiles, personal-care products and laundry additives (Maynard et al., 2006). The use and disposal of materials containing AgNPs and Ag^+ allow these potential contaminants to enter the environment. Another matter of interest as possible environmental contaminants are Co nanoparticles (CoNPs), which are used to produce magnetic polymer microspheres, for information storage and energy, as magnetic resonance imaging contrast agents, in cancer therapy and in anaerobic waste water treatment systems (Florencio et al., 1994; Magaye et al., 2012). The AgNPs and CoNPs forms merit study in natural environments, especially the soil ecosystem where they are subject to transformations like aggregation/agglomeration, redox reactions, dissolution, exchange of surface moieties, and reactions with biomacromolecules (Maurer-Jones et al., 2013). The multiple ways that these NPs may be transformed in the soil environment makes it difficult to predict their fate and toxicity to soil biota, which have a central role in sustaining soil fertility and productivity.

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Direct toxicity of Ag and Co to soil microorganisms is expected to reduce microbial activity and alter the biomass of key groups within microbial communities (Dinesh et al., 2012; Shah et al., 2014). However, soil microorganisms do not respond uniformly to NPs (Vittori Antisari et al., 2015), probably because some microbial groups and genera have mechanisms to sequester metallic and ionic compounds or alleviate cellular stress induced by metal exposure (Nies, 1999; Dinesh et al., 2012). The direct toxicity of Ag and Co to higher trophic groups in the soil food web is another factor that could impact microbial communities. For instance, earthworms are ecosystem engineers that consume and reorganize litter through the soil profile (Bernard et al., 2012; Blouin et al., 2013). Earthworm activities can also consistently stimulate bacterial growth, with higher bacterial populations in the earthworm gut and freshly-deposited faeces (Blouin et al., 2013). Earthworms consume fungal spores and defecate them in nutrient-rich faeces, a favourable microsite for microbial growth. As a consequence, during the passage through the digestive tract of the earthworms, ingested soil microorganisms can be selectively altered (Pedersen and Hendriksen, 1993; Sampedro and Whalen, 2007; Thakuria et al., 2010).

In a previous study (Vittori Antisari et al., 2015), we found that soil microbial biomass and activity is significantly affected by food contaminated with NPs. However, no indications emerged whether microbial community is altered following the passage through earthworms fed with contaminated food.

The aim of this study was to investigate the impact of Ag and Co fed to earthworms (*Lumbricus rubellus*) both as nanoparticles and ions through contaminated food on their health as well as on microbial community of both soil and earthworm faeces. The hypotheses to be tested were that the supply of Ag and Co can alter the microbial community of earthworm faeces and that such alteration in turn can induce changes in the soil microbial biomass, activity and community structure. Furthermore, also earthworms can be directly affected by contaminants so inducing a disorder in their tissues. Finally, we hypothesised that Ag and Co could remain within the body of earthworms also after a depuration period, thus negatively affecting their health. In the first part of the experiment, biochemical soil properties, such as microbial biomass, its activity and community structure, and parameters linked to earthworms stress were investigated in order to contribute to the identification and validation of biomarkers affected by contaminant exposure. Phospholipid fatty acids (PLFAs) and total fatty acids (FAs) were used as biomarkers of shifts in soil microbial community structure and of earthworms stress, respectively. PLFAs were also determined on earthworm faeces to find out possible relationships between microbial communities within soil and earthworm faeces.

Finally, in the second part of the experiment, earthworm health after a period of depuration was assessed by measuring various parameters and using advanced techniques, such as apoptosis frequencies (TUNEL test), Ag and Co contents in earthworm body tissues (ICP-OES), scanning electron microscopy coupled with X-ray probe for microanalysis (ESEM-EDS), and X-ray computed microtomography (X-ray micro-CT).

2. Materials and methods

2.1. Nanoparticles and ions

AgNPs and CoNPs were purchased from Polytech & Net GmbH Germany (AGS-WM1000C) in form of solution (1000 ppm) and from Nanostructured & Amorphous Materials Inc. (USA), in form of powder, respectively. The CoNPs vacuum-sealed bag was opened in a nitrogen-controlled atmosphere cabinet, weighed and mixed with milli-Q water.

The hydrodynamic diameter and zeta potential of nanoparticle fresh suspension were measured by the technique of Photon Correlation Spectroscopy using a Zetasizer Nano ZS (Malvern Instruments, UK). The analyses were performed thrice at 25 °C with an angle of 90° (Table 1). Ionic treatments were prepared from standard solution bought from CPI International (USA): Ag (cod. 4400-1000511), Co (cod. 4400-1000131). The solutions were prepared using high purity metal (99.9%), sub-boiling in distilled nitric acid and diluting in 18 MΩ milli-Q water.

2.2. Experimental design

Mature (with clitellum, two months old) earthworms of the species *L. rubellus* were obtained from a local synchronized culture and weighed individually (average earthworm weight 323 ± 67 mg; $n = 200$). Ten mature earthworms were randomly placed in a box containing 500 g of an artificial soil and allowed to acclimate for two weeks. Soil moisture was maintained at 60% of water holding capacity and temperature at 25 °C. Twenty boxes, each with 10 earthworms, were prepared.

The artificial soil was prepared by mixing neutral sphagnum peat and forest soil (Epileptic Cambisol; sandy clay loam texture; total organic carbon 41.9 g kg^{-1}; total N 3.2 g kg^{-1}) at 1:1 v/v ratio. It had pH 6.9 (in 0.01 M $CaCl_2$; 1:2.5 w/v), cation exchange capacity (CEC) 35.2 $cmol_{(c)}$ kg^{-1}, total organic carbon (TOC) 167.5 g kg^{-1}, total nitrogen (TN) 6.8 g kg^{-1}; microbial biomass carbon (MBC) 863.2 mg kg^{-1}; microbial biomass nitrogen (MBN) 95.7 mg kg^{-1}.

After the acclimation, earthworms were exposed to AgNPs, Ag^+, CoNPs or Co^{2+} via their diet for five weeks. The control treatment differed from the four metal treatments only for earthworms being fed with uncontaminated food. Four boxes per treatment were set-up. Earthworms were fed once a week with ground horse manure (0.5 g dry weight of manure per worm per week). Manure from a non-medicated horse was spiked, 24 h prior to feeding, with a water solution of NPs or ions (as nitrates) to reach the 65% of WHC. The concentration of both NPs and ions was 10 mg of contaminant kg^{-1} dry horse manure. Therefore, each earthworm was exposed to 25 μg of contaminants during the five weeks, i.e. 0.5 μg per gram of soil. The amount of contaminants used in this study was of the same order of our previous and other similar works (Coutris et al., 2012; Dinesh et al., 2012; Vittori Antisari et al., 2015). After the five weeks of exposure to contaminated food (first part), the surviving earthworms of two boxes were transferred to Petri dishes for two days, in order to empty their guts by defecation, and then prepared for further analysis. Faeces produced during the two-days of

Table 1
Main characteristics of Ag and Co nanoparticles (NPs) used to contaminate the horse manure supplied to the earthworms.

Material	Purity (%)	Nominal size (nm)	SSA ($m^2 g^{-1}$)	Morphology	Average hydrated diameter (nm)	Z-potential (mV)	Ratio of occupied volume ($cm^3 mL^{-1}$)	Notes
AgNPs[a]	ND	1–10	–	irregular	60.3	−32.5	13 E-5	Solution in water
CoNPs	99.8%	28	40–60	spherical	102	24.6	77 E-5	Powder: partially passivated w/ [oxygen] about 10%

[a] AgNPs were coated with polyvinyl pyrrolidone (PVP); SSA is the specific surface area.

depuration were collected and frozen at −20 °C. Then, the earthworms, their faeces as well as the artificial soil were separately analysed.

The surviving earthworms of the other two boxes were moved to new boxes with artificial soil, having the same characteristics of the previous one, and fed for another 4 weeks with unpolluted horse manure (second part). Then, earthworms were transferred to Petri dishes for two days in order to empty their guts, and prepared for further analysis.

2.3. Part I: soil and earthworm analyses

After 5 weeks of contaminants exposure, soil was analysed for total organic C (TOC), microbial biomass C (MBC) and basal respiration (CO_2 emission). Both soil and earthworm tissues were analysed for total content of Ag and Co. Moreover, phospholipid fatty acids (PLFAs) were determined on soil and earthworm faeces, whereas earthworm tissues were analysed for the composition in total fatty acids (FAs). Total organic C was determined by CHN analyser (Carlo Erba, mod.1100, Italy). Microbial biomass C was determined by the fumigation-extraction method (Vance et al., 1987). The concentration of K_2SO_4-extractable C from non-fumigated soil was used as a measure of available C (Laudicina et al., 2013). Soil basal respiration (SR) was determined by measuring the cumulative CO_2 evolved from soil incubated under standard conditions. Briefly, 10 g of soil were placed in 125-mL glass bottles at 25 °C, and the CO_2 accumulated in the headspace after three days of incubation was determined by a gas chromatograph (Trace GC, Thermo Electron) equipped with a thermal conductivity detector. Metabolic quotient (qCO_2), i.e. the specific respiration, was calculated as mg CO_2–C h^{-1} g^{-1} MBC (mg CO_2–C evolved in 3 days kg^{-1} d.s.)/72/(g MBC kg^{-1} d.s.).

To determine Ag and Co total contents in soil, finely ground samples (250 mg) were digested by adding nitric acid (65%, w/v, suprapure Merck), hydrochloric acid (37%, w/v, suprapure Merck) at 1:3 (v/v) ratio (Aqua Regia) and subsequent cycle in a microwave oven (Milestone, Start D 1200, USA) (Vittori Antisari et al., 2013). To provide water and saline extracts, finely ground soil samples were shaken on a horizontal shaker for 16 h with deionised water (Milli-Q water, Millipore) or 1 M NH_4NO_3 with a 1 soil: 10 water (w/v) ratio. The suspensions were centrifuged for 15 min at 1200*g* and the supernatants filtered through ø 0.45 μm filter (Millipore). To determine Ag and Co total contents, as well as total Ca and P, in earthworms tissues, 250 mg of lyophilised tissues was finely ground and mineralized with 6 mL nitric acid (65%,w/v, suprapure, Merck) and 1.5 mL hydrogen peroxide (30%, v/v, for electronic use, Carlo Erba) in microwave oven. The elemental total concentrations in both soil and earthworm tissues were measured by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Ametek, Spectro, Germany).

Phospholipid fatty acids (PLFAs) were extracted from soil and earthworm faeces and analysed according to the modified Bligh and Dyer method (Wu et al., 2009). The fatty acid methyl esters were detected on a gas chromatograph (Thermo Scientific FOCUS™ GC) equipped with a flame ionization detector and a fused-silica capillary column Mega-10 (50 m × 0.32 mm I.D.; film thickness 0.25 μm). The GC temperature progression was: initial isotherm at 115 °C for 5 min, increase at a rate of 1.5 °C per minute from 115 to 230 °C, and final isotherm at 230 °C for 2 min. Both injection port and detector were set up at 250 °C and helium at 1 mL min^{-1} in a constant flow mode was used as carrier. The injected volume was 1 μL in a splitless mode. Nonadecanoic acid methyl ester (19:0; cat no. N-5377, Sigma-Aldrich Co.) was used as an internal standard for quantification of the fatty acid methyl esters (FAs). The identification of the peaks was based on comparison of retention times to known standards (Supelco Bacterial Acid Methyl Esters mix cat no. 47080-U and Supelco 37 Component FAME mix cat no. 47885-U). The relative abundance of detected FAs was expressed as mol%. FAs were designated using the nomenclature described in Frostegård et al. (1993). FAs with less than 14 carbon atoms or more than 19 carbon atoms were excluded as considered originating from non-microbial sources (Frostegård et al., 1993; Leckie, 2005). The FAs i15:0, a15:0, 15:0, i16:0, i17:0, 17:0, cy17:0, 18:1ω7, cy19:0 were used to represent bacterial biomass while 18:2ω6,9 for fungal biomass (Frostegård and Bååth, 1996). The FAs i15:0, a15:0, i16:0, i17:0 were chosen to represent Gram-positive (G+) bacteria while 18:1ω7, cy17:0 and cy19:0 for Gram-negative (G-) bacteria (Zelles, 1997; Zogg et al., 1997).

Earthworm tissues were analysed for their total FAs content following the procedure described by Vittori Antisari et al. (2015). Briefly, earthworm tissue sub-samples (about 150 mg) were weighed in 10 mL glass test tubes, 1 mL of 4 M NaOH in 50% methanol was added and then the mixture was heated for 30 min in a boiling water bath. After cooling at room temperature, 2 mL 6 M HCl in methanol was added for methylation of dissolved fatty acids in a water bath at 80 °C (10 min). Then 1 mL hexane:methyl-*tert*-butyl ether (1:1, v/v) was added and lipids extracted by shaking for 10 min. The organic phase was transferred to a new test tube and the extraction was repeated. The combined organic phase was washed once with 0.25 M NaOH, and subsequently transferred to 2-mL vials for analysis on the gas chromatograph as above described. The unsaturation degree of the identified FAs (UD) was calculated as follows:

$$UD = \sum (\%\ \text{mono-unsaturated} + 2 * \%\ \text{di-unsaturated} + 3 * \%\ \text{tri-unsaturated} + \ldots)/100.$$

2.4. Part II: X-ray micro-CT and histological investigation of earthworms

After both first and second part of the experiment, two earthworms from each box (i.e 4 earthworms per treatment) were stored at 4 °C in a test tube containing 4% paraformaldehyde in 0.1 M phosphate buffered solution (pH 7.4). Then, the earthworms were put into small polystyrene containers and analysed using X-ray computed micro tomography (X-ray micro-CT) system "phoenix nanotom s ®" by GE Sensing and Inspection Technologies, Germany. X-ray micro-CT is able to reconstruct 3D structures by processing several 2D X-Ray projection images, allowing the user to reproduce earthworm virtual slices in order to see what is inside without opening it by cutting (Boone et al., 2004), i.e. the presence of health anomalies (e.g. haemorrhages, surface and internal morphological anomalies, calcifications, etc.). Briefly, the earthworms were analysed at the following experimental conditions, optimized for low absorbing materials: molybdenum target, high tension and beam current of 70 kV and 140 μA, respectively, with a frame time of 750 msec giving an overall acquisition time of about 2 h. In this way both good contrast and image quality were assured. The nominal spatial resolution was 32 μm.

For the histological investigation, four earthworms from each box (i.e. 8 earthworms per treatment) were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered solution (pH 7.4), dehydrated in ascending concentration alcohols (70, 80, 95, and 100%), and Paraplast (Bio-Optica, Italy) embedded for sectioning. Groups of four longitudinal serial sections (5 μm thick), from the zone just posterior to the clitellum, were stained for histological observations by Hematoxylin-Eosin (Bio-Optica, Italy). Apoptosis was assessed through a fluorescein-conjugated TUNEL test (Roche, Germany), nuclei were counterstained through 4′,6-DiAmidino-2-Phenyl-Indole (DAPI, 1:1000, Molecular Probes, NL), that is a fluorescent staining that binds strongly to A-T rich regions in DNA.

A negative control was performed by incubating sections with the Label Solution, containing the nucleotide mixture without the transferase enzyme. Sections were examined under a Leica light and epifluorescence microscope (Leica, Germany) and visualized with a Leica software program.

Moreover, one of the dehydrated earthworms was longitudinally sectioned to study the gut and the internal organs. The observation was carried out under a Field Emission Gun Environmental Scanning Electron Microscope (FEG-ESEM, 200 QUANTA, FEI Company, USA) coupled with an X-ray microprobe for the elemental analyses [Energy Dispersive Spectroscopy (EDS) by EDAX, USA]. Finally, three earthworms from each box (i.e. 6 earthworms per treatment) were used to determine total concentration of Ag, Co, Ca and P as before described.

2.5. Statistical analysis

Data were subjected to one-way ANOVA with contaminant as factor. Before performing parametric statistical analyses, normal distribution and variance homogeneity of the data were checked by Kolmogorov–Smirnoff goodness- of-fit and Levene's tests, respectively. Differences among treatments were revealed by the Tukey test at $P < 0.05$. Statistical analyses were performed using Statgraphics (version XV), USA.

3. Results

3.1. Part I: Ag and Co in soil

After exposure to metal contaminants, the total concentrations of both contaminants in soil, regardless of form, increased significantly compared to the control soil (Table 2), whereas for both contaminants the contents under NPs and ion forms did not differ between them (Table 2).

Analyses of available metal content in soil (water and salt extraction; Table S1) were carried out at the end of the five weeks and the Ag concentrations in the water samples from AgNP and Ag^+ treatments were below the detection limit of the ICP-OES (<3 μg Ag L^{-1}) as well as in the NH_4NO_3 samples (<5.4 μg Ag L^{-1}). Slight available Co increases in both water and NH_4NO_3 extracts were detected for CoNP and Co^{2+} treatments compared to the control.

3.2. Part I: soil microbial biomass and activity, and microbial community structure in soil and in earthworm faeces

Extractable organic and microbial biomass C were affected significantly only by food contaminated with Co^{2+}, the former being 13% higher while the latter 24% lower than the control (Table 2). Soil respiration was not significantly affected by contaminants, whereas the qCO_2 (i.e. the biomass-specific respiration rate) was of 0.16 and 0.63 mg CO_2-C kg^{-1} MBC h^{-1} higher in CoNPs and Co^{2+} treatments, respectively, than in the control (Table 2).

Supplying of contaminated food affected significantly the PLFAs extracted from both soil and earthworm faeces.

Sixteen PLFAs were identified in soil samples, accounting for about 70% of those extracted (Table S2). The FAs 16:0, 18:1ω9 and 18:2ω6,9 were the most abundant (>7 mol%) but did not show significant differences among treatments, whereas the two saturated FAs 18:0 and 20:0, and the FAs 17:0cy, 18:1ω7 and 19:0cy changed significantly among treatments. Total fungi and bacteria were not affected by contaminated food. However, compared to control, G- bacteria were increased by CoNPs treatment and by both ion treatments (Table 3) so that the G +/G- ratio was significantly lower in soil where earthworms were fed with contaminated food (Table 3).

Sixteen PLFAs were also identified in earthworm faeces accounting for about 80% of the total extracted PLFAs (Table S3). The 16:0 FA was the most abundant (about 20% of the total extracted PLFAs), followed by the FAs 17:0i, 18:0, 18:1ω7, 18:1ω9. Generally, fungi were not affected by treatments as well as bacteria although they seemed to be increased in faeces of earthworms fed with ion-contaminated food ($F = 3.3$, $P = 0.058$; Table 3). Compared to control, G- bacteria were significantly higher in all treatments

Table 3
Molar percentages of microbial community groups in soils and earthworm faeces after five weeks during which earthworms (*Lumbricus rubellus*) were fed with horse manure contaminated by Ag and Co as ions or nanoparticles (NPs). *, P<0.05; **, P<0.01; ***, P<0.001.

Treatment	Bacteria	Fungi	BacteriaG+	BacteriaG-	Bacteria/Fungi	G+/G-
	Soil					
Control	23.1	7.3	14.3	6.0 b	3.2	2.4 a
AgNPs	23.0	7.6	13.7	6.5 ab	3.0	2.1 b
Ag^+	23.5	7.0	13.4	7.3 ab	3.4	1.8 c
CoNPs	23.7	7.2	12.7	8.2 a	3.3	1.5 d
Co^{2+}	23.7	8.0	13.2	7.8 a	3.0	1.7 cd
F value	ns	ns	ns	4.6*	ns	38.2***
	Faeces					
Control	28.8	4.1	15.8	10.2 c	7.0 c	1.5 a
AgNPs	33.5	3.9	14.4	11.4 bc	8.6 b	1.3 b
Ag^+	38.8	3.8	13.7	12.8 bc	10.2 a	1.1 bc
CoNPs	32.6	4.0	11.9	16.2 a	8.2 b	0.7 d
Co^{2+}	37.1	4.2	13.0	13.6 b	8.9 b	0.9 cd
F value	ns	ns	ns	11.3**	34.0***	44.9***

Table 2
Concentrations of total Ag and Co and chemical and biochemical properties of soil after five weeks during which earthworms (*Lumbricus rubellus*) were fed with horse manure contaminated by Ag and Co as ions or nanoparticles (NPs).*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Treatment	Ag μg kg^{-1}	Co mg kg^{-1}	TOC g kg^{-1}	Extr C mg kg^{-1}	MBC mg kg^{-1}	SR mg CO_2-C kg^{-1} d^{-1}	qCO_2 mg CO_2-C kg^{-1} MBC h^{-1}
Control	17.9 b	3.15 b	193.1	705.2 b	1291.8 a	44.9	1.45 bc
AgNPs	401.3 a	ND	194.3	700.9 ab	1229.3 ab	41.5	1.40 c
Ag^+	444.4 a	ND	183.7	715.7 ab	1257.9 a	42.1	1.39 c
CoNPs	ND	4.19 a	186.8	734.3 ab	1177.3 ab	45.3	1.61 b
Co^{2+}	ND	4.39 a	188.5	797.3 a	975.8 b	48.9	2.08 a
F value	99.9***	749.7***	ns	5.6*	5.2*	ns	47.1***

TOC, total organic carbon; Extr C, extractable organic C; MBC, microbial biomass carbon; SR, soil respiration, i.e. CO_2-C cumulated during 3 days of soil incubation; qCO_2, metabolic quotient; ND, not determined.

except for AgNPs, whereas G+ bacteria were significantly lower only in CoNPs treatment. As a consequence, the bacteria/fungi ratio increased and the G+/G- ratio decreased when the earthworms were fed with contaminated food.

3.3. Part I and II: Ag, Co, Ca and P in earthworms

After the first part of the experiment earthworms surveyed at 90% rate and no differences occurred among treatments. Their average mass was 162 ± 14 mg and 251 ± 31 mg after the depuration period performed, respectively, at the end of the first and of the second part of the experiment. Furthermore, only few cocoons were found in the control and in the treatments during the whole duration of the experiment.

After five weeks of exposure to contaminated food, concentration of Ag and Co in earthworm bodies significantly increased compared to control (Table 4). However, AgNPs more than Ag^+ treatment determined higher concentration of Ag in earthworms tissues, whereas a higher amount of Co was found in Co^{2+} treatment. No significant differences were found, after intoxication, in the concentration of Ca and P as well as in the Ca/P ratio which ranged from 0.59 to 0.67 (Table 4). After the depuration period, the amount of Ag in earthworms bodies previously fed with AgNPs contaminated food was higher than those of the control, whereas that of Co in Co^{2+} treatment was triplicated compared to control (Table 4). Also Ca content in earthworms fed with contaminated food increased compared to the control treatment so that the Ca/P ratio increased from 0.65 in the control to 0.80 and 0.81 in Ag treatments (NPs and ion, respectively) and to 0.77 and 0.78 in Co treatments (NPs and ion, respectively) (Table 4).

3.4. Part I: fatty acids of earthworm tissues

After exposure to contaminants, twenty-eight FAs were detected in earthworm tissues and twelve of them showed significant differences among treatments (Table S4). The most abundant FAs (>10% mol) were 20:5ω3, 20:3ω3 and 18:0. Polyunsaturated FAs (PUFAs) were the richest FAs group ranging from 26.5 to 33.9 mol% (Table 6). Compared to control, Ag^+, Co^{2+} and CoNPs treatments lowered PUFAs and monounsaturated fatty acids (MUFAs) whereas, conversely, increased the saturated fatty acids (SAFAs). As a consequence, the unsaturation degree (UD) was lowered by all the treatments (Table 5).

Table 4
Total Ag, Co, Ca and P concentration, and Ca/P ratio in earthworm tissues after five weeks during which earthworms were fed with horse manure contaminated by Ag and Co as ions or nanoparticles (NPs) and after 28 days of depuration. Within each column, different letters indicate significant differences ($P < 0.05$) among treatments. ND, not determined. **, $P < 0.01$; ***, $P < 0.001$.

Treatment	Ag $\mu g\ kg^{-1}$	Co $mg\ kg^{-1}$	Ca $g\ kg^{-1}$	P $g\ kg^{-1}$	Ca/P
	After intoxication				
Control	<DL	2.7 c	4.1	6.9	0.59
AgNPs	649.3 a	ND	4.6	6.9	0.67
Ag^+	487.3 b	ND	4.5	7.1	0.63
CoNPs	ND	4.3 b	4.7	7.1	0.66
Co^{2+}	ND	12.3 a	4.4	7.2	0.61
F value	259.7***	104.8***	ns	ns	ns
	After depuration				
Control	<DL	3.1 b	4.2 b	6.5	0.65 b
AgNPs	666.2	ND	5.5 a	6.9	0.80 a
Ag^+	<DL	ND	5.6 a	6.9	0.81 a
CoNPs	ND	3.9 b	5.3 a	6.9	0.77 a
Co^{2+}	ND	10.5 a	5.4 a	6.9	0.78 b
F value	643.3***	79.3***	6.6**	ns	13.6***

Table 5
Distribution (mol%) and unsaturation degree (%) of fatty acids (FAs) of *Lumbricus rubellus* after five weeks during which earthworms were fed with horse manure contaminated by Ag and Co as ions or nanoparticles (NPs). The least significant difference (LSD) is reported in bold for those groups of fatty acids that showed significant differences ($P < 0.05$) among treatments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Treatment	Total FAs	SAFAs	MUFAs	PUFAs	UD
Control	85.3	20.3 c	26.3 a	33.9 a	1.42 a
AgNPs	82.7	22.0 bc	24.2 ab	31.3 ab	1.31 a
Ag^+	82.3	24.1 abc	23.7 ab	29.0 bc	1.21 ab
CoNPs	80.9	25.5 a	21.6 b	27.9 bc	1.17 ab
Co^{2+}	82.9	27.6 a	22.3 b	26.5 c	1.12 b
F value	ns	9.7**	5.2*	9.0**	12.5**

SAFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs polyunsaturated fatty acids; UD, unsaturation degree.

3.5. Part II: X-ray micro-CT, ESEM-EDS and histological investigation of earthworms

After the intoxication (Fig. 1A) and the depuration period (Fig. 1B), earthworms were investigated through their structure by volume rendering techniques from 3D obtained by X-ray computed micro tomography (X-ray CT). Generally, high contrast between earthworm tissues and several masses, characterized by different size and position along their bodies, was observed. The 3D images showed denser masses (red areas) within the earthworms body structure placed between the mouth and clitellum in the control treatment of both parts (Fig. 1A and B), while more spots, characterized by great absorption of X-ray, after intoxication emerged along the earthworms body with both NPs and Ag^+ treatments (Fig. 1A). The earthworms of Co^{2+} treatment at the end of an intoxication period were very similar to control ones. After depuration period, a reduction of denser masses was observed for both NPs treatments, although some spots located in both gut and intestine were observed (Fig. 1B); conversely, for both ionic treatments an increase of these huge denser masses was observed.

The investigation on depurated earthworms was completed by ESEM-EDS observations to verify the presence of both Co and Ag in the earthworm tissue and gut. No evidence of Ag and Co was found as well as of soil in their gut. By contrast, ESEM-EDS highlighted the anomalous presence of spherules, with a spectrum made by Ca-P, similar to hydroxyapatite (Fig. 2), next to calciferous glands and between the mouth and clitellum of earthworms fed with contaminated food. Such findings confirmed the ICP-OES results of earthworm tissues in which a significant increase of Ca/P ratio due to an increase of Ca total content in their body tissues was detected (Table 4).

The histological observations on hematoxylin-eosin-stained sections did not show any particular morphological alteration of tissues (Fig. 3a). TUNEL positive nuclei were detectable, although very rare, in the muscular layer of the body wall of virtually all the specimens (Fig. 3b, c and f). Depurated earthworms after the Ag^+ and Co^{2+} exposure showed a situation similar to control (Fig. 3c and e). Depurated earthworms after AgNPs exposure showed numerous apoptotic nuclei in cells of peritoneum, lining the coelomic cavity (Fig. 3d), while those depurated after the treatment with CoNPs had TUNEL-positive nuclei in the epidermis (Fig. 3f).

4. Discussion

4.1. Part I: changes in microbial community of soil and of earthworm faeces

Supply of Ag^+ and both nanoparticles did not show any adverse effect on soil microbial biomass and available C, and on microbial

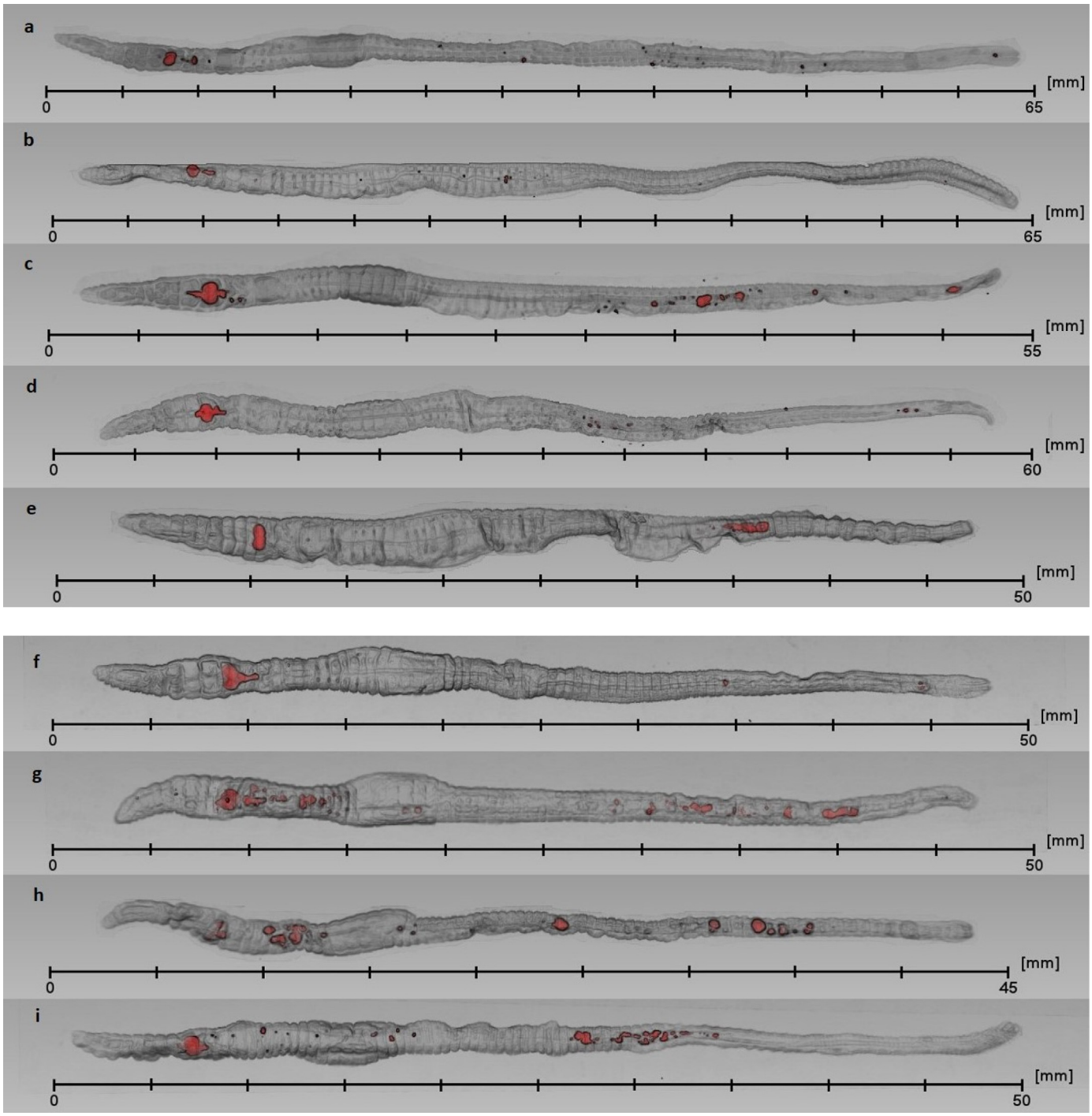

Fig. 1. X-ray computed micro tomography images in 3D of *Lumbricus rubellus* after five weeks of contaminants exposure (a- control, b-Ag^{+}, c-AgNPs, d- Co^{2+}, e-CoNPs treatments) and after four weeks of depuration (f- control, g-Ag^{+}, h-Co^{2+}, I – CoNPs after depuration period). The red area was denser mass in which a great adsorption of X-ray was observed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

activity as Co^{2+} treatment did. The absence of any effect on soil microorganisms by Ag treatment was in contrast with previous results (Dinesh et al., 2012; Vittori Antisari et al., 2015) and might be ascribed to the way by which contaminants were supplied. In fact, the high organic C content of horse manure may have mitigated the availability of Ag (Dinesh et al., 2012) but not that of Co in Co^{2+} treatment. Such different availability of Ag and Co supplied through horse manure agreed with Coutris et al. (2012) who found that the availability of Ag and Co was Co^{2+} > Co NPs >> Ag+ > Ag NPs.

The MBC reduction and the concomitant increase of extractable C suggested that C substrates deriving from the lysis of dead microbial biomass could have been made available to the remaining surviving microorganisms but without being efficiently assimilated, thus explaining the increase of the metabolic quotient (Nannipieri et al., 2003; Novara et al., 2014; Laudicina et al., 2015).

The metabolic quotient represents the quantity of substrate mineralised per unit of MBC and per unit of time. In general, in unsteady ecosystems the qCO_2 value increases in relation to more stable ecosystems (Dalal, 1998; Laudicina et al., 2011). Such an

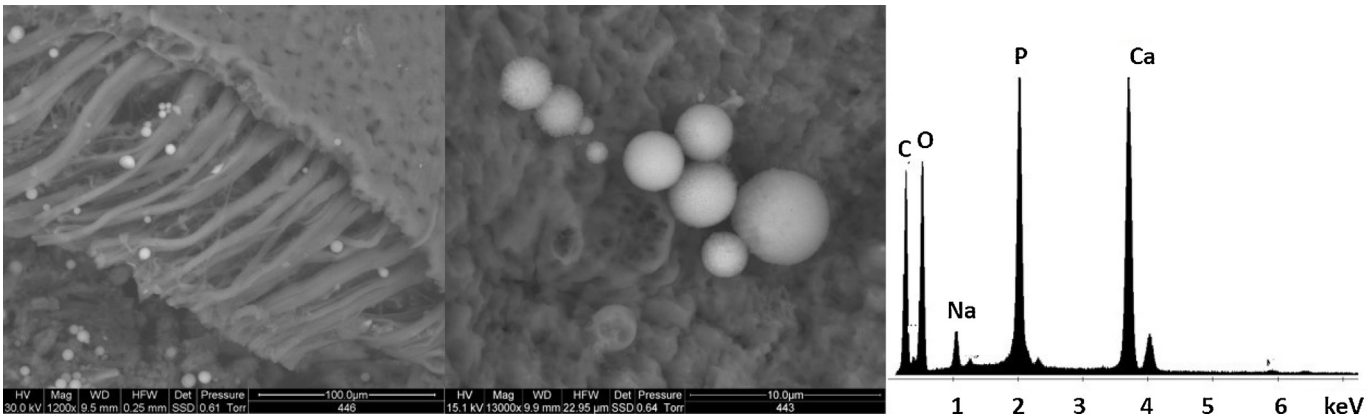

Fig. 2. Field Emission Gun Environmental Scanning Electron Microscope and Energy Dispersive Spectroscopy images of the Ca-P spherules detected in earthworm tissues after the depuration period in Co^{2+} treatment.

increase may be due to several reasons: new input of fresh substrates C, response of the microorganisms to adverse conditions, predominance of the zymogen flora (r-strategists) over the autochthonous one (K-strategists), or alteration of the bacteria/fungi ratio since they have different carbon use strategies (Dilly and Munch, 1998). Here we cannot exclude any hypothesis; however, soil PLFAs did not show changes in the bacterial/fungi ratio (Table 3). On the other hand, Co^{2+} treatment increased G- bacteria and, consequently, reduced the G+/G- bacteria ratio. G+ and G- bacteria have different patterns in C substrate preference. Indeed, Gram-positive bacteria were found to be dominant in soils with low substrate availability such as in deeper soil layers (Fierer et al., 2003), whereas Gram-negative bacteria were found to dominate in soils with high availability of easily decomposable substrate (Kramer and Gleixner, 2006). Indeed, Gram-negative bacteria are often characterized by high nutrient demand and, consequently, low efficiency in utilizing C substrates (Horwath, 2015; Ringleberg et al., 2008).

In addition to Co^{2+} treatment, also all the other treatments affected the soil microbial community structure mainly by decreasing the G+/G- bacteria ratio, but such shifts did not affect microbial activity.

Stimulation/inhibition of specific microbial groups after the passage through earthworms has been studied for *L. terrestris* and *rubellus* following changes in food source (Egert et al., 2004; Knapp et al., 2009), for *Pontoscolex corethrurus* following wheat straw residues addition (Bernard et al., 2012) and for *Eisenia fetida* following habitat diversification (Koubová et al., 2015). To the best of our knowledge, this is the first report assessing the impact of contaminated food on *L. rubellus* faeces. The supplied contaminants increased the G- bacteria in earthworm faeces (although such an increase was significant only in Co treatments) and decreased the G+/G- bacteria ratio. Greater amount of G- bacteria in *L. rubellus* faeces has been previously reported following the supply of different uncontaminated food such as wheat straw residues, dwarf shrub litter, grass litter and horse dung (Bernard et al., 2012; Knapp et al., 2009).

Other studies (Frostegård et al., 1993; Markowicz et al., 2010) investigated the effect of heavy metals on soil microbial community structure and reported that G- bacteria are more resistant to contaminants than other microbial groups. Such a capacity of G- bacteria to survive under stress conditions could be attributed to the presence of the cyclo fatty acids in their membranes and the outer lipopolysaccharide layer which can better counteract with the stress (Guckert et al., 1986).

4.2. Part I: accumulation of contaminants and changes of FAs in earthworms

Accumulation of Co metal in earthworm tissues due to the supply of food contaminated with CoNPs and Co^{2+} was, respectively, two and three times greater than the corresponding Ag treatments. The different availability of the two contaminants, due to their different affinity with soil organic matter (Dinesh et al., 2012), showed that Ag^{+} and AgNPs were mostly immobile and, consequently, extractable only under strong chemical conditions, whereas Co^{2+} and CoNPs were more mobile and so largely extractable with mild extractants such as in water.

Both Co treatments affected strongly FAs of earthworm tissues. The environmental stresses concern the FAs composition in earthworm body; indeed, usually changes in the degree of unsaturation of FAs in earthworm body have occurred following shifts in soil temperature (Petersen and Holmstrup, 2000) and also nanoparticle exposure (Vittori Antisari et al., 2015). In particular, a reduction in the degree of unsaturation of body FAs after NPs exposure has been found in order $Ni > TiO_2 = Co > Ag$ (Vittori Antisari et al., 2015). Saturated fatty acids can pack together better than unsaturated ones, thus rendering the highly abundant lipid layers within membranes more viscous and less permeable (Collins et al., 1990). Our results, in agreement with others (Markowicz et al., 2010; Vittori Antisari et al., 2015), could be ascribed to an earthworm defence response aimed at reducing the ability of Co, supplied as both ionic and NP forms, to generate an oxidative stress on the lipid layers (Howlett and Avery, 1997). Such an effect has been already extensively reported for the yeast *Saccharomyces cerevisiae* and fungi (Avery et al., 1996; Gadd 1993; Ohsumi et al., 1988). Those studies suggested that as a primary mechanism of toxicity, both cadmium and copper, along with other transition metals, cause disruption of cellular and organellar membranes, resulting in rapid impairment of membrane function and loss of membrane integrity.

4.3. Part II: earthworm health

X-ray micro CT analysis of depurated earthworms evidenced the presence of denser masses of which the assignment to residual soil within the earthworm body was excluded by ESEM investigation. These results suggest that the development of the dense spots can be due to the difficulty by the earthworm to excrete the contaminants (Boone et al., 2004).

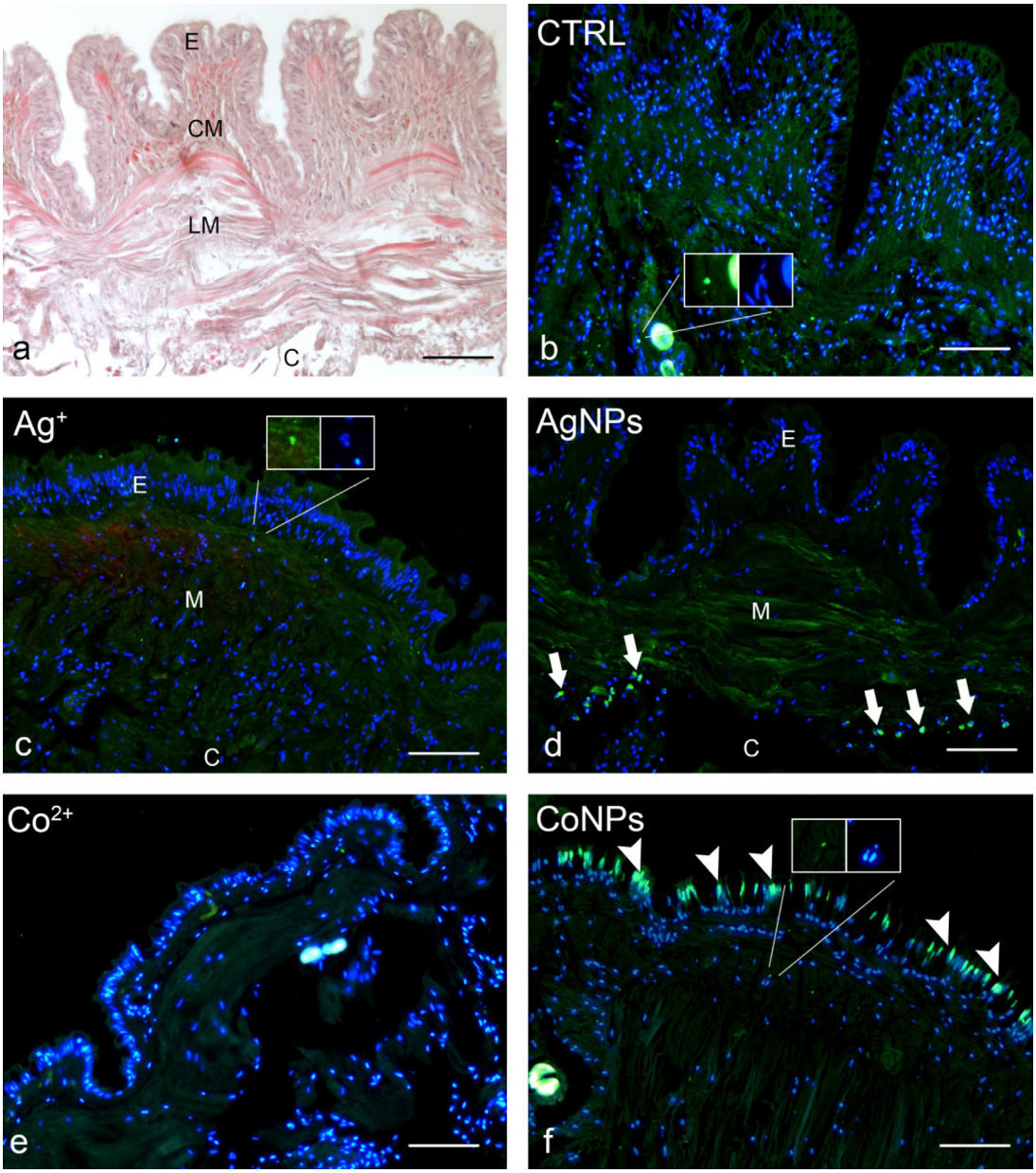

Fig. 3. Histology of *L. rubellus*, body wall, longitudinal section from the zone just posterior to clitellum. a) Hematoxylin-Eosin of a control specimen. The body wall is made up by epidermis (E), circular muscle layer (CM), longitudinal muscle layer (LM), and a layer of cells which line the coelomic cavity (C), named peritoneum. b) TUNEL and DAPI staining in control specimen. An apoptotic nucleus is detectable in the muscle (see frames with a higher magnification and split fluorescence channels), close to a seta, which is autofluorescent. c) TUNEL and DAPI staining in a specimen treated with Ag^+ and depurated. No apoptosis is detected in epidermis (E), nor in the peritoneum lining the coelomic cavity (C), while scattered, rare apoptotic nuclei are present in the muscle (M) (see frames with an higher magnification and split fluorescence channels). d) TUNEL and DAPI staining in a specimen treated with AgNPs and depurated. Numerous apoptotic nuclei are detectable in the peritoneum (arrow) lining the coelomic cavity (C). e) TUNEL and DAPI staining in a specimen treated with Co^{2+} and depurated. f) TUNEL and DAPI staining in a specimen treated with CoNPs and depurated. Numerous apoptotic nuclei are detectable in epidermis (arrowhead) lining the coelomic cavity (C). Scale bars: 100 μm.

In addition, the histological investigations, after the depuration period, showed positive results about the NPs toxicity. In particular, TUNEL-positive nuclei in epidermis and in peritoneum, suggest the presence of toxicosis. Such results agreed with those of van der Ploeg et al. (2014) that found immune cells (coelomocytes) of earthworms showing sensitivity to both AgNP and $AgNO_3$ in vitro. Some metals can replace or mimic essential metals such as magnesium and calcium giving a mechanistic insight into the toxic action of certain metals (Clarkson, 1993). Co^{2+}can be bound by chloragosome granules and can have detrimental effects on the metabolism of essential cations, notably Ca^{2+} (Clarkson, 1993; Morgan and Morris, 1982), so compromising the Ca/P ratio within chloragosomes organelles (Morgan and Morgan, 1989).

Indeed, Ca/P spherical calcifications, as those identified in earthworm tissues, have been previously detected in human pathological tissues affected by cancer (Gatti et al., 2008; Gatti and Montanari, 2008). Based on these findings, the Ca/P ratio in earthworm tissues needs to be further investigated to prove its usefulness as basal biomarker for assessing changes in earthworm health.

5. Conclusions

The addition of polluted horse manure to soil as food for earthworms changed the microbial community structure in earthworm faeces and, in turn, such changes were reflected on

soil microbial biomass and community structure, thereby suggesting a close relationship between the microbial communities of soil and of earthworm faeces. Our results suggest a significant impact of contaminants, supplied via contaminated food to earthworms, on bacterial community structure after its passage through the digestive tract whereas fungi were not affected. PLFAs biomarkers suggested that G- bacteria are those more impacted by contaminants. The absence of changes in microbial basal respiration may suggest a functional redundancy of soil microorganisms. However, such an aspect should be studied more comprehensively as slight but significant shifts in specific respiration rate were observed in Co treatments, indicating some stress on microbial biomass.

The investigation of *L. rubellus* health after four weeks of depuration evidenced the onset of disorders at different degrees in all treatments. The Ca/P ratio in earthworm tissues could become an early biomarker of earthworm health.

To our best knowledge, this study is the first concerning the effect of Ag and Co on the relationship between soil microorganisms and *L. rubellus*. Further studies are needed to better understand the mechanistic way of Ag and Co effects on soil microorganisms and on its bioaccumulation in *L. rubellus*, as well as the identifications of biomarkers able to describe the nano-bio-interactions.

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