Dose effect of Zn and Cu in sludge-amended soils on vegetable uptake of trace elements, antibiotics, and antibiotic resistance genes: Human health implications

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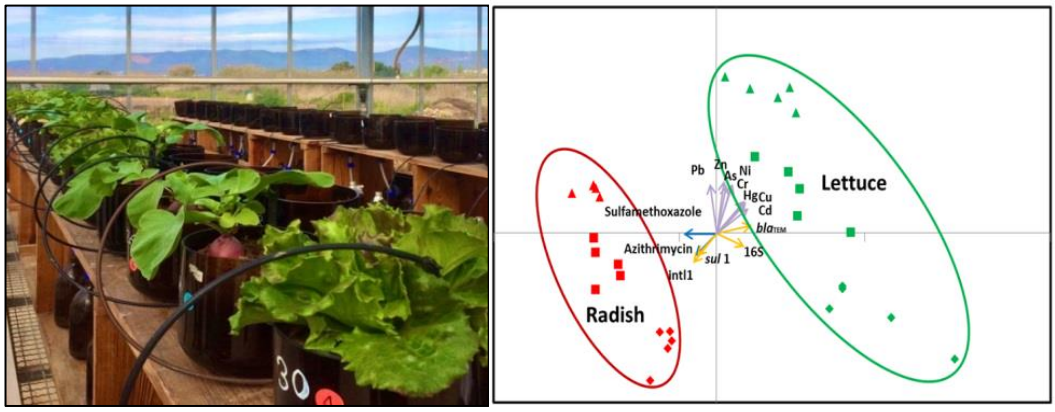
Highlights

- The impact on crops of rising Zn & Cu doses in sludge-amended soils was assessed
- Adding Zn & Cu favored the accumulation of most trace elements (TEs) in vegetables
- Antibiotic and antibiotic resistance gene (ARG) uptake was not significant in crops
- Occurrence of antibiotics and most ARGs was higher in radish roots than lettuce leaves
- TE risk increased 2-3-fold with Zn & Cu, but was still not a risk for consumers

Graphical Abstract



Abstract

The application of sewage sludge to agricultural fields reduces the need for mineral fertilizers by increasing soil organic matter, but may also increase soil pollution. Previous studies indicate that zinc and copper, as the most abundant elements in sewage sludge, affect plant uptake of other contaminants. This paper aims to investigate and compare the effect of increasing amounts of Zn and Cu in sludge-amended soils on the accumulation of trace elements (TEs), antibiotics (ABs), and antibiotic resistance genes (ARGs) in lettuce and radish. The vegetables were grown under controlled conditions, and the influence on plant physiology and human health were also evaluated. The results show that the addition of Zn and Cu significantly increased the concentration of TEs in the edible tissue of both vegetables. According to the hazard quotient (HQ) of the TEs, the human health risk increased 2 to 3 times and was 3 to 4 times greater in lettuce than in radish. In contrast to the TEs, the occurrence of ABs and most of the ARGs was higher in radish roots than lettuce leaves. ABs were not detected in lettuce leaves, and the amount of all ARGs except bla_{TEM} was 10 times lower than in radish roots. On the other hand, the addition of Zn and Cu had no significant effect on the occurrence of ABs and ARGs in the edible part of the vegetables, and no damage was found to plant productivity or physiology. The results show that the consumption of lettuce and radish grown in sewage-sludge-amended soils under our dosage does not pose an adverse human health effect, as the total HQ value was always less than 1, and the presence of ABs and ARGs was not found to have any potential impact. Nevertheless, further studies are needed to estimate the long-term effect on human health of crops grown under frequent application of biosolids in arable soil.

Keywords: lettuce; radish; metals; antibiotics; ARG; sewage sludge

1. Introduction

The implementation of the Urban Waste Water Treatment Directive (91/271/EC) in the European Union led to an increase in the quantity of sewage sludge, a by-product of the wastewater treatment process. According to a forecast by Milieu Ltd (2010), the amount of sludge generated in the EU would exceed 13 million tons by 2020. Furthermore, the safe disposal of sewage sludge accounts for up to 50% of the operating costs of wastewater treatment plants. The European Commission thus proposed an action plan related to the circular economy (European Commission, 2015), which encourages the application of treated sewage sludge to agricultural soil due to its high content of nutrients and organic matter, provided it complies with Directive 86/278/EEC in terms of chemical (trace elements) and biological (pathogens) pollutants.

However, soil amendment with sewage sludge remains a challenging task because of the presence of organic and inorganic contaminants. For example, Iglesias et al. (2018) found a significant increase in the amount of Pb, Hg, Zn, and Ag in sludge-amended soils. Cheng et al. (2014) detected that the concentrations of fluoroquinolones (FQs), tetracyclines (TCs), and sulfonamides (SAs) in 58 sewage sludge samples ranged from 1,569 to 23,825 μg/kg, 592 to 37,895 μg/kg, and 20.1 to 117 μg/kg dry weight (dw), respectively. Therefore, concerns have emerged about the chemical contamination of crops cultivated in sewage-sludge-amended soil, as plants can take up TEs and ABs from the substrate (Guoqing et al. 2019).

In addition to these contaminants, the antibiotic resistome, which results from the application of sewage sludge, has gotten more and more attention. As reported in many studies, sewage sludge is a hotspot for bacteria carrying antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) (He et al., 2019; Zieliński et al., 2019). Owing to the abundant carriers derived from sludge, ARGs could be disseminated in soil by horizontal gene transfer (HGT) after application (Zhou et al., 2019). Furthermore, the presence of TEs and ABs in sewage sludge can exert long-term selective pressure on soil microorganisms and increase the over-expression of ARGs (Urra et al., 2019). Some bacteria carrying ARGs might colonize plants as endophytes or adhere to plant surfaces and manage to survive and persist throughout the vegetable growth stage (Pu et al., 2019). Thus, there will be a notable human health risk of exposure to ARGs through the consumption of contaminated vegetables (Zhao et al., 2019). Yang et al. (2018) detected

numerous ARGs (*catB*8, *php, van*B, and *str*) in lettuce grown in amended soil and discovered that the application of sewage sludge boosts the evolution and dissemination of ARGs in the soil-plant system. Murray et al. (2019) compared the effect of sewage sludge on the occurrence of ARGs in several vegetables and reported that root vegetables carry more abundant ARGs than leafy vegetables.

As the most plentiful elements in sewage sludge, copper and zinc could be crucial for the occurrence and long-term accumulation of other contaminants in crops as a result of three effects or mechanisms. The first is the antagonist effect, which reduces the concentration of other TEs in the crop through competition for the same binding sites on the root. The second is the complexation effect, which affects the fate of ABs as a result of complexing with a large number of amino groups, carboxyl groups, hydroxyl groups, and heterocycles in ABs. One common consequence, a reduction in antibiotic potency, has long been known, with Zn inactivation of penicillin first having been reported in 1946 (Eisner and Porzecansri, 1946) and later having been shown to result from Zn binding to and promoting the hydrolysis of this β-lactam (Gensmantel et al., 1980). Nevertheless, Sayen et al. (2019) revealed that the presence of Cu favored the plant uptake of enrofloxacin (Enro), a fluoroquinolone antibiotic, which could be taken up as both free (mainly in zwitterionic form) and Cu-Enro complexes (as positively charged complexes). Finally, the third is the co-selection mechanism, which triggers the proliferation of ARGs (Liu et al., 2019). ARGs and metal resistance genes (MRGs) are frequently located together on the same genetic elements, such as a plasmid, transposon, or integron. This physical linkage results in an increase in the expression of ARGs in bacteria under the pressure of Cu and Zn.

Many studies have separately examined the levels of TEs, ABs, and ARGs in vegetables grown in soil amended with sewage sludge (Shamsollahi et al., 2019; Wei et al., 2020). However, information on the accumulation and interaction of these contaminants is still limited, especially under the selective pressure of Cu and Zn. Therefore, the present paper aims to assess the effect of different Cu and Zn content in sewage-sludge soil amendment on the uptake of TEs, ABs, and ARGs in the edible part of lettuce (leaf) and radish (root), as well as evaluate the phytotoxicity and human health implications.

2. Materials and method

2.1 Experimental layout

The experiment was conducted in a glass greenhouse located at the Agròpolis-UPC agricultural experiment station (41° 17′ 18″ N, 2° 02′ 43″ E) in Viladecans (Barcelona, Spain) with an average temperature of 21℃ and a relative humidity of 56%. In accordance with the optimum N concentration needed for plant growth (Pomares and Ramos, 2010), experimental units consisted of 2.5 L cylindrical amber glass pots (Ø = 15 cm, 20 cm high) filled with 1652 g fresh soil, 878 g sand, and 70 g wet sewage sludge with a humidity of 79.1%. Soil samples were collected from the adjacent area (<20 cm) to which no antibiotic products had intentionally been applied before this project. Sewage sludge was obtained from the Gavà-Viladecans wastewater treatment plant (Parc del Baix Llobregat, Barcelona). $ZnSO_4 \cdot 7H_2O$ and $CuSO_4 \cdot 5H_2O$ were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) with a purity >99%. Lettuce (*Lactuca sativa* L. cv. Arena) and radish (*Raphanus sativus* cv. Redondo Rojo Vermell) were chosen for the study, because they are some of the most widely consumed vegetables in the Mediterranean region and could represent two different crop types (leafy and root plant). To evaluate the effect of Cu and Zn content in sewage sludge on the uptake of contaminants in vegetables grown in amended soil, the following treatments were established: (i) control: unspiked sewage sludge containing 240 mg/kg Cu and 700 mg/kg Zn (dw); (ii) treatment 1 (T1): sewage sludge spiked with $CuSO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$ to two-fold the background value of Cu and Zn, i.e. to 480 mg/kg and 1400 mg/kg (dw); and (iii) treatment 2 (T2): sewage sludge spiked with more $CuSO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$ to four-fold the original Cu and Zn concentration, i.e. to 960 mg/kg and 2800 mg/kg (dw). All treatments were replicated five times. One lettuce or radish seedling was planted in each experimental unit on March 4, 2019, and irrigated with harvested rainwater (50 mL/pot/day) through a drip irrigation system. Plants were harvested until they reached the commercial size (35 productive days for radish, 56 for lettuce), and the lettuce leaf and the radish root were stored at -20 ℃ until analysis.

2.2 Analytical procedures

2.2.1 Reagents and standards

This information is included in the Supplementary Material.

2.2.2 Analyses of soil and sewage-sludge samples

Physical and chemical parameters: Humidity, pH, electrical conductivity (EC), organic matter content (OM), total nitrogen, and available nitrogen, phosphorus, and potassium (NPK) were determined using standard methods described by Sparks (1996).

Trace elements: Soils and sewage sludge were digested in concentrated HCl–HNO_3 at 95 °C for 30 min. After cooling and filtering (<0.45 µm), samples were analyzed by ICP-MS (Thermo Scientific) (Martin et al., 1994). The mercury concentration was measured using an AMA-254 (Altec, Prague, Czech Republic).

Antibiotics: No products containing antibiotics had ever been intentionally applied to the soil used in the experiment. The extraction method for the sewage sludge sample was as described by Berendsen et al. (2015). Briefly, 500 mg sludge was weighed into a 50 mL centrifuge tube and 4 mL of McIlvain–EDTA buffer and 1 mL ACN were added to the sample. The sample was ultrasonic extracted for 15 min. Then 2 mL of lead acetate solution was added and the sample was vigorously shaken by hand. After centrifugation (3500 g, 10 min) the extract was diluted with 13 ml 0.2 M EDTA solution. A Phenomenex (Torrance, CA, USA) Strata-X RP 200 mg/6 mL reversed-phase solid phase extraction (SPE) cartridge was conditioned with 5 mL MeOH and 5 mL water. The complete extract was applied to the SPE cartridge, which was subsequently washed with 1 mL of MeOH/H_2O (5/95, v/v). The antibiotics were eluted from the cartridge using 5 mL MeOH followed by evaporation to dryness. The residue was redissolved in 1 mL of MeOH/H_2O (5/95, v/v) before being transferred into an LC–MS/MS sample vial.

Antibiotic resistance genes: DNA was extracted from the soil and sewage-sludge samples using a DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNA concentration was measured using a NanoDrop Spectrophotometer 8000 (Thermo Fisher Scientific). Seven ARGs (*sul1, qnrS1,* bla_{TEM}, *tetM,* $bla_{CTX\text{-}M\text{-}32}$, $bla_{OXA\text{-}58}$, and *mecA*), the integron *intI1*, and the 16S ribosomal RNA gene were quantified by qPCR following the protocol described by Cerqueira et al. (2019b).

2.2.3 Analyses of vegetables

Plant phytotoxicity: The length and number of leaves of both vegetables were measured weekly until the end of the experiment. The chlorophyll content in the leaves and weight of the edible part of both vegetables were measured in situ. Chlorophyll was gauged using a chlorophyll meter (Opti-Sciences, Hudson, NH, USA). Each measurement was performed on three leaves per crop. A calibration curve was obtained to correlate the chlorophyll content with the previously measured absorbance. Round leaf samples (4 cm diameter) were then extracted with 5 mL of *N*,*N*-dimethylformamide (DMF) and kept in the dark at 4 °C for 48 h before the spectrophotometric determination. The extracts were measured at two wavelengths, 647 and 664.5 nm, so that the chlorophylls could be calculated using Inskeep and Bloom's coefficients (Inskeep and Bloom, 1985; Porra, 2002). Lipid extraction was carried out by adding 15 mL of ethanol/hexane (1:1, v/v) to a glass tube with 3 g of fresh sample (Margenat et al., 2018). The sample was then sonicated for 15 min and centrifuged at 2500 rpm for 15 min. It was further filtered through a 0.22 μm nylon filter (Scharlab, Barcelona, Spain). After the solvent was removed by purging and drying the sample with nitrogen gas, the sample remaining in the tube was weighed and operationally defined as lipid content.

Trace Elements: The edible part of the vegetables (the leaf of the lettuce and root of the radish) was freeze-dried and digested with a microwave oven (Milestone Ethos), as described by Llorente-Mirandes et al. (2010). Briefly, 0.1 g of powdered sample was weighed into the PTFE vessels, and 8 mL of HNO_3 and 2 mL of 31% H_2O_2 were added. The digestion program carried out was as follows: 15 min from room temperature to 90 ℃; 10 min at 90 ℃; 20 min from 90 ℃ to 120 ℃; 15 min from 120 ℃ to 190 ℃; 20 min at 190 ℃. After cooling to room temperature, the digested sample was analyzed by ICP-MS. The mercury concentration was measured with an AMA-254 (Altec, Prague, Czech Republic).

Antibiotics: A modified QuEChERS method (Martínez-Piernas et al., 2018) was performed. Briefly, 5 g of homogenized vegetable sample was placed in a 50 mL PTFE tube, 10 mL of 1% acetic acid in ACN was added to the sample, and the tube was vigorously shaken for 5 min. Then, 6 g of anhydrous $MgSO_4$ and 1.5g of NaOAc were added to the tube, and the tube was vigorously shaken for another 5 min and centrifuged at 3500 rpm for 5 min. After that, 5 mL of aliquot of the upper organic phase of the extract

was transferred to a 15 mL centrifuge tube and cleaned up through the addition of 450 mg of $MgSO_4$ and 60 mg of C18. The tube was then shaken vigorously for 30 s in a vortex and centrifuged at 3500 rpm for 5 min. Finally, 100 µL of the final extracts were evaporated to dryness under a gentle N_2 stream and reconstituted with 1 mL of H_2O:ACN (95:5, v/v) before being injected in the LC–MS/MS system.

Antibiotic resistance genes: The preliminary procedure for DNA extraction from vegetables was performed as described by Cerqueira et al. (2019a). Briefly, the edible part of the vegetable (90-100 g) was processed in a grinder (Grindomix GM200, Retsch, Inc). The crushed vegetable matter was transferred to a beaker along with 50 mL of sterile phosphate-buffered saline (PBS), mixed gently, and then filtered through a sterile gauze to remove the pulp. The resulting filtrate was transferred to 50 mL tubes through a 100 µm nylon mesh cell strainer (Corning® Cell Strainer) and centrifuged at 4000 rpm for 15 min. DNA extraction was carried out from the pellets and absolute quantification of ARGs (*sul*1, *qnr*S1, bla_{TEM}, *tet*M, $bla_{CTX\text{-}M\text{-}32}$, $bla_{OXA\text{-}58}$, and *mec*A), *intI*1, and the 16S genes was performed as indicated in the soil and sludge section. The 16S values were subsequently corrected as determined by the 16S sequencing (Cerqueira et al. 2019b) to remove plastidial (chloroplasts, leucoplasts, and mitochondria) 16S sequences.

2.3 Estimated daily intake

The estimated daily intake (EDI) of TEs and ABs were determined based on both the content in the vegetables and the consumption amount of the respective crop. The EDI for a Spanish adult was calculated as follows (Equation 1):

$$EDI = \frac{\mathrm{DI} \times C_P \times \mathrm{F}}{\mathrm{BW}} \qquad (1)$$

Where DI is the daily intake of vegetables (according to the EFSA's Comprehensive Food Consumption Database, the average weights of lettuce and radish consumed by a Spanish adult are 0.045 and 0.022 kg/day (wet mass), respectively); C_P is the concentration of each pollutant in the crop (mg/kg dw); and F is a factor (0.091) to convert fresh weight (fw) to dry weight. BW is body weight, which is assumed to be 70 kg.

2.4 Human health risk assessment

To evaluate the human health risk, the hazard quotient (HQ) was calculated.

The HQ of TEs (HQ_{TE}) is the ratio between the EDI and the reference oral dose (RfD), as shown in Equation 2.

$$HQ_{TE} = \frac{\mathrm{EDI}}{\mathrm{RfD}} \qquad (2)$$

Where RfD is the maximum tolerable daily intake (μg/kg/day) of a specific element that does not result in carcinogenic effects for human beings, obtained from IRIS (2020). An HQ＞1 implies a potential risk to the population; otherwise, the consumer is safe.

Finally, the total hazard quotient (THQ_{TE}), used to assess the total risk of all chemicals to which an individual might be exposed, was calculated as the sum of the HQ_{TE} of all the elements.

The HQ of ABs (HQ_{AB}) was calculated using the acceptable daily intake (ADI), as shown in Equation 3.

$$HQ_{AB} = \frac{\mathrm{EDI}}{\mathrm{ADI}} \qquad (3)$$

Two antibiotic ADIs were calculated based on different endpoints. For therapeutic purposes, ADI1 was calculated by dividing the lowest daily therapeutic dosage for an adult (mg/day) by a safety index (1000) and body weight (70 kg) (Prosser and Sibley, 2015), while ADI2 was adopted from provisional values established in the literature or derived using toxicological, microbiological, or therapeutic approaches (Wang et al., 2017). Consumption of vegetables contaminated with antibiotics is only one pathway of human exposure. Therefore HQ_{AB}＞0.1 indicates a potential hazard.

2.5 Data analysis

A non-parametric Mann-Whitney U test was performed and Spearman's correlation coefficient was calculated for multiple comparisons or to analyze interactive effects between different factors. Principle component analysis (PCA) was conducted on the concentrations of TEs, ABs, and ARGs. Varimax rotation was applied because orthogonal rotation minimizes the number of variables with a high loading on each component and

facilitates the interpretation of results. Statistical significance was defined as $p < 0.05$. The data analysis was performed with the SPSS v25 package (Chicago, IL, US).

3. Results and discussion

3.1 Characterization of soil and sewage sludge

Some physical and chemical properties of soil and sewage sludge are shown in Supplementary Table S2. Due to its significantly higher content of NPK and organic matter, sewage sludge has proven to be a useful source of nutrients for vegetables. However, the higher electrical conductivity of sludge also poses a potential threat, namely, possible salt toxicity to plants and soil organisms (Zhou et al., 2005).

The TEs were selected according to the applicable regulation of the European Parliament and European Council (PE-CONS 76/18, 2019). A total of 8 TEs need to be tested for in sewage sludge used for fertilizer. Supplementary Table S3 shows their concentrations in the soil and sewage-sludge samples, as well as the corresponding maximum admissible limits for agricultural soil and biosolids fertilizer. In the present soil sample, TE concentrations (dw) ranged from 0.025 mg/kg (Hg) to 92 mg/kg (Cu), and all content was below the generic reference value for contaminated soil in Catalonia. In the sewage sludge, the most abundant element was Zn (700 mg/kg), followed by Cu, Cr, Ni, Pb, As, Hg, and Cd. The TE contents were similar to the abundance of TEs reported in sludge from other sewage treatment plants in Barcelona, Spain (0.12-1270 mg/kg) (Husillos Rodríguez et al., 2013). Only the level of Ni was found to be slightly higher than allowed under fertilizer regulations (see Table S3).

The concentration of ABs (dw) in the sewage sludge ranged from undetected to 5790 μg/kg (ciprofloxacin), which is 45 times higher than the other detected ABs. Supplementary Table S4 shows that 6 of the 16 ABs analyzed had values over the limit of detection in sewage sludge. According to previous studies, AB concentrations in sewage sludge vary from ng to mg per kg dw depending on the treatment techniques, operational conditions, and sources (Li et al., 2013). In the present sludge, the concentration of ciprofloxacin was similar to that in sludge from 11 Swedish sewage treatment plants (1600–11000 μg/kg dw)（Östman et al., 2017).

Quantification of *mec*A in both soil and sewage sludge samples was not possible since it was found to be under detection limits. The rest of the targeted genetic elements

were all detected, but *bla*$_{CTX-M-32}$ in sewage sludge samples and qnrS1, *bla*$_{TEM}$, *bla*$_{CTX-M-32}$, and *bla*$_{OXA-58}$ in soil samples were found to be below the quantification limits (Supplementary Fig. S1). Since ARGs are more abundant in sewage sludge than soil, this indicates a potential risk of increased antibiotic resistance in soil after amendment. In addition, the high values of *intI*1 in sewage sludge imply that the application of sludge may accelerate the dissemination of ARGs in the matrix through horizontal gene transfer. Among the ARGs, *sul*1 was detected at levels around 100 times higher than the others, except for *tet*M. This is consistent with the report that the sulfonamide-resistant gene is prevalent in sewage sludge, due to the very low removal of the *sul* gene during sewage treatment plant processes (Xu et al., 2015).

3.2 Effect of Zn and Cu addition to sludge on plant uptake of trace elements

The addition of Zn and Cu to sludge resulted in significant changes in most of the TEs in the edible part of both vegetables (Fig. 1). The difference became clear at higher spiking levels, except for Cd (in lettuce and radish) and Hg (in radish). Zn–Cd interactions are controversial, since both antagonism and synergism between the two elements have been reported. One field experiment showed that the interaction mechanism of these two metals was synergistic, such that increased Cd and Zn content in soils could increase the bioaccumulation of Zn or Cd in crops (Nan et al., 2002). Later findings, however, found antagonism between Zn and Cd in the uptake–transport process (Wu et al., 2003). In the present study, the changes observed in Cd with increasing Zn were not steady. This may be caused by the interaction of these two mechanisms. Hg has comparatively high electronegativity values and easily forms bonds with other elements, especially with S anions. Hg compounds are unaffected by hydrolysis and hardly used by plants. Therefore, the addition of Zn and Cu neither favored nor inhibited Hg uptake by radish. Unsurprisingly, the quantity of Zn grew notably in plant tissue when extra Zn was added to the substrate. Specifically, Zn increased from 28.75 mg/kg to 46.54 mg/kg in lettuce, and from 17.50 mg/kg to 36.42 mg/kg in radish. In contrast, Cu tends to be more strongly absorbed in soil, and plants regulate its uptake more effectively than Zn (Kabata-Pendias, 2011). In the present study, Cu only increased from 4.02 mg/kg to 4.45 mg/kg in lettuce, and from 2.17 mg/kg to 2.69 mg/kg in radish. Thus, plant tissue concentrations and availability of Cu are usually much lower and less sensitive to soil inputs of this element as a component of sludge compared with more mobile elements such as Zn. On the other

hand, a Zn–Cu antagonistic interaction has been reported, due to the involvement of the same carrier sites in their absorption mechanisms (Kabata-Pendias, 2011). The concentrations of other TEs increased to different extents. For lettuce, the increase of TEs was as follows: Pb (344%) > As (289%) > Ni (243%) > Cr (235%) > Zn (162%) > Hg (137%) > Cu (111%) > Cd (103%). In the radish root, the sequence was Pb (332%) > Ni (288%) > As (266%) > Zn (208%) > Cr (187%) > Cu (124%) > Cd (105%) > Hg (97%).

As shown in Figure 1, the accumulation of TEs was significantly ($p<0.05$) greater in the edible part of lettuce than in radishes. The concentration in lettuce leaves ranged from 0.012 to 46.54 mg/kg vs. 0.005 to 36.42 mg/kg in radish root. The relatively longer growth period of lettuce compared to radish (2 months vs. 1 month) makes it easier for lettuce to absorb more TEs. In addition, the higher transpiration and translocation rates of lettuce facilitate element transport to aerial parts (Gupta et al., 2019). The sequential increase in the abundance of TEs in both crops was equivalent (Zn > Cu > Cr > Ni > Cd > As > Pb > Hg), indicating that although the absolute amount of TEs in vegetable tissue differs between plants, the relative abundance of TEs is more dependent on the type of trace elements. In this sense, Zn and Cu, the essential metals in plants, are constituents of several key enzymes and also play important functions in physiological processes. In contrast, there is no evidence to date that the rest of the TEs play an essential role in plant metabolism, or that they can even be considered phytotoxic elements for plants (As, Pb, and Hg). Accordingly, their uptake was limited.

3.3 Effect of Zn and Cu addition to sludge on plant uptake of antibiotics

Table 1 shows the occurrence of ABs in plant samples grown under different concentrations of Zn and Cu. Target ABs were below the LOD in lettuce samples, and 2 (azithromycin and sulfamethoxazole) out of 16 ABs were close to or slightly above the LOQ in radish samples.

It was somewhat surprising to find sulfamethoxazole in the radish samples, as its value in the sludge was lower than the LOD. There are two possible explanations for this. The first might be related to the possibility that the sulfamethoxazole in the sewage sludge is in its conjugate form and, consequently, was not detected. Once in the soil, enzyme activity could release the parental product, which could be accumulated in the radish. The second possible explanation is that sulfamethoxazole simply has a very high bioconcentration factor, which would explain its accumulation in radish even though its concentration was below the LOD in the sludge (Table S4). In this sense, a recent study

found sulfamethoxazole in all the vegetables tested, with the highest contamination levels being found in lettuce leaves compared to tomato fruits, cauliflower, and broad bean seeds (Tadić et al, 2019). This is also consistent with a previous study that found that sulfamethoxazole has high leaching potentials from soil and sludge due to the low K_d value (1.7 L/Kg) (Höltge and Kreuzig, 2007).

The AB concentrations varied slightly across the treatments ($p > 0.05$), which may be due to their low abundance levels in vegetables. In fact, most were below the LOQ, suggesting they may not be able to express a precise fluctuation. The sulfamethoxazole increased along the gradient of Cu and Zn concentrations by around two-fold; however, the difference was not significant. Liu et al. (2017) reported that the presence of Cu^{2+} inhibited the sorption of sulfamethoxazole by competing in the hydrophobic adsorption region in soils. Desorbed sulfamethoxazole could be taken up by plants along with pore water. In the present study, AB concentrations in plant tissues were fairly low. This is probably due to three factors: (a) the background AB value in the substrate was much lower than in other studies (Qian et al., 2016; Ye et al., 2016); (b) the poor mobility of ABs such as ciprofloxacin, which is considered non-mobile (Tolls, 2001) and has a high affinity to soil particles (K_d 430 L/kg), hinders their uptake by vegetables from soil, despite their relatively high abundance in the substrate; and (c) the plant's detoxification mechanism, which may lead to undetectable AB accumulation in plant tissue (Farkas et al., 2007).

3.4 Effect of Zn and Cu addition to sludge on plant uptake of antibiotic resistance genes

Absolute values of 16S rDNA sequences in lettuce and radish ($0.7\text{-}10.2 \times 10^8$ copies/g and $0.9\text{-}4.4 \times 10^8$ copies/g, respectively) (Fig 2.) were slightly higher than in other vegetables (e.g., in broad beans, the range is from 140 to 5.2×10^7 copies/g) treated with chemical fertilizers in peri-urban plots of Barcelona (Cerqueira et al., 2019c). These results suggest that the application of sewage sludge may favor the proliferation of endophytic bacteria in crops. In contrast, *intI*1 was found at lower levels than in other comparable studies (10^4 for lettuce, 10^5 for radish in Lau et al., 2017), which suggests a relatively low potential for horizontal gene transfer linked to group I integrons, especially for lettuce.

Among the ARGs, abundance levels varied by several orders of magnitude, with *sul*1 being the most abundant ARG in radish and bla_{TEM} in lettuce. Quantification of *mec*A

and *tet*M was not possible since they were found to be under detection limits. On the other hand, *bla*$_{TEM}$, in the case of radish samples, and *qnr*S1, *bla*$_{CTX-M-32}$, and *bla*$_{OXA-58}$, for both radish and lettuce, were found below quantification limits. The distribution of quantified genetic element abundance in vegetable samples was evaluated using the Mann-Whitney U test. The analysis shows that ARG abundance was more influenced by crop type than additional Cu and Zn. Radish may pose a higher ARG risk, except in the case of *bla*$_{TEM}$. This finding is consistent with prior studies showing radishes had a greater load of ARGs than lettuce (Guron et al., 2019; Tien et al., 2017). Both growth time and vegetable species could affect the occurrence of ARGs in vegetable tissues. For instance, the *tet*AP gene was detected in the endive phyllosphere at 30 d, but not at 60 d (Wang et al., 2015). Crops could shape the overall rhizosphere and phyllosphere microbiota through the secretion of various proteins, amino acid, phenols, etc. (Berg and Smalla, 2009; Bulgarelli et al., 2013). Another study (Fogler et al., 2019) found differences in the lettuce and radish resistomes and suggested that the extent of soil contact should be considered. Furthermore, studies carried out in our group have established that 0.01 to 1% of the ARG loads present in the soil can be found in the edible parts of the plant (Cerqueira et al., 2019a, 2019b, 2019c).

In the present study, no significant differences in ARGs in plant tissue were observed with the additional dose of Zn and Cu. Metal cations have a broad ability to complex with antibiotics to decrease their bioavailability (Uivarosi, 2013). Hence, following amendment with extra Zn and Cu, soil bacteria may face less pressure from antibiotics, thereby preventing the spread of ARGs in the matrix microbial community. Owing to the low abundance of *intI*1 in plant tissue, the possibility for ARGs to be transferred from soil bacteria to endogenous bacteria in plants is relatively rare. On the other hand, bacteria have developed a variety of resistance mechanisms to counteract TE stress, including altered gene expression (increasing metal resistance genes, which may induce the expression of ARGs) and changes in the physiological state (Teitzel and Parsek, 2003).

Supplementary Figure S2 shows the relationship among quantified genetic elements in the edible tissue of both vegetables. In radish samples, *sul*1 was significantly related to bacterial 16S and *intI*1. This indicates that the occurrence of *sul*1 in radish was strongly dependent on the number of endophytic bacteria and positively related to the ability of horizontal transfer of bacterial genes, whereas this relationship is weak in lettuce. ARGs in air particulates have been reported to be diverse and abundant (Li et al., 2018) and may be deposited on the surface of plant leaves and invade them via leaf stomata.

The airborne ARGs may impair the association of ARGs-*intI*1-bacterial abundance in microbiomes in the aerial part of the plant.

3.5 Distribution and relationship of contaminants

The correlations between the TE, AB, and ARG variables were further analyzed by PCA, as shown in the biplot (Fig. 3). Two principal components explain 79% of the variation in the data. The first principal component (PC1), which accounted for 61% of the variance, clearly separated the samples into two distinct groups, lettuce and radish. This trend indicates that vegetable type is the key factor for the accumulation of contaminants in edible tissue. Cd and *bla*$_{TEM}$ have high positive loading values (>0.80) in PC1， while sulfamethoxazole has a high negative loading value (-0.873) (Table S5). This result indicates that the occurrence of these three contaminants is significantly different between vegetables and that sulfamethoxazole may inhibit the absorption of Cd and the expression of *bla*$_{TEM}$.

Additionally, PC2 accounted for 18% of the total variance. As can be seen in Figure 3, this second component grossly separated the control and the treatments applied to vegetables. Negative values correspond to the control and higher positive values are related to the highest concentrations of Cu and Zn, in treatment T2. The results for PC2 also indicate some associations between TEs and suggest that the addition of Cu and Zn favored the accumulation of other TEs. More specifically, high positive loadings (>0.80) were found for As, Zn, Pb, Cr, and Ni, indicating the same trend as the observed variables. In this sense, adding Zn significantly favored the accumulation of most TEs in vegetables. In general, the main factor affecting the uptake and distribution of TEs was their speciation. The increased TEs in the vegetable tissue may originate from the substrate-bound compounds. When the Zn and Cu are added, they compete for the binding sites in the soil and sludge with other elements, resulting in many more TEs being released into pore water as free ions, which are easily absorbed by plants (Norvell et al., 2000).

Finally, the individual scores for each vegetable in the biplot showed a clearly separate pattern for lettuce and radish, as well as for each treatment. A first cluster made up of the lettuce treatments can be observed (lower and upper right), with a pattern distributed along the PC2 axis for the TEs and *bla*$_{TEM}$. In a second cluster, radish scores (lower and upper left) were scattered toward positive and negative values along the PC2

axis. This is consistent with the lower TE values, but higher AB and genetic element values here than in lettuce.

3.6 Effect on phytotoxicity

Supplementary Figure S3 shows the change in the length and number of leaves according to the different studied conditions. No significant differences were found between the two growth indices in T1 and T2 and the control, indicating that extra Zn and Cu did not hinder the development of either vegetable. This finding is contrary to that reported by Wolf et al. (2017), who found that equivalent Zn and Cu hampered lettuce growth. This was due to the strong complexing ability of the sewage sludge in the present case, which prevented plants from absorbing overdoses of Zn and Cu.

At the end of the experiment, the total chlorophyll content, lipids, and carbohydrates in the leaves, the fresh weight of the edible potion, and the height of the aerial part of both vegetables were analyzed to evaluate the effect of contaminants on productivity (Table S6). No significant differences were observed among the different treatments in either vegetable, indicating that added Zn and Cu have no influence on crop productivity.

3.7 Potential human health risk

3.7.1 Risk assessment for trace elements

The estimated daily intake (EDI) of elements was calculated according to the concentration in edible tissues and daily consumption amount, which is shown in Figure 4. The EDI value was dependent on the element, treatment, and vegetable type. Generally, consuming vegetables grown under treatment T2 led to the highest intake of TEs (3.1×10^{-3} mg/kg/bw/day for lettuce and 1.1×10^{-3} mg/kg/bw/day for radish). As for quantity, Zn accounted for the majority in both crop tissues (2.8×10^{-3} mg/kg/bw/day for lettuce and 0.9×10^{-3} mg/kg/bw/day for radish). According to Commission Regulation (EC) No 1881/2006, the maximum accepted levels of Pb and Cd in lettuce are 0.3 and 0.2 mg/kg, respectively. In radish, this level is 0.1 mg/kg for both metals. Therefore, according to Equation (1), the maximum acceptable EDI value for lettuce is 1.9×10^{-4} mg/kg/bw/day for Pb and 1.3×10^{-4} mg/kg/bw/day for Cd. For radish, the maximum acceptable EDI value

is 3.1×10^{-5} mg/kg/bw/day for both metals. In the present study, the highest EDI values for these two elements were both lower than the limit.

HQ_{TE} was calculated based on the EDI value and the oral reference dose for non-carcinogenic effects (Table 2). Arsenic had the highest HQ_{TE} value in the edible parts of the vegetables and thus posed the highest health risk to humans. This finding is consistent with that reported in Shamsollahi et al. (2019), in which the risk of As was around 20 times higher than Pb and Cd in lettuce samples grown under sewage sludge amendments. In the present study, the level was around 10 times lower than in previous work, due to different background values of TEs in the sewage sludge. The additional pressure of Zn and Cu influenced the uptake of TEs, but it did not change the order of health risks posed by the elements, which was As > Cd > Zn > Pb > Cu > Hg > Cr.

The THQ_{TE} was <1 for all the samples. The THQ_{TE} values were around 3-4 times higher in lettuce than in radish. This is due not only to the higher TE values in lettuce, but also to its higher consumption in Spain. Vegetables grown with the extra pressure of Cu and Zn showed a higher risk, although, since the values were still <1, no risk should be assumed. Nevertheless, the risk was 2-3 times higher in T1 and T2, respectively, compared to the control.

3.7.2 Risk assessment for antibiotics

The consumption of vegetables containing ABs is one pathway of human exposure. Generally, the daily intake of ABs varies considerably between reports and depends on the AB and the vegetable growing conditions. Table 3 lists the EDI values of ABs detected in the vegetables in the present study and other similar studies. The daily intake value of azithromycin in radish varied from 3.1×10^{-6} to 6.3×10^{-6} μg/kg/day, whereas the value of sulfamethoxazole was relatively higher, varying from 9.4×10^{-6} to 1.9×10^{-5} μg/kg/day. As can be seen, compared with other reports, the EDI of antibiotics associated with the consumption of organic vegetables was significantly lower, probably due to the significant difference in substrates.

Table 4 lists the HQ value of the detected antibiotics (azithromycin and sulfamethoxazole) in radish, as well as their acceptable daily intake (ADI) values based on two evaluation methods. Although the risk of both antibiotics varied according to different targets, the HQ_{AB} values of both ABs were significantly lower than the HQ of $\geqslant$ 0.1, identified as a potential hazard to human health for the current assessment. Therefore, consumption of radish and lettuces grown in soil amended with sewage sludge

with high concentration levels of Zn and Cu has no hazardous effect in terms of antibiotics. However, further research is required, as many other exposure pathways for human beings to antibiotics, e.g. farm fish, drinking water, or inhaled particles, are plausible.

3.8 Risk assessment of antibiotic resistant genes

The human health concern posed by foodborne antibiotic resistance is derived from the fact that pathogenic bacteria may acquire antibiotic resistance genes from strains in ready-to-eat food, thereby jeopardizing the future of antibiotic therapy (e.g., due to the loss of the option of clinical antibiotic use) and increasing the severity of infection as manifested in the prolonged duration of disease.

The mechanism of resistance of *bla*$_{TEM}$ is the production of β-lactamases, which are able to hydrolyze the four-membered β-lactam ring present in some antibiotics, such as penicillin and cephalosporin. *bla*$_{TEM}$ is the predominant gene in lettuce leaves but at levels slightly above the quantification limit. Even at these low values, the presence of this gene may be cause for some concern regarding lettuce leaf consumption, although the real importance of this potential risk has yet to be determined.

On the other hand, *sul*1 accounted for the largest share of the target ARGs in radish samples. This gene codes for resistance to sulfonamide antibiotics, which can be related to the detected presence of sulfamethoxazole in radish. In this regard, it is worth considering the possibility that the actual antibiotic selective pressure may occur in the soil, rather than in the root, as the plant resistome is highly influenced by the soil (Cerqueira, 2019b). The similar trends observed in *sul*1 and *intI1* in both matrices is probably linked to the fact that *sul*1 is a group I integron-associated gene (Ma, et al., 2020). This further supports the hypothesis that some selective pressure for sulfonamide resistance exists in either soil or the plant.

This study thus demonstrates that foodborne antibiotic resistance depends on the type of vegetable consumed and that Cu and Zn accumulation in the soil does not affect its spread. However, the question of the extent to which foodborne ARGs could affect human health remains. Appropriate models for translating foodborne antibiotic resistance to measures of human health risk are needed. The present study provides data that could be useful for making such a determination in the future, as they suggest that the target antibiotic resistance differs among different vegetables.

4. Conclusions

Increased doses of Zn and Cu in sludge-amended soils influence TE accumulation in vegetables; however, crop type, rather than increased Zn and Cu concentrations, seems to be the key factor. This is especially evident for the ABs and ARGs slightly detected in vegetables, at least, at the Zn and Cu spiking doses used in this study. Although the addition of Zn and Cu increased the uptake of TEs in the vegetables, ABs and ARGs did not significantly change. In this sense, TE uptake and the health risk were greater (3-4 times) in lettuce than in radish, probably because of the high consumption rate among the Spanish population. However, there was no health risk for the consumer. In contrast, ABs were not detected in lettuce and the abundance of ARGs was 10 times higher in radish. On the other hand, only two ABs, sulfamethoxazole and azithromycin, were quantified and efficiently accumulated in radish root, which had a greater number of gene copies of endophytic bacteria and ARGs than lettuce leaves, except for *bla*$_{TEM}$. Finally, the health risk posed by ABs in the edible tissue of vegetables in this study was relatively lower than reported elsewhere, and the main risk of antibiotic resistance depended on vegetable type. Although the present study showed that lettuces and radish grown under sewage-sludge-amended soil with different accumulated levels of Zn and Cu did not significantly affect the accumulation of ABs and ARGs in the edible part of vegetables, and that increased TEs likewise had no hazardous effect on human health, long-term monitoring is necessary. Frequent amendments with sewage sludge inevitably result in the accumulation of other TEs and ABs in the soil, and increasing environmental pressure could trigger an outbreak of ARGs in vegetables, especially the selection pressure of non-biodegradable TEs.

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Table 1. Concentration of detected ABs (μg/kg, fw) in vegetable tissue

	Lettuce					**Radish**				
	Control	T1	T2	LOD	LOQ	Control	T1	T2	LOD	LOQ
AZM	＜LOD	＜LOD	＜LOD	0.029	0.054	0.02± 0.01[a]	0.02± 0.01[a]	0.01± 0.01[a]	0.010	0.019
SMZ	＜LOD	＜LOD	＜LOD	0.018	0.036	0.03 ± 0.01[a]	0.05±0.01[a]	0.06±0.02[a]	0.030	0.059

Azithromycin (AZM) and sulfamethoxazole (SMZ). Different letter indicates significant difference ($P<0.05$).

Table 2. Hazard quotient of TE (HQ_{TE}) and total hazard quotient (THQ_{TE}) in vegetable samples

	Lettuce			Radish			RfD (mg/kg bw/day)
	Control	**T1**	**T2**	**Control**	**T1**	**T2**	
As	0.0218	0.0437	0.0631	0.0063	0.0129	0.0165	3×10^{-4}
Cu	0.0006	0.0007	0.0007	0.0002	0.0002	0.0003	0.4
Ni	0.0015	0.0028	0.0036	0.0003	0.0005	0.0009	0.02
Zn	0.0057	0.0068	0.0090	0.0017	0.0027	0.003	0.3
Pb	0.0011	0.0028	0.0039	0.0005	0.0011	0.0011	3.5×10^{-3}
Cd	0.0078	0.0076	0.0090	0.0021	0.0021	0.0033	1×10^{-3}
Cr	3.7×10^{-5}	5.8×10^{-5}	8.7×10^{-5}	1.0×10^{-5}	1.2×10^{-5}	2.5×10^{-5}	1.5
Hg	0.0004	0.0004	0.0005	0.0001	0.0001	0.0002	2×10^{-3}
THQ	0.0390	0.0648	0.0900	0.0112	0.0195	0.0251	

Table 3. Estimated daily intake of detected antibiotics of vegetables in several reports

Crop species	Treatment	Antibiotics	EDI (μg/kg/day)	Reference
Radish	Control	AZM	6.3×10^{-6}	Our study
Radish	T1	AZM	6.3×10^{-6}	Our study
Radish	T2	AZM	3.1×10^{-6}	Our study
Radish	Control	SMZ	9.4×10^{-6}	Our study
Radish	T1	SMZ	1.6×10^{-5}	Our study
Radish	T2	SMZ	1.9×10^{-5}	Our study
Lettuce	Manure-amended	SMZ	5.1×10^{-3}	Dolliver et al., 2007
Radish	Manure-amended	OTC	2.4×10^{-4}	Hu et al., 2010
Radish	Manure-amended	LIN	8.9×10^{-5}	Hu et al., 2010
Lettuce	Sewage sludge-amended	CIP	1.3×10^{-2}	Lillenberg et al., 2010
Lettuce	Sewage sludge-amended	AZM	4.7×10^{-5}	Sidhu et al., 2019

AZM: azithromycin; SMZ sulfamethoxazole; LIN: Lincomycin; OTC oxytetracycline; CIP: ciprofloxacin

Table 4. Acceptable daily intake (ADI) and hazard quotient of ABs (HQ_{AB}) of radish based on different effect endpoints

	*ADI1	HQ_{ADI1}			**ADI2	HQ_{ADI2}		
	(μg/kg/day)	Control	T1	T2	(μg/kg/day)	Control	T1	T2
Azithromycin	7.1[a]	8.9×10^{-7}	8.9×10^{-7}	4.4×10^{-7}	1.7[c]	3.7×10^{-6}	3.7×10^{-6}	1.8×10^{-6}
Sulfamethoxazole	5.7[b]	1.6×10^{-6}	2.8×10^{-6}	3.3×10^{-6}	130[c]	7.2×10^{-8}	1.2×10^{-7}	1.5×10^{-7}

* Based on therapeutic purpose

** Based on microbiological and toxicological effect

[a] For ADI1 calculations therapeutic dosage for azithromycin is 500 mg/day (https://www.healthline.com/health/azithromycin-oral-tablet#dosage)

[b] For ADI1 calculations therapeutic dosage for sulfamethoxazole is 400 mg/day (Prosser and Sibley, 2015)

[c] Wang et al., 2017

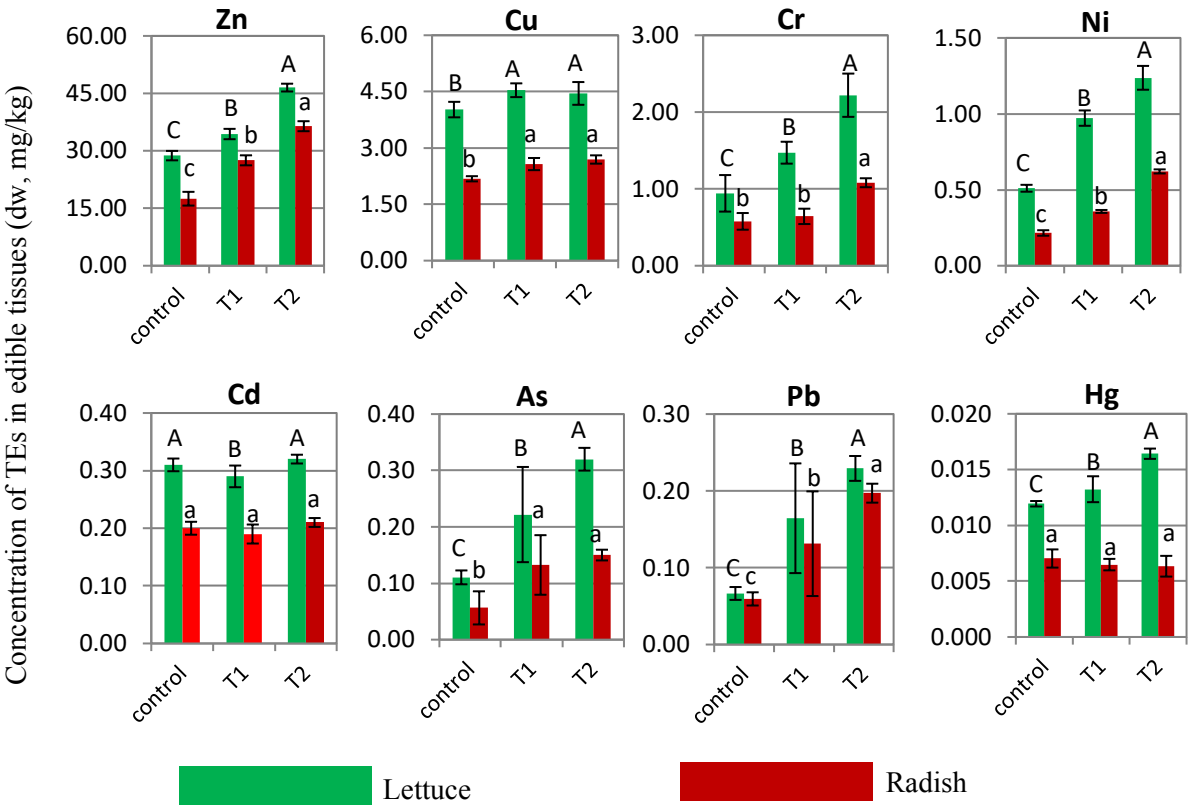

Figure 1. Effect of Cu and Zn on the uptake of TEs by leaves of lettuce and root of radish (Mean ± SD, N = 5). Uppercase and lowercase letters refer to significance ($p < 0.05$) for lettuce and radish, respectively.

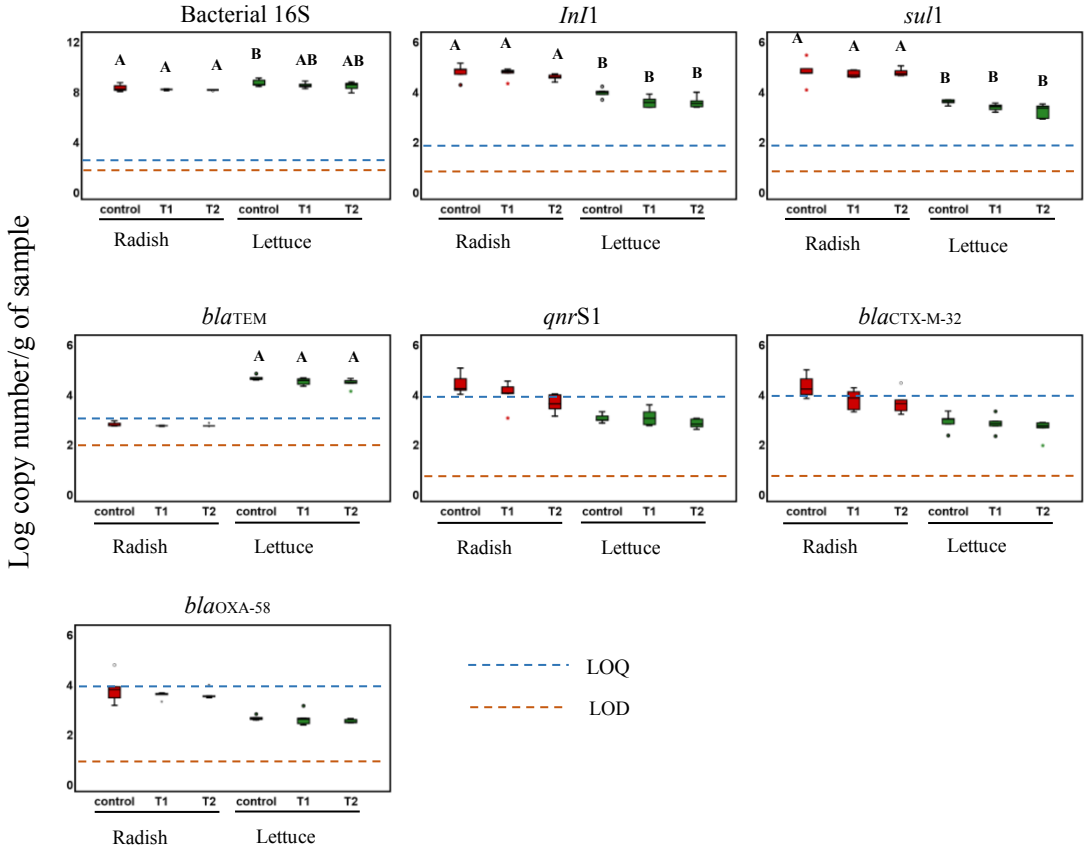

Fig 2. Relative abundances of the different genetic elements in vegetable samples. Data are expressed as copies of each sequence per g of tissue (log10 values), and code colored by vegetable type: green for lettuce and red for radish. Different uppercase letters indicate statistically difference ($p<0.05$).

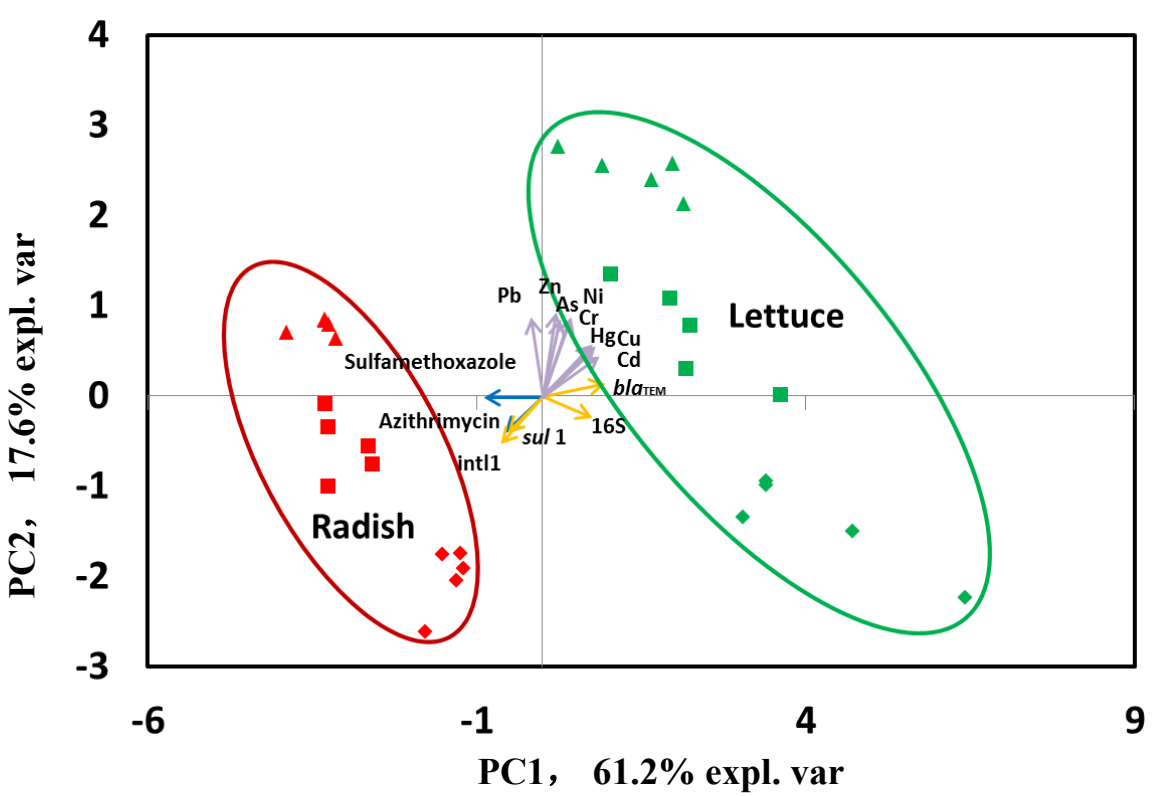

Figure 3. The PCA biplot of lettuce and radish data showing the loading of each variable (arrows) and the scores of each treatment (symbols). Treatments are expressed as rhombus (control), square (T1), triangles (T2). Green and red symbols correspond to lettuce (L) and radish (R). TEs, ABs and genetic elements loading are represent by violet, blue and yellow arrow. The length of the arrows approximates the variance of the variables, whereas their angles among them estimate their correlations

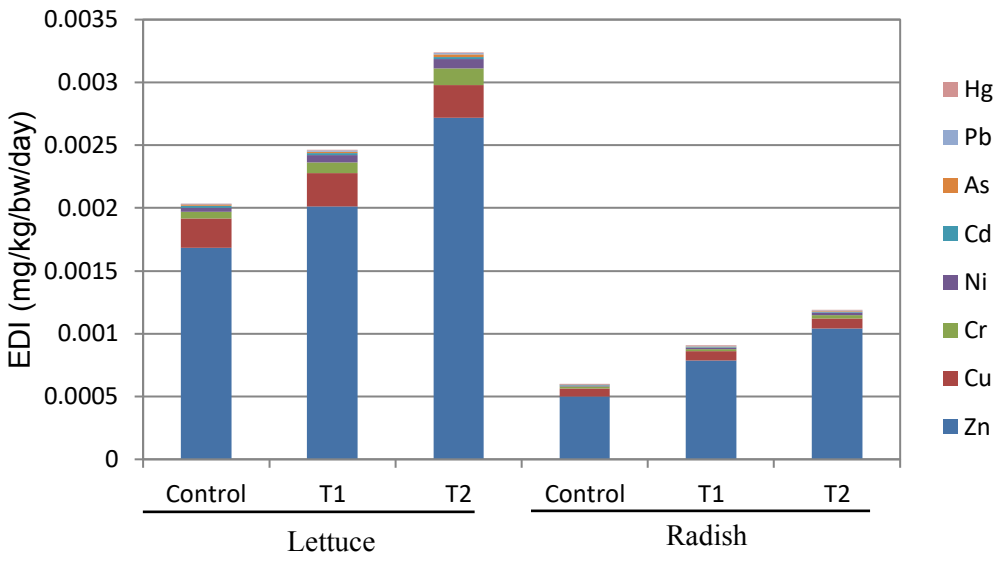

Figure 4. Estimated daily intake of trace elements

SUPPLEMENTARY MATERIAL FOR THE MANUSCRIPT:

Dose effect of Zn and Cu in sludge-amended soils on the vegetable uptake of trace elements, antibiotics and antibiotic resistance genes: Human health implications

2.2.1 Reagents and standards

Trace elements. The reagents for extraction were prepared using nitric acid (69%) (Panreac), 37% hydrochloric acid (Merck, Darmstadt, Germany), and 31% hydrogen peroxide (Merck). All solutions were diluted with doubly deionized water obtained from Millipore water purification systems (Elix&Rios) (18.2 MΩ/cm resistivity and TOC, Total Organic Carbon <30 µg/L). CRM 1570a, supplied by the National Institute of Standards and Technology (USA), has certified values for As, Cd, Pb, Cr, Cu, Hg and Zn. Digestion reagents were also used as a blank matrix. For quality control purposes, in each sample digestion series, CRM and laboratory reagent blank were added. A limit of detection (LOD) of in the solution analyzed was determined from three times the standard deviation obtained from the analysis of ten runs of blank samples on the same day as the determinations. Similarly, the limit of quantification (LOQ) was calculated by multiplying the standard deviation by ten times.

Antibiotics: Sulfathiazole, sulfamethizole, sulfadiazine, sulfamethazine, sulfamethoxazole, ofloxacin, enrofloxacin, ofloxacin-d3, enrofloxacin-d3 hydrochloride, lincomycin, tetracycline, ciprofloxacin, and azithromycin were purchased from Sigma-Aldrich whereas ofloxacin methyl ester, and ofloxacin ethyl ester were purchased from LGC Standards S.L.U. (Barcelona, Spain). Clindamycin-d3 hydrochloride was purchased from Toronto Research Chemicals (Toronto, Canada), and sulfamethoxazole-d4 was purchased from Analytical Standard Solutions – A2S (Saint Jean d'Illac, France), respectively. All standards were high purity (95% or higher). Methanol and water (both LC-MS grade), ethyl acetate (GC-ECD/FID grade), and formic acid (98–100%, pro-analysis) were obtained from Merck, whereas acetonitrile (LC-MS grade) was obtained from Fisher Scientific UK (Loughborough, UK). SPE Strata-X cartridges (100 mg/6 mL) were obtained from Phenomenex (Torrance, CA, USA), and 0.22-µm pore nylon filters were purchased from Sigma-Aldrich. In order to enable equilibration, 100 ng/g (dw) ofloxacin-d3, clindamycin-d3, enrofloxacin-d3, and sulfamethoxazole-d4 were spiked one hour prior to extraction into the samples (1 g) as surrogate standards. LOD and LOQ were calculated as the mean background noise in a blank triplicate plus three and ten times.

Antibiotic resistance gene: Primers for sul1, tetM, qnrS1, mecA, blaTEM, blaCTX-M-32, blaOXA-58, and bacterial 16S rDNA sequences are listed in supplementary Table S1. To ensure the absence of bacteria on the surface of vegetable, the last rinsing water was plated in LB agar media for 72 hours at 28 ℃. For blank control of qPCR reactions, complete reaction mixes plus nuclease free water, but no template, were used. PNORM1 plasmid and pUC19 plasmids were used as quantification standards for ARGs. The limit of quantification (LOQ) was defined as the lowest point on the linear part of the standard curve: 100 gene copies per reaction for *sul*1, $bla_{\text{CTX-M-32}}$ and *intI1* and 1000 gene copies per reaction for 16s rDNA, *tet*M, bla_{TEM}, $bla_{\text{OXA-58}}$ and *mec*A .

Table S1: Primers utilized for detection and quantifications of antibiotic resistance genes in soil, sewage sludge and plant parts

Target gene	Primers	Sequence (5'-> 3')	Amplicon size (bp)	Tm(°C)	Reference
intI1	intI1LC5 intI1LC1	GATCGGTCGAATGCGTGT GCCTTGATGTTACCCGAGAG	196	60	Barraud et al, 2010
16s rDNA	331F 518R	TCCTACGGGAGGCAGCAGT ATTACCGCGGCTGCTGG	195	60	Bräuer et al., 2011
blaTEM	blaTEM-F blaTEM-R	TTCCTGTTTTTGCTCACCCAG CTCAAGGATCTTACCGCTGTTG	113	60	Di Cesare et al., 2016
blactx-m-32	ctx-m-32-FW ctx-m-32-R	CGTCACGCTGTTGTTAGGAA CGCTCATCAGCACGATAAAG	156	60	Hembach et al., 2017
blaOXA-58	OXA58F OXA58R	GCAATTGCCTTTTAAACCTGA CTGCCTTTTCAACAAAACCC	152	63	Laht et al., 2014
mecA	mecAF mecAR	AAAAAGATGGCAAAGATATTCAA TTCTTCGTTACTCATGCCATACA	185	63	Szczepanowski et al., 2009
qnrS	qnrSrtF11 qnrSrtR11	GACGTGCTAACTTGCGTGAT TGGCATTGTTGGAAACTTG	118	60	Marti & Balcázar, 2013
sul1	sul1-FW sul1-RV	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	162	60	Pei et al., 2006
tetM	tetMF tetMR	GCAATTCTACTGATTTCTGC CTGTTTGATTACAATTTCCGC	186	60	Tamminen & Karkman, 2011

Table S2. Physical and chemical parameters of soil and sewage sludge sample

Parameters	Soil	Sewage sludge
Humidity (%)	1.33	79.1
pH	8.48	8.40
EC (dS/m)	0.24	2.56
OM (%)	1.27	51.5
Total N (mg/kg)	890	27700
Available N (mg/kg)	298	2800
Available P (mg/kg)	33	15500
Available K (mg/kg)	344	2070

Table S3. Content of TEs in soil and sewage sludge (dw, mg/kg). The generic reference levels of these elements for contaminated soil in Catalonia and legislation for fertilizer.

Element	Soil	Standard for soil	Sewage sludge	Legislation for sludge
Cu	92.45 ± 1.78	-	240.12 ±2.14	300 [b]
Zn	74.32 ± 2.17	170 [a]	700.78 ± 1.14	800 [b]
Pb	58.12 ± 0.99	60 [a]	30.85 ± 1.09	120 [b]
Ni	33.01 ± 0.32	45 [a]	53.01 ± 1.08	50 [b]
Cr	21.01 ± 0.12	400 [a]	57.96 ± 0.21	100 [c]
As	19.61 ±1.01	30 [a]	7.71 ± 0.01	40 [b]
Cd	＜0.5	2.5 [a]	0.51 ± 0.01	1.5 [b]
Hg	0.0252 ± 0.0001	2 [a]	0.67 ± 0.09	1 [b]

[a] Generalitat de Catalunya (2017); [b] PE-CONS 76/18 (2019); [c] Saveyn and Eder. (2014).

Mean ± SD (N = 3).

Table S4. Concentration of ABs in sewage sludge.

Compound	LOD (dw, μg/kg)	LOQ (dw, μg/kg)	Concentration (dw, μg/kg)	Molecular weight	Log K_{ow}	Solubilty (mg/L)	pKa	K_d (L/kg)
ciprofloxacin	1.29	4.35	5789.47	331.3	0.28	30000	6.09	430 [a]
doxycycline	1.39	4.69	130.57	444.4	0.63	50	3.09	na
sulfathiazole	1.39	4.69	126.32	255.3	0.05	373	7.2	4.9 [b]
tetracycline	1.20	4.02	104.78	444.4	-1.37	231	3.3	1140 [c]
azithromycin	0.72	2.49	102.39	749	4.02	2.37	8.74	na
lincomycin	0.29	1.05	15.87	406.5	0.20	927	7.6	66 [d]
sulfamethoxazole	0.77	2.49	<LOD	253.28	0.89	610	1.6	1.7 [e]

[a] Nowara et al., (1997); [b] Tolls, (2001); [c] Sithole and Guy, (1987); [d] Li et al., (2019); [e] Höltge and Kreuzig, (2007)

na: not available.

Table S5. Loadings for PCA with relatively high scores in bold typeface

	Component	
	1	2
Cd	**.856**	.444
As	.254	**.851**
Cu	.792	.552
Cr	.400	**.852**
Zn	.203	**.930**
Pb	-.186	**.877**
Hg	.751	.577
Ni	.420	**.880**
Sulfamethoxazole	**-.873**	-.119
Azithromycin	-.577	-.412
16S	.746	-.234
blatem	**.945**	.141
intI1	-.640	-.525
sul1	-.539	-.401

Table S6. Vegetable quality parameters (n=5)

	LC	LT1	LT2	RC	RT1	RT2
Chl_T (μg/cm^2)	0.19 ± 0.06	0.20± 0.06	0.20± 0.06	0.31± 0.05	0.26± 0.06	0.31± 0.07
Lipids (%)	0.27 ± 0.03	0.26 ± 0.04	0.34 ± 0.14	0.07 ± 0.01	0.11 ± 0.08	0.07 ± 0.01
Carbohydrates (%)	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.01	0.06± 0.01	0.06± 0.02	0.07± 0.02
Fresh weight (g)	215.8 ± 51.4	223.7 ± 22.9	247.5 ± 48.0	50.3± 18.2	35.7± 35.7	55.5 ± 14.6
Height (cm)	13.8 ± 0.3	14.4 ± 0.4	13.9 ± 1.2	15.90 ± 1.0	16.7 ± 1.0	16.7 ± 1.3

LC= lettuce in control; LT1= lettuce in T1; LT2= lettuce in T2; RC= radish in control;RT1= radish in T1; RT2= radish in T2

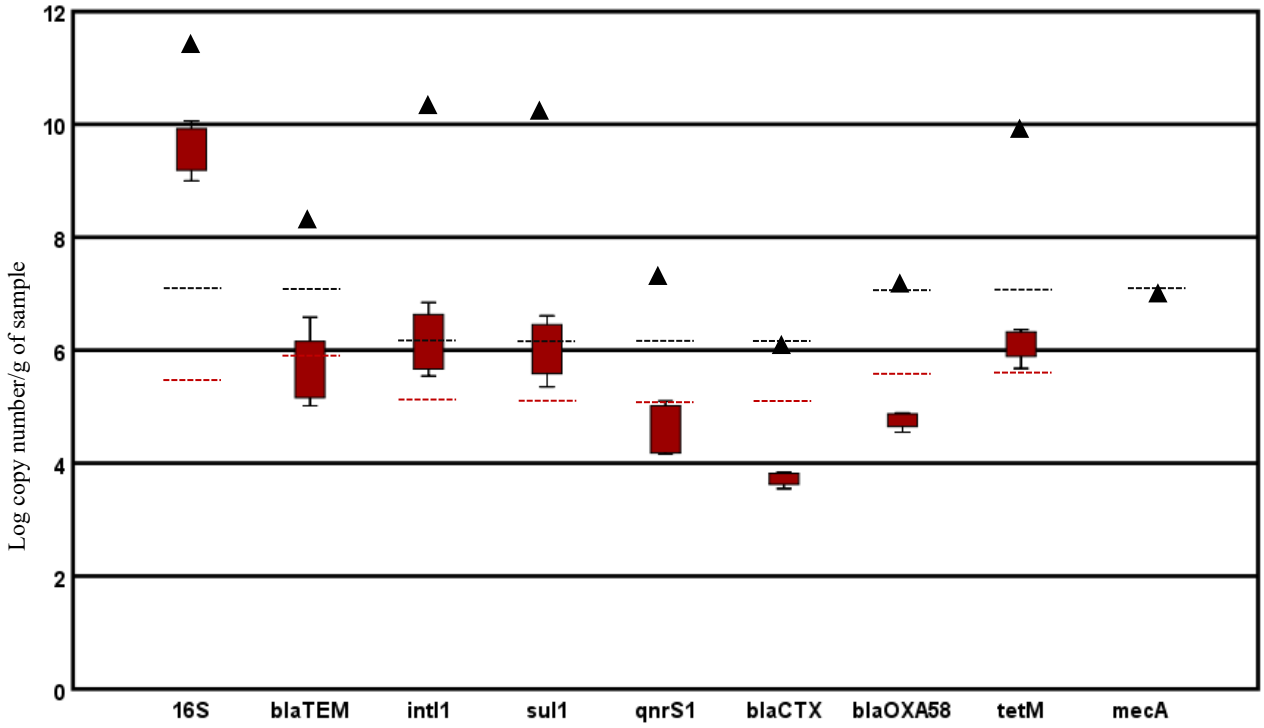

Figure S1. Abundance of genetic elements in fresh soil and sewage sludge. Data are expressed as copies of each sequence per g of sample (log 10 values). Box represents soil samples (n=4), while triangle is the abundance in sewage sludge (n=1). Dotted line is LOQ of genetic element (black for sludge, red for soil).

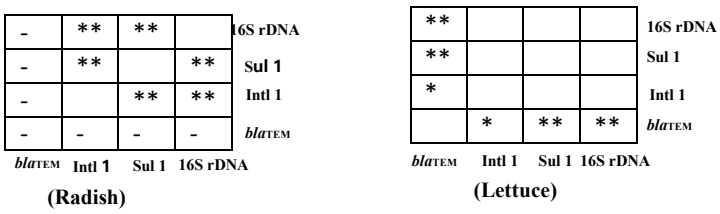

Figure S2. Correlation maps between the different genetic elements. Asterisks indicate significant Pearson correlations (*, p<0.01, **, p<0.05), while white corresponds to no correlation.

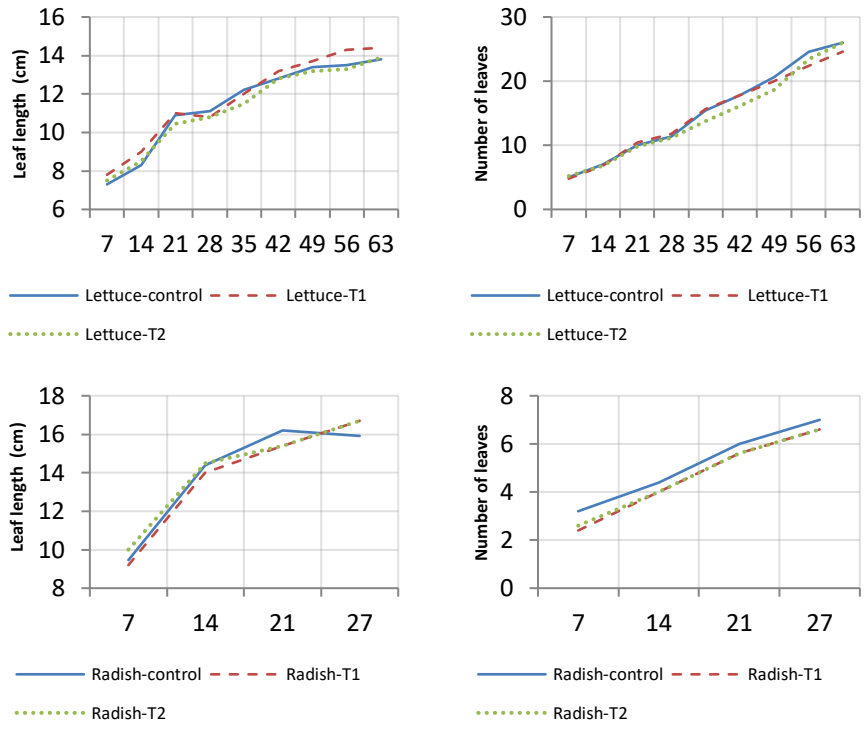

Figure S3. The change of growth indexes of vegetables over time

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