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Assessment of the Combined Inputs of Antimicrobials from Top Soil Improvers and Irrigation Waters on Green Leafy Vegetables Fields

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Abstract: Sustainable food systems imply the re-use of biowastes and water. In this paper we characterize 30 top soil improvers of anthropogenic, animal and green waste origin, and 11 irrigation waters from rivers, channels, and civil wastewater treatment plants (cWWTPs) for the presence of antimicrobials. Liquid chromatography coupled to hybrid High Resolution Mass Spectrometry (LC-HRMS/MS) was applied to determine 50 different drugs belonging to the classes of sulfonamides (11), tetracyclines (7), fluoroquinolones (10), macrolides (12), amphenicols (3), pleuromutilins (2), diaminopyrimidines (1), rifamycines (1) and lincosamides (1). Biowastes from cWWTPs and animal manure, slurry and litter revealed the highest loads for sulfonamides, tetracyclines, fluoroquinolones, and macrolides (80, 470, 885, and 4,487 ng/g wet weight, respectively) with nor- and cipro-floxacin as marker of anthropogenic sources. In composts and digestates antimicrobials were almost below the determination limits. Re-used waters for irrigation in open field lettuce production resulted contaminated in the range of 12-221 ng/L for sulfonamides, tetracyclines, and fluoroquinolones, against very few detects in channels and surface waters. Antimicrobials Hazard Index (HI) based on Predicted No Effect Concentration for Antimicrobial Resistance ($PNEC_{AMR}$) was largely >100 in contaminated top soil improvers from urban and animal sources. Accounting for worst-case inputs from top soil improvers and irrigation water and dilution factors of amended soil, fluoroquinolones only showed a HI around 1 in open fields for lettuce production. The origin of top soil improvers plays a pivotal role for safe and sustainable leafy vegetable productions, to abate the risk of AMR onset in food-borne diseases, as well the transfer of AMR elements to human gut flora.

Keywords: antimicrobials; top soil improvers; reused waters; high resolution mass spectrometry; analysis; Hazard Index; antimicrobial resistance

1. Introduction.

Climate changes and an overall improvable environmental sustainability of food production systems (Holden et al., 2018) represent the main drivers to consider the regular use of bio-wastes from anthropogenic sources and of reclaimed water in agriculture (EEA 2020a; EEA 2020b).

The necessity to provide agriculture soil of sufficient amount of organic matter to guarantee food security in the past relied on the use of animal manure, slurry, litter dressed on soil; now, such organic carbon supplementation recognise also inputs from composts and digestates from urban wastes, such as sludges from civil Wastewater treatment plants (cWWTPs), green wastes, and food-related household wastes (Anderson et al., 2021).

Moreover, recently the direct use in agriculture of effluents from cWWTPs has been allowed to reduce the water footprint (EU Regulation 2020/741). Authorised plants should be able to provide a third phase treatment to run-off waters, able to abate microbial loads and pathogens. No mention at this time about pharmacological residues end-of-waste criteria, even if the Commission is consolidating a strategic approach to human and veterinary pharmaceuticals in the environment, that could embrace also the presence of Antimicrobial Resistance Bacteria and of related genetic elements (EU Commission notice, 2022)

Within a One Health approach, the environmental input of pharmaceuticals – in this context antimicrobials, from the above mentioned sources in agriculture may lead to: a) alteration of soil microbial communities, of relevance within the frame of "safe soil" assessment (Cycon et al., 2019; Patyra et al., 2023); b) uptake of antimicrobials by plants, where green leaf vegetables may be acknowledged as most sensitive, thus determining residues in ready-to-eat vegetables (Matamoros et al., 2022); c) elict a environmental pool of antimicrobial genetic determinants that could be transferred to animals and humans also via non pathogenic bacteria (FAO and WHO, 2023).

Owing to the above, in this paper we consider a production cycle of lettuce in open field as model to compute the associated inputs of selected antimicrobials in agriculture soils from top soil improvers (TSI) of and irrigation waters of different origin.

2. Materials and Methods

2.1. Sampling

A convenient sampling of top soil improvers intended for agricultural use has been performed in the October 2020 – March 2021 Period. The representative panel considered was composed by the following main categories: bio-solids from cWWTPs (N = 7) and dairy processing plant (N = 1); mixed composts (N = 8) mixed digestates (N = 5) and top soil improvers from animal sources (bovine manure, pig slurry, poultry litter; N = 9). Samples were drawn from the market, from the production plants, from farms, according to national guidelines (Mecella 2001; ISPRA, 2011), and stored at + 4°C in the dark, until analysed.

Independent irrigation waters samples (N = 8) were drawn from a primary Italian regional district devoted to open field green leafy vegetables production in Northern Italy during summer 2022, according to national guidelines (Mecella 2001), and acknowledge the following typologies: a) surface waters from Appennine mountains rivers, as baseline samples (N = 2); irrigation channel waters from the Po River plain, that acknowledge contribution both from civil and animal farms effluents (N = 4); re-used waters from the third phase process of a civil wastewater treatment plant, receiving both civil and animal farming wastewater inputs (N = 3). Sampling sites did not acknowledge the presence of a animal farm in a 1000 m radius (**Figure 1**).

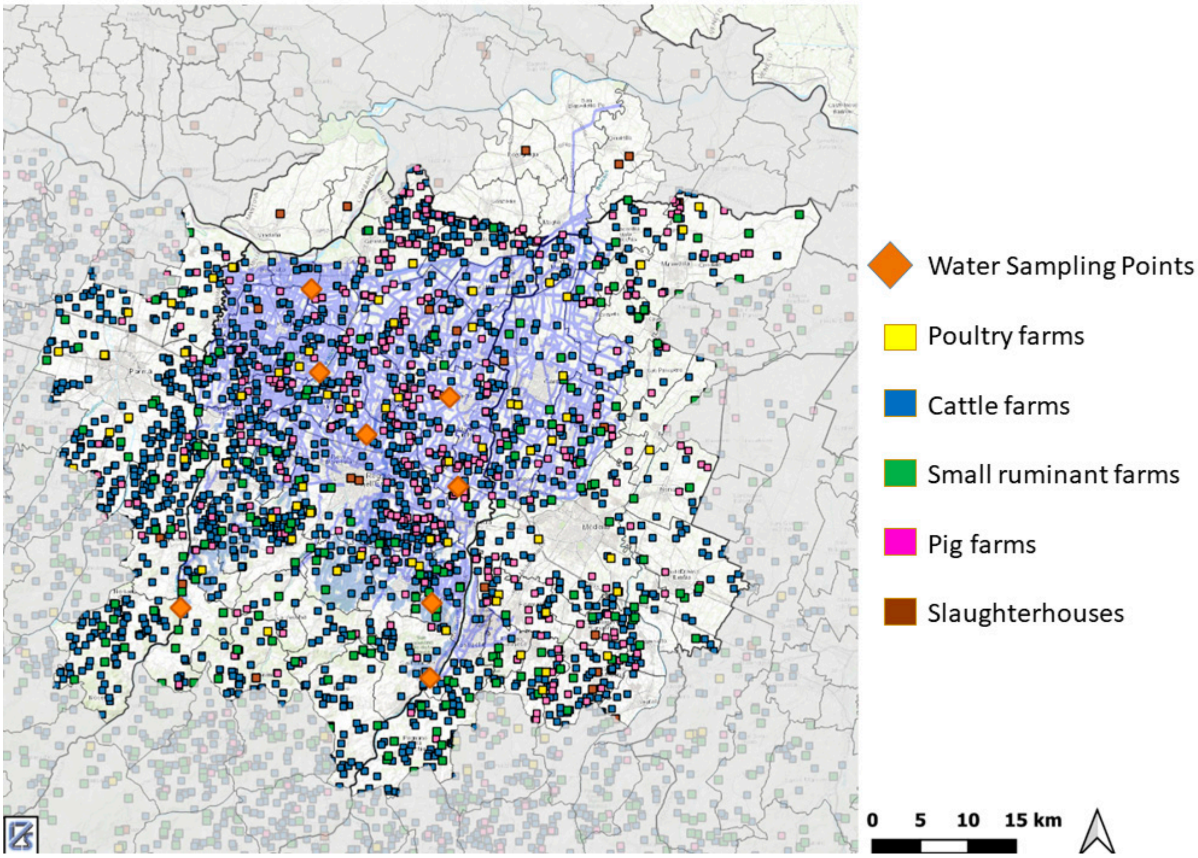

Figure 1. Inventoried animal farms and slaughterhouses pressures on the water sampling points. Data kindly provided by Regione Emilia Romagna Veterinary Office.

2.2. Agriculture parameters for antimicrobial inputs estimates

Lettuce salads producers were interviewed to recover informations about the open field agricultural cycle. Soft top soil for lettuce production (10% clay, 70% sand; 0.771 tons/cubic meter) is routinely amended with TSI for a 20 cm depth, just before the transplantation of small lettuce plants. According to these parameters, the overall amount of 1 hectare of soil intended for lettuce production with a 0.20 m depth weights 1,542 tons.

The amount of top soil improver dressed per hectare could vary on the basis of moisture content of the product, as well of its Nitrogen content, whose inputs is limited to prevent nitrate pollution in groundwater (EU Council Directive 91/676), and non compliant nitrate presence in edible leaves (EU Regulation 1258/2011). Such constrains limit the TSI use up to 0.03 tons of pelletted compost (16-18% of moisture), or poultry litter, or pig slurry per hectare, on wet weight (ww) basis. Composts and digestates (40 – 60 % moisture) find use up to 0.15 tons ww, and animal manure up to 0.30 tons ww. Biosolids from cWWTPs (95% moisture) can not be disposed directly on field intended for horticulture, but they can be used up to 40% when inserted in the so called “compost from sludge” to be used in conventional horticulture, only. This information has been considered in modelling antimicrobials inputs from cWWTP biosolids to lettuce production. Irrigation water demand ranges from 5 to 10 L/kg lettuce product harvested. For an average open field lettuce production of 35 tons per hectare, under a conservative approach tailored on the summer season water needs, we computed a 350,000 L water use/ha for a 2 month long production cycle.

Total antimicrobial inputs in cultivated soils were expressed as ppb (ng/g top soil ww) basis under the lower-bound approach (not determined results posed equal to 0). Estimated environmental concentration are affected from uncertainties related to the different half lives of antimicrobials, according to soil texture, the environmental conditions, the daily inputs from irrigation water, and the fraction uptaken by lettuce.

2.3. Antimicrobial Analysis of top soil improvers and irrigation waters

Chemicals: Acetonitrile LC-MS grade (ACN) and ammonia (25-30%) were purchased from Carlo Erba Reagents (Milan, Italy). Methanol LC-MS grade (MeOH) was purchased from Honeywell (Charlotte, NC, USA). Formic acid and ammonium acetate LC-MS grade were purchased from VWR (Radnor, PA, USA). Acetic acid LC-MS grade and EDTA disodium salt were purchased from Merck (Darmstadt, Germany). OASIS HLB cartridges (200mg/6 mL) were purchased from Waters (Milford, MA, USA) and Strata X-C 200 (mg/6 mL) from Phenomenex (Torrance, CA, USA).

Sample preparation: A panel of fifty antimicrobials belonging to the classes of sulfonamides (11), tetracyclines (7), fluoroquinolones (10), macrolides (12), amphenicols (3), pleuromutilins (2), diaminopyrimidines (1), rifamycines (1) and lincosamides (1) were analysed in the selected top soil improvers and irrigation water samples according to Sargenti et al. (2020) with some modifications. Because of the very different origin and composition, for biosolids it was necessary to develop two different sample processing protocols as summarized in **Table 1 SM.** Limits of Detection (LODs) have been assessed case by case during analytical sessions, via the insertion of both blank and fortified control samples.

LC-HRMS analysis: LC-HRMS conditions were the same described by Moretti et al. (2016). Briefly, a Thermo Ultimate 3000 High Performance Liquid Chromatography system (Thermo Scientific, San Jose, CA, USA) coupled to Q Exactive Plus high resolution mass spectrometry (Thermo Scientific) operating in positive heated electrospray ionization (HESI) mode was used. Sheath and auxiliary gas were 35 and 15 arbitrary units, respectively. Spray voltage was set at 3.00 kV, capillary temperature at 300°C, HESI temperature at 320°C and S-Lens RF at 50.0 V. For acquisition a Full MS/dd-MS^2 experiment was performing setting the following parameters: full scan: resolution 70000 FWHM (@200 *m*/*z*), AGC target 3e6, maximum injection time 300 ms and scan range 150-1200 *m*/*z*; dd-MS^2: resolution 17500 FWHM (@200 *m*/*z*), AGC target 5e5, maximum injection time 80 ms, loop count 20, isolation window 2.4 *m*/*z*, minimum AGC targeted 1.00e3 and dynamic exlusion 2.0 s. The selected precursor and fragment ions as well as the retention times (RT) are listed in **Table 2 SM**.

Chromatographic separation was performed on a Poroshell 120 EC-C18 column (100 × 3.0 mm; 2.7 µm, Agilent Technologies, Santa Clara, CA, USA) equipped with a pre-column (2.1 × 5 mm, 2.7 µm, Agilent Technologies). Mobile phases were an aqueous solution of formic acid 0.1% (A) and MeOH (B). Flow rate was set at 0.25 mL min^{-1}, injection volume at 5 µL and column temperature at 30 °C. The gradient was initiated with 5% eluent B for 1 min, continued with linear increase to 95% B in 19 min. This condition was maintained for 5 min. The system returned to 5% B in 1 min and was re-equilibrated for 4 min (run time: 30 min). Injection volume was 5 µL.

Estimated LODs for top soil improvers and irrigation waters, as well as HRMS/MS acquisition parameters are reported in **Table 3 and 4 SM**.

2.4. Risk assessment for the onset of Antimicrobial Resistance

Hazard Intex for the onset of antimicrobial resistance was computed as ratio between the environmental concentration in top soil, irrigation water, and amended and watered soil, respectively and the available Predicted No Effect Concentration for antimicrobial resistance ($PNEC_{RES}$) derived from Bengtsson-Palme and Larsson (2016).

3. Results

In **Table 1 and 2** we report the occurrence of the determined antimicrobials in the different top soil improvers and irrigation waters considered. **Table 3** illustrates the computed inputs on agriculture fields according to the inventoried lettuce cultivation practices, under the worst case scenarios. The Hazard Index for antimicrobial resistance (HI_{RES}), accounting for worst case concentration found in top soil improvers and irrigation water and for modelled inputs in soil rare shown in **Table 4.** The Extracted Ion Chromatograms of a standard solution of ciprofloxacin (A), a spiked sample (B) and a blank sample (C) are shown in **Figure 2.**

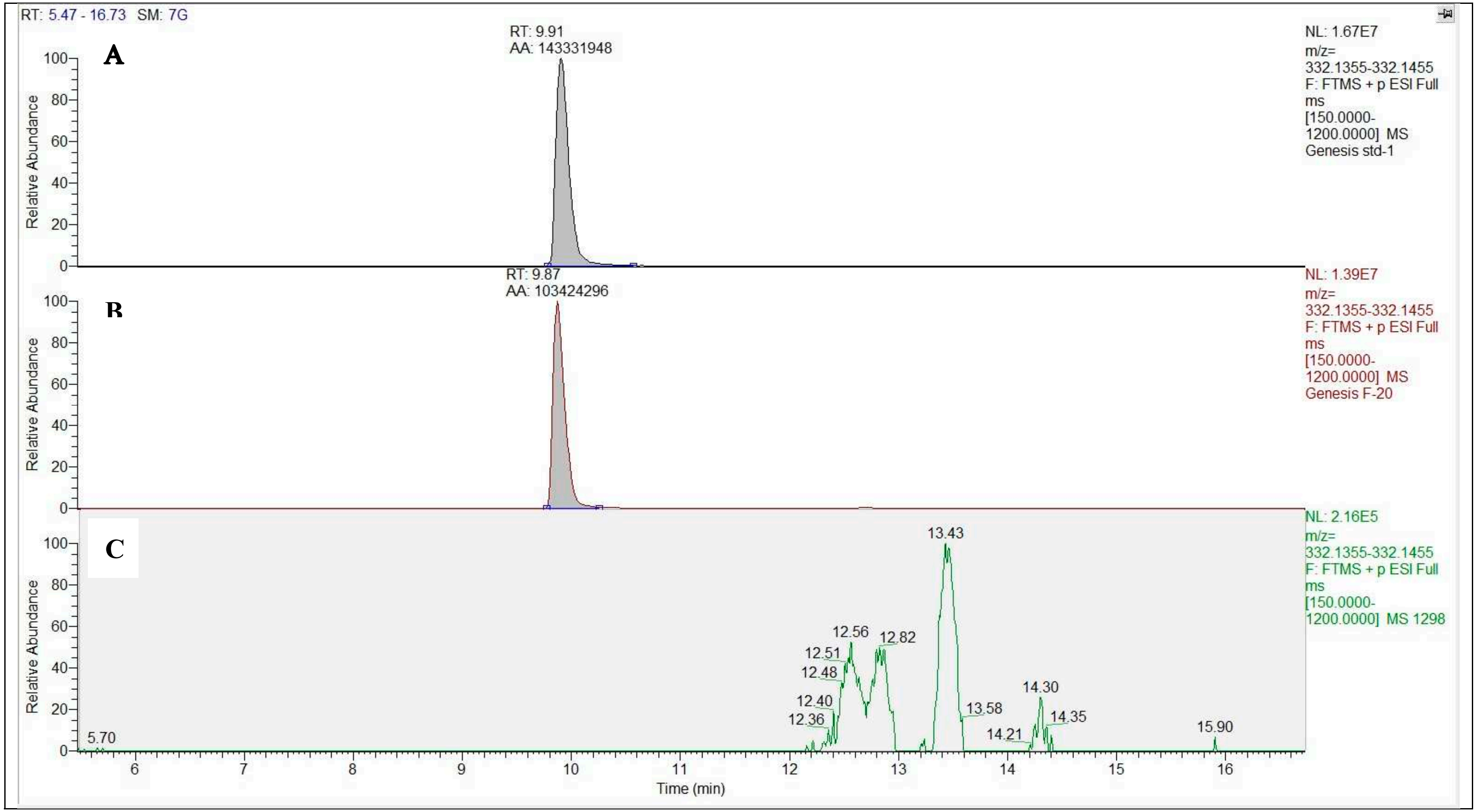

Figure 2. Extracted Ion Chromatograms referred to analysis of ciprofloxacin (fluoroquinolone) in top soil improvers and irrigation waters. A) Standard solution (10 ng/g) ; B) WWTP biosolid sample ID 20 table 1; C) River freshwater sample ID 1298 Table 2.

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Table 1. Determined antimicrobials (ng/g wet weight) in top soil improvers, according to the different origin.

		Sulfonamides								**Tetracyclines**			**Fluoroquinolones**						
ID	top soil improver	**GUA**	**ANI**	**DIA**	**MERA**	**META**	**MMTX**	**DMTX**	**TRMP**	**Tetra***	**Oxy***	**Doxy**	**Nor**	**Cipro**	**Flum**	**Enro**	**Marbo**	**TYLA**	**LIN**
1.0	**mixed compost**	<LOD	**1832**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
4.0	**mixed compost**	<LOD	**1710**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
5.0	**mixed compost**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
5.2	**mixed compost**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
6.0	**mixed compost**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
11	**mixed compost**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
14	**mixed compost**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
19	**mixed compost**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
2.0	**Bovine manure and meat and bone meals**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	**27**	**57**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	**111**	<LOD	<LOD	<LOD
3.0	**Bovine + equine manure**	<LOD	<LOD	<LOD	<LOD	**20**	<LOD	**51**	**178**	<LOD	<LOD	**95**	<LOD	<LOD	<LOD	**95**	<LOD	<LOD	<LOD
7.0	**Bovine manure from organic farm**	**37**	<LOD	**41**	<LOD	**33**	<LOD	**80**	**274**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	**179**	<LOD	<LOD	<LOD
8.0	**Pig Slurry**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	**190**
10	**Pig Slurry**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	**76**	**54**	<LOD	<LOD	<LOD	<LOD	**14**	<LOD	**959**
13	**Poultry Litter**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
15	**Bovine manure**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
26	**Bovine manure**	<LOD	<LOD	**36**	**14**	**14**	**20**	<LOD	<LOD	<LOD	58	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
30	**Poultry Litter**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	4487	<LOD
9.0	**Digestate**	<LOD	<LOD	**12**	<LOD	**11**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
24	**Digestate**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	**13**	<LOD	<LOD

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25	**Digestate**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
28	**Digestate**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
29	**Digestate**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
16	**WWTP biosolid**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	**202**	<LOD	<LOD	<LOD	<LOD	<LOD
17	**Cheese plant biosolid**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
18	**WWTP biosolid**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	**22**	<LOD	**63**	<LOD	**353**	<LOD	<LOD	<LOD	<LOD	<LOD
20	**WWTP biosolid**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	**34**	**29**	**885**	<LOD	<LOD	<LOD	<LOD	<LOD
21	**WWTP biosolid**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	**41**	**36**	**632**	<LOD	<LOD	<LOD	<LOD	<LOD
22	**WWPT biosolid + lime**	<LOD	<LOD	**58**	<LOD	<LOD	<LOD	<LOD	<LOD	**81**	<LOD	**470**	<LOD	**406**	**22**	<LOD	<LOD	<LOD	<LOD
23	**WWPT biosolid + lime**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	**572**	**58**	<LOD	<LOD	<LOD	<LOD
27	**WWPT biosolid + lime**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	**307**	<LOD	<LOD	<LOD	<LOD	<LOD

Abbreviations: GUA = Sulfaguanidine; ANI = Sulfanilamide; DIA = Sulfadiazine; MERA = Sulfamerazine; META = Sulfamethazine; MMTX = sulfamomnometoxine; SDMX = Sulfadimetoxine; TRMP = Trimethoprim; Tetra = Tetracycline; Oxy= Oxytetracycline; Doxy = Doxicycline; Nor = Norfloxacin; Cipro = Cipofloxacin; Flum = Flumequine; Enro = Enrofloxacin; Marbo= Marbofloxacin; TYL A = Tylosin A; LIN = Lincomycine. * and its epimer

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Table 2. Determined antimicrobials (ng/L) in irrigation waters, according to the different origin.

Origin	**Sampling date** **dd/mm/yy**	**ID**	**GUA**	**DIA**	**PYRI**	**ANI**	**MTX**	**TRM**	**CIPR**	**SPIR**	**DOX**	**TIA**	**FLU**
Reused waters	23/05/2022	129	**22**	**D**	**26**	<LO	**136**	**50**	**12**	**30**	**61**	**11**	**10**
River freshwater	23/05/2022	129	<LO	<LO	<LO	<LO	**D**	<LOD	<LOD	<LOD	<LO	<LO	<LO
Irrigation channel	23/05/2022	129	<LO	<LO	<LO	<LO	**D**	<LOD	<LOD	<LOD	<LO	<LO	<LO
Irrigation channel	23/05/2022	130	**14**	**D**	**13**	<LO	**77**	**18**	<LOD	<LOD	<LO	**D**	**D**
Reused waters	20/06/2022	155	**25**	**11**	**22**	**11**	**181**	**35**	<LOD	<LOD	<LO	<LO	<LO
Irrigation channel	20/06/2022	155	<LO	<LO	<LO	<LO	<LO	<LOD	<LOD	<LOD	<LO	<LO	<LO
River freshwater	20/06/2022	155	<LO	<LO	<LO	<LO	<LO	<LOD	<LOD	<LOD	<LO	<LO	<LO
Irrigation channel	20/06/2022	155	<LO	<LO	<LO	<LO	<LO	<LOD	<LOD	<LOD	<LO	<LO	<LO
Reused waters	30/08/2022	221	**28**	<LO	**17**	<LO	**221**	<LOD	<LOD	<LOD	<LO	<LO	<LO
Irrigation channel	30/08/2022	221	<LO	<LO	<LO	<LO	<LO	<LOD	<LOD	<LOD	<LO	<LO	<LO
Irrigation channel	30/08/2022	221	**16**	<LO	<LO	<LO	**15**	<LOD	<LOD	<LOD	<LO	<LO	<LO

Abbreviations: GUA = Sulfaguanidine; DIA = Sulfadiazine; PYRI = Sulfapyridine; ANI = Sulfanilamide; MTX = Sulfametoxazole; MERA = Sulfamerazine; META = Sulfamethazine; TRMP = Trimethoprim; Doxy = Doxicycline; Cipro = Ciprofloxacin; Flum = Flumequine; TIAM = Tiamuline. D = Detected but not quantified

Table 3. Averaged computed inputs (mg) of antimicrobials from different classes of top soil improvers in soils intended for lettuce production, accounting for the considered standard agriculture practices. Sludge compost values computed as 40% of the recorded contamination in the biosolid from cWWTP.

Inputs			**Sulfonamides**								**Tetracyclines**		**Macrolides**			**Fluoroquinolones**				
			G	**A**	**DI**	**M**	**SD**	**M**	**PY**	**TR**	**DO**	**OX**	**TY**	**SP**	**LY**	**EN**	**N**	**CI**	**MA**	**TI**
TSI	ID	to																		
manure	7	0.3	**11.**	N	**12.**	**9.9**	**24**	N	N	**82.**	ND	ND	N	N	N	**53.**	N	ND	ND	N
Mixed Compost	1	0.1	ND	**275**	N	N	ND	N	N	N	ND	ND	N	N	N	N	N	ND	ND	N
Sludge compost	15	0.1	ND	N	N	N	ND	N	N	N	**2.0**	ND	N	N	N	N	**1.7**	**53.**	ND	N
Poultry Litter	30	0.0	ND	N	N	N	ND	N	N	N	ND	ND	**89.**	N	N	N	N	ND	ND	N
Pig Slurry	10	0.0	ND	N	N	N	ND	N	N	N	**1.0**	**1.52**	N	N	**19.**	N	N	ND	**0.28**	N
Waters		to																		

Reused (N=3)		35	**8.7**	**1.3**	**1.3**	N	ND	**62.**	**7.7**	**9.8**	**7.0**	ND	N	**3.5**	N	N	N	**1.4**	ND	**1.3**
Rivers (N=2)		35	ND	N	N	N	ND	N	N	N	ND	ND	N	N	N	N	N	ND	ND	N
Channels (N=6)		35	**1.7**	N	**0.6**	N	ND	**5.2**	**0.7**	**1.0**	ND	ND	N	N	N	N	N	ND	ND	N
Worst case																				
Top soil + water		m	**12.**	**276**	**12.**	**9.9**	**24.**	**62.**	**7.7**	**82.**	**2.0**	**1.52**	**89.**	**3.5**	**19.**	**53.**	**1.7**	**53.**	**0.28**	**1.3**
Dilution factor	15	to																		
final input in soil		ng	**0.0**	**0.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.00**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.00**	**0.0**

Abbreviations: GUA = Sulfaguanidine; ANI = Sulfanilamide; DIA = Sulfadiazine; MET = Sulfamethazine; SDM = Sulfadimetoxine; MTX = Sulfametoxazole; PYR = Sulfapyridine; TRP = Trimethoprim; DOXY = Doxicycline; OXY= Oxytetracycline; TYL = Tylosin A; LIN = Lincomycine; ENR = Enrofloxacin; NOR = Norfloxacin; CIPR = Ciprofloxacin; MARB = Marbofloxacin; SPIR= Spiramycin; TIA = Tiamulin; * and its epimer; ND = Not detected.

Table 4. Hazard Index for antimicrobial resistance (HI res), accounting for worst case (wc) concentration (ppb) found in top soil improvers (TSI), irrigation water (IW) and for modelled inputs in soil. Predicted No Effect Concentration (PNEC) for antimicrobial resistance derived from Bengtsson-Palme and Larsson (2016).

Antimicrobials		**PNEC res**	**TSI wc**	**IW wc**	**Soil wc**		**HI res**	**HI res**	**HI res**
							TSI	**IW**	**Soil**
Ciprofloxacin		0.064	885	0.012	0.034		13828	0.19	0.53
Enrofloxacin		0.064	179	na	0.035		2797	na	0.55
Ciprofloxacin + Enrofloxacin		0.064	na	na	0.069		na	na	1.08
Norofloxacin		0.500	470	na	0.001		940	na	< 0.01
Doxicycline		2.000	470	0.061	0.001		940	0.03	<0.01
Oxytetracycline		0.500	76	na	0.001		235	na	<0.01
Tetracycline		1.000	81	na	0.010		81	na	0.01
Sulfamethoxazole		16.00	na	0.221	0.042		na	0.01	<0.01
Trimethoprim		0.500	274	0.050	0.053		548	0.1	0.11
Sulfamethoxazole + trimethoprim		0.500	na	0.216	0.095		na	0.43	0.19
Tylosin A		4.000	4487	na	0.058		1122	na	0.01
Lincomycin		2.000	959	na	0.012		479.5	na	<0.01

4. Discussion

In this paper we tried to characterize the environmental pressure from antimicrobials in the production of a ready-to-eat leafy vegetables, as lettuce. This would represent the basis to support a olistic genomic and metagenomic based approach on the potential presence of foodborne pathogens and related virulence factors as AMR, in the considered ready-to-eat food production environment (Gigliucci et al., 2017; Barbieri et al., 2023?).

The presence of antimicrobials in irrigation waters (Slobodiuk et al., 2021; Solaiman et al., 2022; Seyoum et al., 2022) and top soil improvers (Concilosi et al., 2021; Wang et al., 2023; Ferreira et al., 2023) has been largely described in previous papers, and addressed to: a) eco-toxicity effects such as the alteration of soil functions as matter of the disruption of microbial communities; b) food safety aspects related to to the tranfer of the pharmaceuticals to the edible portion of green leaf vegetables and human exposure via food intake (Bhalsod et al., 2018; Albero et al., 2019); and last but not least, c) the induction of Antimicrobial Resistance Genes in the soil microbioma, and possibly their transfer to food-born pathogens (Mathews et al., 2022). Among the above adverse effects, the induction of AMR genetic elements today represents the most sensitive health-based end-point, within a One Health Approach (ECDC 2022).

4.1. Analysis

Limits of detection (LODs) of methods developed and applied to irrigation waters were in the range 10 ng/L -20 ng/L (see Supplementary Material), satisfying the requirements of European Union Commission Implementing Decision 2022/1307, which established the following maximum acceptable method detection or quantification limits for the monitoring of three antimicrobials in freshwaters: sulfametoxazole (100 ng/L), trimethoprim (100 ng/L) and ofloxacin (26 ng/L). This would guarantee the re-use of our evidences in irrigation waters within other legislative frames focused on priority substances relevant for ecotoxicity. Currently, in the European Union, no legislative requirements for antimicrobial monitoring have been enacted in soil improvers (European Commission Regulation 2019/1009); however, several national regulations on organic food production prohibit the use of sludge, sludges-derived compost and digestate (Lasaridi et al., 2018). About the analytical performances, a positive list of active substances licensed in animal feeds (EU Regulation 2019/4) frames the following antimicrobials, with a final concentration from premixtures integration up to 1 g/kg feed w/w: amoxicillin, amprolium., apramycin, chlortetracycline, colistin, doxycycline, florfenicol, flumequine, lincomycin, neomycin, spectinomycin, sulfonamides with the exception of sulfametoxazole, tetracycline, oxytetracycline, oxolinix acid, paromomycin, penicillin V, tiamulin, thiamphenicol, tilmicosin, trimethoprim, tylosin, and valnemulin. Therefore, accounting for the toxicokinetics of the above antimicrobials, LODs in the range 10 ng/g-100 ng/g ww top soil improvers, seem appropriate for their analysis. It is worth pointing out that during the development of analytical methods, colistins, high-priority polypeptide antibiotics for antimicrobial resistance, had also been included (Saluti et al. 2018). Unfortunately, in biosolids poor recoveries and low precision were observed forbidding to furnish acceptable results also for these very polar compounds (Binsker et al., 2022).

4.2. Antimicrobials profiling in top soil improvers and irrigation waters

Among fluoroquinolones, ciprofloxacin seems clearly a marker of human sources, as far as its presence is constant in biowastes from cWWTP (7/7) (**Table 1)** and reused waters (1/3) (**Table 2**); such findings are in good agreement with evidences from other studies (Wang et al., 2023).

Among top soil improvers of animal origin, animal manure reveals the almost systematic presence of sulfonamides coupled to the synergic Trimethoprim. However, it is worth noting the presence of sulfametoxazole, an active principle present only in human medicines coupled to trimethoprim (such as in the Bactrim™ speciality) is absent, while it is present in re-used water only. As for ciprofloxacin, we could assume sulfamethoxazole as tracer of the anthropogenic inputs/pressures.

On the other side, lincomycin seems distinctive of pig slurry, as well as tylosin A of poultry litter. Such antimicrobials recognize a target medicated feed administration against respiratory diseases caused by mycoplasma, that routinely affect intensive pig and poultry farming systems, respectively.

Mixed compost and digestates, whose composition is mostly based on green and food household wastes are not so heavy affected by the presence of antimicrobials, with the exception of two samples that revealed the unexpected presence of sulfanilamide at ppm level. Sulfanilamide is an old dated antimicrobial, and its presence could be reasonably addressed as break-down product of methyl 4-aminophenylsulfonylcarbamate (Asulam™) a phytosanitary product used as herbicide. Therefore, a carry-over of a phytosanitary contamination associated to green wastes used for composting could be not excluded (EFSA 2021).

Last but not least, the only bio-solid waste recovered from a dairy milk processing plant (**Table 1**, sample No. 17), indicates such category of waste could be not framed in the biosolid from cWWTP category, as currently done by the pertinent legislation (Anderson et al., 2021), because of its different origin from raw milk already compliant for the presence of antimicrobial residues in dairy products.

Our results in top soil improvers are in good agreement with recent evidences provided by Matamoros et al., (2023) where almost the same antimicrobial classes were found in biosolids from cWWTPs (sulfonamides, fluoroquinolones, tetracyclines) even if in the Spanish paper reported at concentration 10 fold higher as matter of a high density urban settlement. In swine slurry, the presence of lincomycin is confirmed as marker of the intensive pig farming system pressure. On the same line, the no detectable presence of antimicrobials in mixed compost and digestate from green and household wastes, confirms their choice as safer tool for soil fertilisation intended for vegetable food production.

From irrigation water dataset (Table 2), it seems clear the difference in the antimicrobial load between reused waters, originating directly from cWWTPs after a third phase treatment (combined UV and Oxygen Hydroperoxide treatment to abate microbial loads) and the other sources from rivers and agriculture channels. Re-used waters could directly irrigate green-leafy vegetables, according to their total E. Coli load (EU Regulation 2020/741); sparse of paper deals with antimicrobial presence in reused waters: from a recent publication referred to 8 different Italian WWTPs, a mean concentration of 27 ng/L for sulfamethoxazole with a RSD of 105 % was reported (Palumbo et al., 2022). This result is aligned with our evidences with concentrations from 77 ng/L to 221 ng/L (N =3). Freshwater from low-impact rivers (samples taken upstream of urban settlements) and canals experienced severe drought during sampling in summer 2022, thus reducing the dilution factor of urban and animal discharges. In the case of re-used waters, it is worth noting that the above mentioned third phase oxidation process is able to break-down several antimicrobials (Sagaseta et al., 2022). Therefore, the presence/absence and amounts of the determined substances in reused waters could be not assumed as proxy for the occurrence of AMR bacteria and of related genetic factors. Of some interest, the presence of tiamulin, a diterpene antimicrobial with a pleuromutilin chemical structure similar to that of valnemulin, whose administration is largely focused in pig farms to fight against respiratory diseases caused by gram-positive micro-organisms and mycoplasma. Tiamulin presence in reused waters is consistent with the urban and animal farming wastewater inputs in the considered cWWTP where sampling activities were carried out.

In a review carried out on three Italian rivers heavily impacted from anthropogenic pressures (Grenni et al., 2018), freshwater resulted contaminated in the range of 1.30 - 124 ng/L for ciprofloxacin, 0.66 – 74.2 ng/L for spiramycin, 1.20 - 14.4 ng/L for oxytetracycline, 1.83 - 236 ng/L for sulfonamides, and 1.20 - 249 ng/L for lincomycin. In our study (Table 2), such loads were not found because most of the sampling sites belonged to rural and rural-urban areas, which do not experience the heavy contribution of high-density urban settlements. This suggests a smart urbanisation design of areas devoted to food production could represent a factor for safe and sustainable food systems.

In waters, the presence of ciprofloxacin could be addressed both to the metabolization of Enrofloxacin, a fluoroquinolone prescribed in intensive animal farms, as well as to the active principle present in human antimicrobials. The distinctive presence of ciprofloxacin in biosolids derived from water purification plants, together with its absence (<LOD) in animal-derived soil improvers suggest that its origin is mainly anthropic.

4.3. Antimicrobial Inputs in lettuce cultivation cycle

In this paper, we consider a cycle of lettuce production (2 month-long during the summer season) accounting also for the combined presence of antimicrobials from different sources (top soil improvers and irrigation waters). In doing this, a target multi-residue method has been used, that, even if not exhaustive of all the potential antimicrobials other than those usually considered in animal farming, gave the following evidences.

From the results of Tables 1 and 2, it could be possible to propose a risk ranking about the antimicrobials inputs in agriculture soils from top soil improvers and irrigation waters, respectively, according to their origin. Clearly, with the exception of two mixed compost samples with Sulfanilamide, the regular use of mixed composts from green and household wastes, as well from sludges originating from dairy milk process, could be considered safe and sustainable, as well as irrigation from rivers and channels freshwaters from not high population density areas.

On the contrary, animal manure and cWWTPs sludge –based composts, when combined with re-used waters inputs, can determine the association of antimicrobials of different classes (sulfonamides + trimethoprim, tetracyclines, macrolides, quinolones), associations not always present in specific human and veterinary medicines, and consequentely, not framed in the pre-marketing Environmental Risk Assessment of medicinal products for human or veterinary use.

This point could be addressed in the next future, within the frame of a comprehensive soil health strategy, based on soil functions (Cycon et al., 2019).

The computed final concentration in soils after the 2 months long lettuce cycle of cultivation (Table 3) recognises the following sources of uncertainties: the halflives of the different antimicrobials in soil, after their binding to the organic matter (Cycon et al., 2019); the subtraction of antimicrobals via the uptake from plants (Albero et al., 2019). The degradation rate variation recorded in the literature accounts for differences in soil composition and weather conditions, as well as the potential residual antimicrobial load in the same soil, after different cycles of production with the use of contaminated top soil improvers and irrigation waters. The overall balance seems a little bit complicated due to the daily inputs from irrigation waters that are able to provoke a so called drug pseudo-persistence (Kumiriska 2020): regular inputs compensate the overall losses, thus provoking an environmental persistence for the considered contaminant.

The assessment of Antimicrobial Resistance has been recently proposed in the European legislation of wastewaters (EU Commission notice, 2022): in Table 4, under the worstcase approach, it is clear that the risk of AMR tranfer in food productions basically derives from the use of top soil improvers originating from cWWTPs sludges, animal manure and slurry for almost all the class of antimicrobials determined (HI >1). In amended and irrigated soil, according to the related dilution factors, the only concern is related to the associated presence of fluoroquinolones.

In terms of risk ranking, the presence of fluoroquinolones should deserve a great attention, as far as AMR resistance to such class of antimicrobials is inserted among the priorities of the World Health Organization (WHO), also on the evidence-based resistance in food-borne pathogens such as Campylobacted and Salmonella (high risk) and Shigellia (medium Risk) (https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed).

To conclude, the health-realted risk assessment of safe and sustainable food systems request an olistic approach based on the origin and provenience of environmental resources such as top soil improvers and irrigation waters. Antimicrobials as drivers of AMR transfer from environment to humans via food could be minimised via the use of mixed composts and irrigation water from not anthropogenic impacted freshwaters, thus suggesting a safe-by-design approach of agricultural settings. In perspective of a progressive limitation of the antimicrobial administration to farmed animals via medicated feeds as well medicated drinking water, we could presume reused waters and top soil improvers based on the recycling of cWWTPs sludges would represents the main pharmaceutical inputs in soils devoted to agricultural practices. Further work is in progress to associate the antimicrobials in selected matrices with the presence of antimicrobial resistant food-borne pathogens and antimicrobial resistence genetic elements, via microbiological, genomic, and metagenomic approaches.

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List of abbreviations:

AMR	Anti-Microbial Resistance
cWTTPs	Civil wastewater treatment Plants
HI	Hazard Index
LOD	Limit of Detection
PNEC	Predicted No Effect Concentration
SM	Supplementary Materials
TSI	Top Soil Improvers
ww	wet weight

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