Environmental Science Advances

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
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Cite this: *Environ. Sci.: Adv.*, 2025, **4**, 252

Treated wastewater reuse for crop irrigation: a comprehensive health risk assessment†

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The use of treated effluent/wastewater (TWW) for crop irrigation is gaining prominence globally due to growing freshwater scarcity. However, there are still questions about the safety of such a practice. This study sought to investigate and evaluate the health risks associated with the use of TWW for crop irrigation by assessing the potential risks arising from pathogens, heavy metals/potentially toxic elements (PTEs), micropollutants or pharmaceuticals and antibiotic resistance genes (ARGs), using tomato, carrot and cabbage as test crops. The levels of copper bioaccumulated in TWW irrigated crops were 25 mg kg^{-1} for tomato, 30 mg kg^{-1} for carrot and 20 mg kg^{-1} for cabbage, while those of the control (tap water) were 30 mg kg^{-1} for tomato, 40 mg kg^{-1} for carrot and 65 mg kg^{-1} for cabbage, respectively. Arsenic, cadmium and lead levels were below the detection limit for all treatments. The hazard quotient (HQ) and hazard index (HI) of copper and zinc were below 1 (adults) for TWW irrigated crops. *Escherichia coli*, *Clostridium perfringens*, coliform and thermotolerant bacteria were not detected on the fruits of tomato plants irrigated with TWW. All analysed pharmaceuticals were below the limit of detection except gabapentin, which was 3 μg kg^{-1} in TWW irrigated tomatoes. *tetA*, *ermB*, bla_{TEM}, *sul2*, *sul3* and *qnrS* genes were found in the metagenomic DNA extracted from TWW- and tap-irrigated cabbage. The results indicate no potential non-carcinogenic health risk for adult consumers and no microbial contamination of the tomato fruits under TWW irrigation. No difference was observed in the presence and distribution of the ARGs between TWW- and tap-irrigated crops, suggesting no contribution to the diffusion of different ARGs due to irrigation. Altogether, these findings highlight that health risk assessment of TWW for crop irrigation should focus on the quality of the TWW and on soil characteristics, which may contribute to risk exposure of different types of contaminants.

Received 11th July 2024
Accepted 17th November 2024

DOI: 10.1039/d4va00274a

rsc.li/esadvances

Environmental significance

The growing scarcity of freshwater for agricultural use calls for the use of alternative water sources aside from freshwater resources to help achieve environmental sustainability. Treated wastewater is considered a viable substitute for freshwater for crop irrigation. However, due to the perceived potential risks associated with treated wastewater, its use for crop irrigation should be done with care to protect public health and maintain the environmental integrity of the different environmental compartments. The study improves our understanding of the risks associated with water reuse, an important component in the promotion of environmental sustainability. It also sheds light on how the practice of water reuse could impact human health taking into account the different environmental compartments such as water, soil and biota.

1 Introduction

The use of treated wastewater (TWW) for irrigation is gaining prominence globally due to growing freshwater scarcity. Rapid population growth, urbanization and industrialization have led to increased demand for and depletion of freshwater resources.[1] Various water management strategies have been developed to meet the growing water demands and to ensure water sustainability. These include sea or saline water de-salinization, water conservation measures such as efficient water use technologies (for example, pressured irrigation systems) and treated wastewater irrigation. As almost 70% of the global water resources are abstracted for agricultural use, the reuse of treated wastewater (TWW) for crop irrigation can significantly reduce the amount of water extracted from freshwater sources.[2] Globally, the history of using treated wastewater reveals the feasibility of wastewater reuse in agriculture[3] and countries such as Israel and Tunisia

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† Electronic supplementary information (ESI) available. See DOI: https://doi.org/10.1039/d4va00274a

have demonstrated the sustainable use of TWW in agriculture.[4,5] TWW irrigation could be essential for maintaining food security and promoting agriculture, especially in semi-arid and arid regions with limited freshwater sources.[6] Reusing TWW for irrigation presents many benefits such as the supply of nutrients, an increase in crop yield and constant irrigation water supply.[1] Such practice could help protect environmental quality and alleviate or minimize the pressure on limited freshwater sources for agricultural irrigation.[7] However, there is a perceived risk associated with the practice, which has fueled a lack of public acceptance in some regions of the globe.[8,9] There is a reluctance to accept agricultural products produced using TWW.[10] This perceived risk is associated with the composition or the quality of the water. TWW could be a reservoir of pathogens, organic, non-biodegradable and persistent pollutants such as potentially toxic elements (PTEs) or heavy metals, bacteria, viruses and contaminants of emerging concern (CECs) that could potentially enter the human food chain through irrigation.[9,11–13]

Metallic elements with a density greater than 5 g cm^{-3} are referred to as heavy metals and these include lead (Pb), copper (Cu), cadmium (Cd), arsenic (As), chromium (Cr), *etc.*[14] Irrigating crops with TWW could lead to the accumulation of these elements in arable soils and their bioaccumulation and biomagnification in the food chain.[1,15] Rezapour *et al.* investigated the bioavailability and accumulation of five heavy metals (zinc (Zn), nickel (Ni), copper, lead and cadmium) in winter wheat crops and calcareous soils irrigated with TWW.[16] The authors reported a significant accumulation of heavy metals in the soil and a considerable build-up in the wheat crops. Significant accumulation was noted in the wheat roots when compared to the shoot and grains. In vegetables, heavy metals could be taken up by the roots and accumulate in the edible parts.[17] Such bioaccumulation may pose a threat to public health since the human body could absorb the heavy metals/PTEs through food ingestion and skin contact with the soil.[14,18] Bioaccumulation of these elements in the bones, liver and kidneys to harmful levels could lead to serious health problems.[14] Malfunctions of cell respiration, nerves, kidneys and muscles are all associated with heavy metal/PTE toxicity.[19]

Another risk associated with the use of TWW for irrigation is microbial contamination of food. Depending on the nature of the treatment processes, TWW could harbour a significant amount of pathogenic and indicator microorganisms such as *Enterococci*, *Escherichia coli*, *Coliforms*, *Clostridium perfringens*, *Salmonella* spp., *etc.*, which could pose serious health risks to humans and agricultural animals.[20] Several studies involving TWW irrigation have reported higher microbial content above the local or international wastewater reuse guidelines.[21–23] A significant number of faecal enterococci, *E. coli* and coliforms were found in a secondary effluent used for irrigating tomatoes and broccoli plants.[23] Pathogen internalization could occur through root uptake and leaf contact as a result of exposure of the crops to pathogens by the irrigation water. Interactions between the irrigated crops and the exposed pathogens vary among different cultivars of the same crop species.[24]

The exposure of humans to pathogens under treated wastewater irrigation occurs through direct contact with the water (in the case of farm workers) and mouth ingestion of contaminated food crops (consumers). Diarrhea and extraintestinal diseases are some health risks the public could encounter if *E. coli* contamination of food occurs through irrigation.[25] With health risk barrier management strategies such as disinfection, drip irrigation and post-harvest food washing, these health risks could be eliminated or reduced to the barest minimum.

In recent times, the risk of exposure to CECs associated with TWW irrigation has gained attention. These are groups of organic compounds and substances with known or perceived ecological and health risks, comprising antibiotic-resistant genes (ARGs) and antibiotic-resistant bacteria (ARB), antibiotics, personal care products, endocrine disrupting compounds (EDCs), pharmaceuticals and their metabolites.[6,9,13,26] The presence of CECs in TWW has been reported in the literature.[27–29] Diaz-Sosa *et al.* detected atenolol, caffeine, carbamazepine, tramadol and sulfamethoxazole together with other pharmaceuticals in the secondary TWW of the Prague central wastewater treatment plant.[28] ARGs such as *sul1*, *ermB*, *uidA*, *mefC*, and *tetX* have also been detected in TWW from Portugal, Denmark, the Czech Republic, the Netherlands and Israel.[29] These findings elucidate the biological safety risk associated with the practice of TWW irrigation. Studies have shown that TWW irrigation could lead to ARG dissemination in soil microbiota, while others have reported the opposite.[6,30] A study by Fatta-Kassinos *et al.* highlighted the bioaccumulation of CECs in soil and crops irrigated with TWW.[26] The accumulation of antibiotics in agroecosystems and their potential uptake by food crops is a public health concern.[13]

Several studies have been conducted on risk assessment of TWW irrigation. Sallach *et al.*, Cerqueira *et al.*, Marano *et al.*, Gudda *et al.*, and Liu *et al.* focused on antibiotics and ARGs; Razapour *et al.*, Chen *et al.*, and Mosa *et al.* focused on heavy metals or PTEs; Forslund *et al.*, Farhadkhani *et al.*, and Tripathi *et al.* focused on pathogens or microbial contamination; Yan *et al.* focused on heavy metals and CECs; and Sallach *et al.* focused on antimicrobials and pathogens.[6,13,16,24,31–40] However, due to the complexity of the potential risks associated with the practice, no single study has evaluated the comprehensive risk of the practice from the point of view of heavy metal/PTE risk, microbial risk and CEC risk, to the best of our knowledge. Previous studies have focused on either one or two of these risk areas. It is hypothesized that TWW irrigation presents little or no health and environmental risk, and incorporation of the aforementioned risk areas in a single study provides a better option for risk assessment. The study, therefore, provides a comprehensive health risk assessment of TWW irrigation considering the potential risk of exposure to heavy metals/PTEs, microbial contamination and contaminants of emerging concern. The objectives are to evaluate (i) the bioaccumulation and bioaccessibility of heavy metals in soil and edible parts of crops (tomato, cabbage, and carrot), (ii) the potential risk of microbial contamination of crops (tomato), (iii) the bioaccumulation of antibiotics or pharmaceuticals in the edible parts of crops and (iv) the presence of ARGs in the edible parts of crops (cabbage), under TWW irrigation. The outcome of the study will contribute greatly to the assessment of the suitability

of TWW for irrigation and ensure public safety concerning the practice.

2 Methods

2.1 Experimental design

The study involved irrigating three different vegetable crops with secondary effluent and tap water cultivated in a greenhouse under a tropically mimicked environment. Secondary effluent was obtained from a municipal wastewater treatment plant (WWTP) in the Czech Republic and tap water from the University of Chemistry and Technology, Prague. Crop treatments were made up of secondary effluent-irrigated crops (SE) and tap water-irrigated crops (Tap). Tap water-irrigated crops (Tap) represented the control group. The test crops were cabbage (*Brassica oleracea* L.), carrot (*Daucus carota* subsp. *sativus*) and cherry tomato (*Solanum lycopersicum*) and the seeds were purchased from a commercial supermarket. Cherry tomato and cabbage seeds were subject to seeding on a filter paper placed in a Petri dish. The sprouted seeds were transferred to a nursing substrate in the laboratory and later transported to the greenhouse for planting. Carrot seeds were sown directly into the soil in the greenhouse without nursing. Each treatment (tomato and cabbage) consisted of six pots, three for each plant except carrot which consisted of 4 pots. Each pot had a height of 20 cm and a volume of 3.4 L (0.0034 m^3) and was filled with soil to about 85% of the volume. The soil is composed of clay (14.0%), silt (37.2%), and sand (48.7%) fractions, falling into the loam soil classification. The pots were set up on a growth bench in a greenhouse and illuminated for 12 hours during the day with a Growth Spectrum Advanced 600 W lamp (GIB Lighting, Berlin, Germany). The time was adjusted to a 9 h daytime setting after 66 days. This was done to reduce the rapid rate at which water evaporated from the soil and plants. The lamp has a photon flux (100 h) of 740 μmol s^{-1}, a color temperature of 8000 K, a light intensity of 48 000 lm, and a nominal power of 600 W. Since the lamp created a high rate of evaporation, irrigation was often done once a day to provide a sufficient supply of water. The average growth temperature and relative humidity were 26 °C and 35%, respectively. No compost, manure, fertilizer, or other soil amendment was employed during the experiment. The sowing of the seeds occurred in September 2021, and the matured crops were harvested in February of the following year. The experimental design is from the studies of Ofori *et al.*[41,42] and a detailed description can be found in these articles. A second study on antibiotic resistance gene dissemination involved irrigating cabbage crops with secondary effluent and tap water. The experiment was conducted in a growth room at 24 °C temperature and 75% relative humidity. The seeds were sown directly in loam soil contained in a pot (the same dimensions as aforementioned) and the growth period lasted for about three months. The young cabbage plants were harvested after this period and analysed for the presence of ARGs. Evaluation of the health risk in both studies focused primarily on oral ingestion of the edible part of the vegetable crops and exposure to contaminated soil.

2.2 Determination of the potential risk of heavy metals or potentially toxic elements

2.2.1 Analysis of heavy/PTEs in irrigation water, soil and plant biomass. Lead (Pb), copper (Cu), zinc (Zn), cadmium (Cd) and arsenic (As) were the elements of health interest and therefore were analysed in irrigation water, soil before irrigation, irrigated soil and plant biomass matrices. Secondary effluent and tap water samples were collected in washed bottles, preserved with nitric acid (pH < 2) and kept in a fridge (4 °C) until the analyses. Heavy metals in the water samples were measured using atomic absorption spectroscopy (AAS). Soil samples consisting of irrigated soil and non-irrigated soil (soil before the application of water) were collected by the composite sampling method, air-dried and sieved with a 2 mm sieve. Using a 10 : 1 (v/w) ratio, a 0.01 M $CaCl_2$ extractant was used to extract the elements from the soil according to the procedures of Houba *et al.* and Motsara and Roy.[43,44] $CaCl_2$ solution was added to the soil sample, shaken mechanically for about 2 hours and filtered through a filter paper of 150 mm diameter with a pore size of about 15 μm (Papírna Perštejn s.r.o., Czech Republic). The Pb, Zn and Cd levels in the soil extract were analysed by atomic absorption spectroscopy (AAS-Agilent 280FS AA, Agilent Technologies).

The procedures for the plant biomass sample preparation and extraction followed those of Hunt, and Motsara and Roy.[44,45] Harvested biomass was rinsed several times with tap water, followed by 0.2% detergent solution to remove dirt and waxy or greasy coatings. Biomass samples were then washed with 0.1 M HCl, followed by thorough washing with tap water and final rinsing (twice) with distilled water. The samples were air-dried at room temperature in a dust-free environment for about 72 hours and oven-dried at 70 °C for about 48 hours. The dried samples were then ground with a mill, ashed in a furnace and stored for heavy metal analyses. The ash was then dissolved in a 0.5 M HCl solution, shaken and filtered through a filter paper with a pore size of about 15 μm (Papírna Perštejn s.r.o., Czech Republic) into clean 50 mL tubes. Estimation of the concentration of the heavy metals (Pb, Cu, Zn, As and Cd) in the extract was performed by atomic absorption spectroscopy (AAS-Agilent 280FS AA, Agilent Technologies). The limit of detection was the smallest possible signal that could be differentiated and was determined from the initial phase of the calibration curve. The calibration parameters of the heavy metals are presented as ESI files.†

2.2.2 Health risk exposure assessment. Health risk posed by heavy metals in crops irrigated with TWW was assessed using hazard quotients (HQs) and the hazard index (HI) and comparing the levels with World Health Organization (WHO) guidelines. The HI is the summation of multiple hazard quotients (HQs) for multiple contaminants (heavy metals) and exposure pathways (eqn (1)). The HQ refers to the ratio of a single contaminant exposure level over a defined duration to a reference dose for that substance obtained from a similar exposure duration (eqn (2)).[46] The HQ represents a numerical assessment of the potential risk posed by a single contaminant and the HI represents the overall risk of exposure to all the

contaminants.[14,17,47] Since the study focuses primarily on the edible part of the irrigated crops, the ingestion pathway was adopted. Irrigated soil was not included in the HI assessment due to the lower likelihood that people would be exposed to heavy metals *via* the ingestion pathway. This consideration is in line with the literature. The equations below used for the assessment were adopted from the US EPA and Adam *et al.*[14,46]

$$\mathrm{HI} = \sum_{i=n}^{n} \mathrm{HQ} \quad (1)$$

$$\mathrm{HQ} = \frac{I}{\mathrm{RfD}} \quad (2)$$

I (mg per kg per day) refers to contaminant intake or exposure (eqn (3)); RfD (mg kg^{-1}) refers to the reference dose of the ingested contaminant. An HI or HQ < 1 implies no potential for health effects; An HI or HQ > 1 implies concern for potential health risk or effects; An HQ >>> 1 implies greater health risk concern. RfD values for Cu and Zn were 0.04 and 0.3, respectively, adopted from Rezapour *et al.*[16]

$$I = \frac{C_f \times I_R \times F_I \times E_F \times E_D}{B_W \times A_T} \quad (3)$$

$$I = \frac{C_f \times I_R \times E_F \times E_D}{B_W \times A_T} \quad (4)$$

C_f (mg kg^{-1}) is the contaminant concentration in food; I_R (kg per meal) is the ingestion rate; F_I (unitless) is the fraction ingested from the contaminated source; E_F (meals per year) is the exposure frequency; E_D (years) is the exposure duration; B_W (kg) is the bodyweight; A_T (days) is the average time. Taking a conservative approach, F_I was assumed to be 1.0, leading to eqn (4).[48] HI assessments for As, Cd and Pb were not performed because the C_f was below the detection limit. The risk exposure assessment was partitioned between adults and children since their responses to health risks differ. The input data used for the computation are presented below in Table 1.

The risks of bioaccessibility and bioaccumulation of heavy metals in irrigated soil were evaluated using a modified enrichment factor (EF) from Rezapour *et al.*[16] EF estimation (eqn (5)) was performed using the initial level of heavy metals in the soil (before irrigation) as the reference.

$$\mathrm{EF} = \frac{C_{\mathrm{SE\ or\ Tap\ soil}}}{C_{\mathrm{Bf\ soil}}} \quad (5)$$

C_{Bf} is the concentration of heavy metals in the soil before irrigation. $C_{SE\ soil}$ and $C_{Tap\ soil}$ are the concentrations of heavy metals in tap water and TWW irrigated soils, respectively. EF > 1 was interpreted as heavy metal accumulation; EF = 1 was interpreted as no accumulation of heavy metals; EF < 1 was considered as depletion of heavy metals. Due to data availability, EF was performed for only zinc.

Table 1 Input data used for the computation of intake, health quotient and health hazard[a]

Input parameters	Cu	Zn
$C_{f\text{-Tap-Tom}}$ (mg kg^{-1})	30	230
$C_{f\text{-Tap-Carr}}$ (mg kg^{-1})	40	200
$C_{f\text{-Tap-Cabb}}$ (mg kg^{-1})	65	85
$C_{f\text{-SE-Tom}}$ (mg kg^{-1})	25	215
$C_{f\text{-SE-Carr}}$ (mg kg^{-1})	30	165
$C_{f\text{-SE-Cabb}}$ (mg kg^{-1})	20	65
$I_{R\text{-Tom Raw}}$ (kg per day)	0.044	0.044
$I_{R\text{-Tom Cooked}}$ (kg per day)	0.046	0.046
$I_{R\text{-Carr Raw}}$ (kg per day)	0.018	0.018
$I_{R\text{-Carr Cooked}}$ (kg per day)	0.03	0.03
$I_{R\text{-Cabb Raw}}$ (kg per day)	0.027	0.027
$I_{R\text{-Cabb Cooked}}$ (kg per day)	0.05	0.05
E_F	365	365
$E_{D\text{-Adult}}$ (year)	70	70
$E_{D\text{-Child}}$ (year)	6	6
$B_{W\text{-Adult}}$ (kg)	70	70
$B_{W\text{-Child}}$ (kg)	15	15
$A_{T\text{-Adult}}$ (days)	25 550	25 550
$A_{T\text{-Child}}$ (days)	2190	2190
RfD	0.04	0.3
$B_W \times A_{T\text{-Adult}}$	1 788 500	1 788 500
$B_W \times A_{T\text{-Child}}$	32 850	32 850

[a] Tap-Tom, Tap-Carr, and Tap-Cabb refer to tap water irrigated tomatoes, carrots and cabbage, respectively. SE-Tom, SE-Carr and SE-Cabb refer to secondary effluent irrigated tomatoes, carrots and cabbage, respectively. Tom-Raw, Carr-Raw and Cabb-Raw refer to uncooked tomatoes, carrots and cabbage, respectively. Tom-Cooked, Carr-Cooked and Cabb-Cooked refer to cooked tomatoes, carrot and cabbage, respectively. B_W, E_D, E_F, I_R and RfD were obtained from US EPA, Rezapour *et al.* and Adam *et al.*[14,16,46]

2.3 Determination of the potential risk of exposure to pathogens

2.3.1 Analysis of microbial pathogens in irrigation water and plant biomass. To determine the risk of exposure of humans to pathogens, four indicator microorganisms were analysed in the irrigation water and the harvested tomato fruits. The tomato fruits were thoroughly rinsed with ultra-pure water and the rinsed water was collected in a sterilized container. Secondary effluent, tap water and rinsed water from the tomato fruits were analysed for the presence and quantification of coliform bacteria, thermotolerant bacteria, *E. coli* and *C. perfringens*. The procedure was based on the Czech Republic's norms ČSN 757837, 757835 and Decree 252/2004, respectively.[49–51] The water samples were filtered through a 0.45 μm membrane filter paper. The filter paper was then transferred to an Endo agar medium on a Petri dish. The filter paper together with the dish were placed upside down in a thermostat and cultured at 36 °C for about 18–24 h. The filter was then transferred to a cytochrome oxidase solution-saturated medium. After 120 s, the colonies of coliform bacteria formed were counted.[49] Detection of *C. perfringens* in the water samples followed the Czech Decree 252/2004. The water samples were filtered using a membrane filter and the filter was placed on an m-CP agar medium. Cultivation was performed in an anaerobic environment at 44 °C for about 21 h. After cultivation, the yellow-grown colonies were counted and later exposed to ammonia vapour for 20–30 s for the determination of *C. perfringens*.[51] In the evaluation of the presence of thermotolerant coliform and *E. coli* in the water samples, the water samples were filtered with a 0.45 μm filter paper. Cultivation of the

bacteria occurred on m-FC agar at 44 °C for 18–24 h. After cultivation, the filter was transferred onto a liquid culture medium for 2–4 h in the dark at 36 °C. Visualization of the formed colonies was performed under a UV lamp at 360 nm.[50] A detailed description of the various procedures has been outlined in previous work.[42]

2.3.2 Health risk exposure. The risk of exposure focused primarily on the ingestion of the tomato fruits and the exposure of farm employees to pathogens through contact with irrigation water. The exposure risk of the public to the irrigation was excluded as this pathway is very unlikely. The quantification of the indicator microorganisms was used to evaluate the risk by comparing the microbial loads of the study matrix with threshold values stipulated in reputable guidelines such as European Union (EU) and World Health Organization (WHO) guidelines.

2.4 Determination of the potential risk of contaminants of emerging concerns

2.4.1 Analysis of pharmaceuticals in irrigation water and plant biomass. A total of 30 pharmaceutical products were analysed in the irrigation water samples and 82 in the biomass samples. These products comprised antibiotics, painkillers, antidepressants, *etc.* Fourteen (14) of these pharmaceuticals are presented in the text while the rest are presented as ESI files.† Water samples were collected in glass bottles and refrigerated at 4 °C. The samples were then analysed within 5 days after collection. The quantification of the targeted pharmaceuticals in the irrigation water samples was performed by LC-MS using a 1290II HPLC system (Agilent Technologies) with a 6460 triple quadrupole mass spectrometer (Agilent Technologies). A Zorbax Eclipse Plus C18 RRHD (2.1 × 50 mm; 1.8 μm) as a delay column and an analytical column Zorbax Eclipse Plus C18 RRHD (2.1 × 100 mm; 1.8 μm) with a guard column Zorbax Eclipse Plus C18 precolumn (2.1 × 5 mm; 1.8 μm) at a flow rate of 0.40 mL min^{-1} were used for separation. The temperature of separation was 40 °C. Eluent A was aqueous 0.5 mM ammonium fluoride and eluent B was methanol. The following gradient elution was used: 0–8 min, 5–100% B; held for 5.5 min at 100% B; from 13.50–14 min, a decrease to 5% B, and 3.5 min starting conditions before the next injection. The injection volume of samples was 100 μL. The ESI source with Agilent Jet Stream technology was operated under the conditions given in Table 2. The data recorded were processed with MassHunter software. For quantification and confirmation two multiple reaction monitoring (MRM) transitions were monitored for each analyte in dynamic MRM mode. Parameters for QQQ are in Table 8 of the Appendix.

$$\mathrm{IDL} = t \times \frac{\mathrm{RSD}}{100\%} \times \text{amount measured}$$

RSD is the relative standard deviation of the peak area obtained from 8 measurements (injection of standards); t (2.999) is the critical value of Student's t-distribution at the 99% confidence level for seven degrees of freedom.

Quantification of the targeted pharmaceutical products in the biomass was performed by an external laboratory (Povodi Vltavy) in Pilsen, Czech Republic. Sample preparation involved rinsing the fresh cherry tomatoes with distilled water, storing them in clean tubes and freezing them until transportation to the laboratory for the analyses. The samples of biomass were lyophilized and homogenized by grinding. Subsequently, 0.1 g of each sample was weighed in a 4 mL vial on an analytical balance. Then 1 mL of acetonitrile was added to each sample. An isotope dilution was performed in the next step. Analytes were extracted from biomass by sonification in an ultrasonic bath for 30 minutes. The second extraction of pharmaceuticals was done with 1 mL acid water (water + 0.3% formic acid) for 30 minutes. Both extracts were joined together and centrifuged in vials for 10 min at about 3500 rpm. The last step of preparation was the dilution of the extracts with acid water (1 : 3) and transferring the aliquot part of each extract to a 2 mL autosampler vial. Two independent standards with certified concentrations were used for the determination of each analyte, one for calibration and another for standard addition. Standard solutions were prepared both from neat analytes and from commercial solutions with certified concentrations.

Table 2 ESI source parameters[a]

Parameter	Value (+)	Value (−)
Gas temperature (°C)	230	230
Gas flow (L min^{-1})	8	8
Nebulizer pressure (psi)	40	40
Sheath gas heater (°C)	380	380
Sheath gas flow (L min^{-1})	12	12
Capillary voltage (V)	3000	3000

[a] The limit of detection (LOD) was estimated using the instrument detection limit (IDL).

Pharmaceuticals were separated and detected by the LC-MS/MS method based on direct injection of the sample extract into the 1290 ultra-high-performance liquid chromatography (UHPLC) coupled with an Agilent 6495B Triple Quad Mass Spectrometer (MS/MS) of Agilent Technologies, Inc. (Santa Clara, CA, USA). The separation was carried out on a Waters Xbridge C18 analytical column (100 mm × 4.6 mm, 3.5 μm particle size). The mobile phase consisted of methanol and water with 0.5 mM ammonium fluoride and 0.02% acetic acid as the mobile phase additives. The flow rate was 0.5 mL min^{-1}. The injection volume was 0.010 mL.

Quantification was done using MassHunter Workstation Version 10.1 software. Each series of samples was verified through calibration control and by maintaining a clean laboratory environment, equipment, and agents. The performance of the analytical system was validated using blank and spiked samples. Every third sample in every batch was processed by the method of standard addition for all analytes, which was used to check the effect of the matrix of the sample and to reset the actual recovery ratio of a specific analyte. The measurements were performed according to the Czech standard ČSN ISO 20179.[52] The LOD was estimated from either the calculation of the signal-to-noise ratio or the blank calculation (the mean and standard deviation of a set of 10–15 blanks). Table 3 presents

Table 3 Quality characteristics of the irrigation water used for irrigating the cabbage, carrot and tomato plants[a]

Parameter	Tap water	Secondary effluent
Heavy metals/PTEs		
Arsenic (mg L^{-1})	<LOD	<LOD
Cadmium (mg L^{-1})	<LOD	<LOD
Copper (mg L^{-1})	<LOD	<LOD
Lead (mg L^{-1})	<LOD	<LOD
Zinc (mg L^{-1})	0.04 ± 0.01	0.13 ± 0.04
Contaminants of emerging concern		
Ibuprofen (ng L^{-1})	<LOD	<LOD
Naproxen (ng L^{-1})	<LOD	113.75 ± 18.87
Diclofenac (ng L^{-1})	<LOD	1950 ± 100.00
Paracetamol (ng L^{-1})	<LOD	<LOD
Carbamazepine (ng L^{-1})	11.25 ± 2.47	460 ± 14.14
Gabapentin (ng L^{-1})	79 ± 1.41	1900 ± 81.65
Tramadol (ng L^{-1})	0.41 ± 0.01	2625 ± 170.78
Caffeine (ng L^{-1})	12.50 ± 0.71	3.90 ± 2.55
Estriol (ng L^{-1})	<LOD	<LOD
Testosterone (ng L^{-1})	<LOD	<LOD
Sulfamethoxazole (ng L^{-1})	0.39 ± 0.10	770 ± 112.25
Trimethoprim (ng L^{-1})	<LOD	675 ± 64.55
Saccharin (ng L^{-1})	<LOD	645 ± 242.83
Warfarin (ng L^{-1})	<LOD	<LOD
Microbial characteristics		
Thermotolerant coliform (CFU mL^{-1})	nd	$5.80 \pm 4.15 \times 10^4$
Escherichia coli (CFU mL^{-1})	nd	$2.76 \pm 2.33 \times 10^4$
Coliform (CFU mL^{-1})	nd	$9.85 \pm 6.52 \times 10^4$
Clostridium perfringens (CFU mL^{-1})	nd	$1.22 \pm 0.87 \times 10^3$

[a] Values are concentrations expressed in means plus the standard deviation; LOD is the limit of detection. LOD of arsenic (0.01 mg L^{-1}), cadmium (0.01 mg L^{-1}), copper (0.01 mg L^{-1}), lead (0.05 mg L^{-1}), zinc (0.01 mg L^{-1}), ibuprofen (5.0 ng L^{-1}), naproxen (2.0 ng L^{-1}), diclofenac (0.5 ng L^{-1}), paracetamol (0.08 ng L^{-1}), carbamazepine (0.08 ng L^{-1}), gabapentin (0.2 ng L^{-1}), tramadol (0.2 ng L^{-1}), caffeine (2.0 ng L^{-1}), estriol (5.0 ng L^{-1}), testosterone (0.2 ng L^{-1}), sulfamethoxazole (0.08 ng L^{-1}), trimethoprim (0.2 ng L^{-1}), saccharin (20.0 ng L^{-1}) and warfarin (0.2 ng L^{-1}). Heavy metal and microbial data were obtained from Ofori *et al.*[41,42]

the water quality characteristics of the two irrigation water streams.

2.4.2 Analysis of the presence of antibiotic resistance genes in irrigation water, soil and plant biomass. Nine ARGs commonly found in wastewater and the environment were considered as target genes (*sul1*, *sul2*, *sul3*, *ermB*, *tetA*, *tetW*, *qnrs*, *blaTEM*, and *blaZ*) for the ARG dissemination study. The presence of all the targeted ARGs was investigated in secondary effluent, tap water, soil (before and after irrigation) and biomass. Irrigation water samples were filtered using 0.22 μm filter paper to collect microbial biomass for DNA isolation. The filter papers were then frozen at −20 °C. Soil samples from each pot were collected by pouring the soil onto a plastic bag and homogenizing the soil. After homogenization, the samples were randomly taken from six locations to constitute a replicate sample per pot. Biomass samples were washed multiple times with sterilized distilled water after which the samples were lyophilized. The lyophilized biomass samples were then macerated with a pestle and mortar to obtain pulverized biomass. DNA was then obtained from the filter papers, soil samples and the pulverized plant biomass for polymerase chain reaction (PCR) analyses.

DNA was extracted from the samples using the phenol–chloroform method. The extraction of DNA from the soil and biomass was performed according to the method described by Islam *et al.* and Di Leto *et al.* with slight modifications.[53,54] The pellet of crude nucleic acids was precipitated by centrifuging at 16000*g*. On the other hand, the extraction of DNA from water samples was done using a Thermo Scientific Genomic DNA purification kit. The manufacturer's protocol was followed. The concentration of the extracted DNA was measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) at 260 nm. The purity of the isolated DNA was assessed using the 260/230 and 260/280 ratios for organic and protein contaminations, respectively. Extracted DNA was used as a template for the PCR analyses of ARGs' genes and of the V3–V4 region of the 16S rRNA gene to verify the amplifiability.[55] The obtained amplicons were visualized by agarose gel electrophoresis (1.5–2.5% depending on amplicon size). Gels were stained using GelRed (Invitrogen) and then visualized using UV light.

2.5 Statistical analyses of data

The confidence factor t was determined using Student's t-distribution with a 99% confidence level and $n - 1$ $(8 - 1)$ degrees of freedom. The critical value of Student's t-distribution at the 99% confidence level for seven degrees of freedom was 2.999. Chart and table representations were created and data analyses were performed using Microsoft Excel 2019. The test of

significance of the difference of means between two study variables was performed by Student's *t*-test (paired) at a confidence interval of 95% ($p < 0.05$). Analysis of variance (ANOVA) was used to compare the means among multiple variable datasets. To establish the significance (ANOVA) of the difference observed among the means of the different treatments, a confidence level of 95% ($p < 0.05$) was adopted.

3 Results and discussion

3.1 Bioaccessibility and bioaccumulation of heavy metals/PTEs in irrigated crops

The amounts of As, Cd and Pb taken up and bioaccumulated in the edible tissues or biomass of tomatoes, carrots and cabbage were below the detection limit of 0.04 mg L^{-1}, 0.006 mg L^{-1} and 0.4 mg L^{-1}, respectively. These levels are consistent with the concentrations found in the irrigation water (Table 3) and the soil (refer to Ofori *et al.*).[42] The relatively low levels of As, Cd and Pb in the irrigation water and soil may account for the insignificant amount accumulated in the edible tissues of the test crops. Copper levels in tap water and TWW were also below the limit of detection while those of Zn were 0.04 ± 0.01 mg L^{-1} and 0.13 ± 0.04 mg L^{-1}, respectively. The quantity of Cu accumulated in crops irrigated with tap water was 0.06 mg $L^{-1} \approx 30$ mg kg^{-1} for tomato, 0.08 mg $L^{-1} \approx 40$ mg kg^{-1} for carrot and 0.13 mg $L^{-1} \approx 65$ mg kg^{-1} for cabbage, while that of treated effluent was 0.05 mg $L^{-1} \approx 25$ mg kg^{-1} for tomato, 0.06 mg $L^{-1} \approx 30$ mg kg^{-1} for carrot and 0.04 mg $L^{-1} \approx 20$ mg kg^{-1} for cabbage, respectively. Tap water irrigated crops accumulated more Cu than treated effluent irrigated crops, with the difference in accumulation being significant for cabbage. The order of accumulation differed between the two irrigation water streams; tap water was cabbage > carrot > tomato; and effluent was carrot > tomato > cabbage. Crop species or physiology, metal translocation and transpiration rate may have influenced the amount of Cu uptake by the different test crops leading to the observed differences in bioaccumulation.[15]

A similar trend of bioaccumulation was also observed for Zn, with tap-water irrigated crops showing higher Zn accumulation. Tap water irrigated crops accumulated 0.46 mg L^{-1} Zn $\approx$ 230 mg kg^{-1} for tomato, 0.4 mg L^{-1} Zn $\approx$ 200 mg kg^{-1} for carrot and 0.17 mg L^{-1} Zn $\approx$ 85 mg kg^{-1} for cabbage, while treated effluent irrigated crops accumulated 0.43 mg L^{-1} Zn $\approx$ 215 mg kg^{-1} for tomato, 0.33 mg L^{-1} Zn $\approx$ 165 mg kg^{-1} for carrot and 0.13 mg L^{-1} Zn $\approx$ 65 mg kg^{-1} for cabbage, respectively. The order of accumulation was the same for both irrigation water, tomato > carrot > cabbage. It was evident that the phenomenon of heavy metal (Cu and Zn) uptake and its accumulation in plant tissues occurred in this study. In a similar study conducted in Iran, the authors also observed an accumulation of Cu and Zn in cabbage after irrigation with wastewater. They reported a higher accumulation of Zn (51.2 mg kg^{-1}) than Cu (10.0 mg kg^{-1}) in wastewater-irrigated cabbage, which is consistent with our results.[15] However, the bioaccumulation of these PTEs in the control water (freshwater) irrigated crops was lower than that in the wastewater irrigated cabbage, which is contrary to our findings. Razapour *et al.* also reported bioaccumulation of Cu and Zn in wheat crops after TWW irrigation, but the mean concentrations were significantly lower compared to the results obtained for the present study.[16]

The results of the present study suggest a potential risk of exposure of consumers to Cu and Zn. Consuming these irrigated crops could lead to the absorption of Cu and Zn and their biomagnification in the human body. Continuous accumulation in the human body could reach toxic levels and cause serious health problems. Cu and Zn are needed by the human body for vital biological processes, but toxic levels could result in liver cirrhosis, brain damage, anaemia, lethargy and risk of prostate cancer, respectively.[56,57]

The results of the study showed that the use of the treated effluent posed a relatively lower risk of heavy metal/PTE accumulation in plants than the control (tap water) and that the concentration of Cu and Zn in the irrigation water did not have a direct relationship with the quantity of Cu and Zn accumulated in the edible parts of the crops. We therefore postulate that the main driver that influenced the uptake and the subsequent bioaccumulation of these elements was not the irrigation water but rather other drivers such as plant physiology and soil properties. Factors such as soil properties may have strongly accounted for the uptake and translocation to the edible parts. Such a conclusion is partly consistent with the findings of previous studies. Jalil *et al.* cited soil characteristics (pH, electrical conductivity, and cation exchange capacity), crop roots and soil interface, metal bioavailability, transpiration rate and metal translocation as factors that may affect the uptake and translocation of metals from soil to crops.[15] Other authors cited plant physiology, the quantity of bioavailable heavy metals in the soil, and soil physicochemical attributes as factors that influence the uptake and bioaccumulation of heavy metals or PTEs.[58,59] The findings of our study highlight the importance of considering soil, plant and heavy metal characteristics in the evaluation of the risk of bioavailability and bioaccumulation of heavy metals under TWW irrigation.

3.2 Zinc accumulation in tap water and TWW irrigated soil

Tap-Tom, Tap-Carr and SE-Tom irrigated soils recorded accumulation of Zn in the order Tap-Carr > SE-Tom > Tap-Tom (Fig. 1). There was a significant ($p < 0.05$) buildup of Zn in SE-Tom irrigated soil. However, accumulation of Zn did not occur in most of the TWW irrigated soils (SE-Carr and SE-Cabb); instead, a depletion was observed. Comparatively, TWW irrigated soils exhibited no or relatively little accumulation and pose a lower risk of Zn uptake compared to tap water irrigated soils. These findings do not support the general notion held by many that TWW irrigation leads to the accumulation of heavy metals in the soil and will lead to higher uptake of PTEs by crops. Contrary to our results, Rezapour *et al.* found a significant accumulation of heavy metals in the soil after five years of TWW irrigation.[16] Therefore, the results of the current study elucidate an important finding that arable lands subjected to TWW irrigation may not spontaneously make PTEs bioavailable for crops. The order of Zn enrichment or accumulation was Tap-Tom > SE-Tom > Tap-Cabb > Tap-Carr > SE-Carr > SE-Cabb.

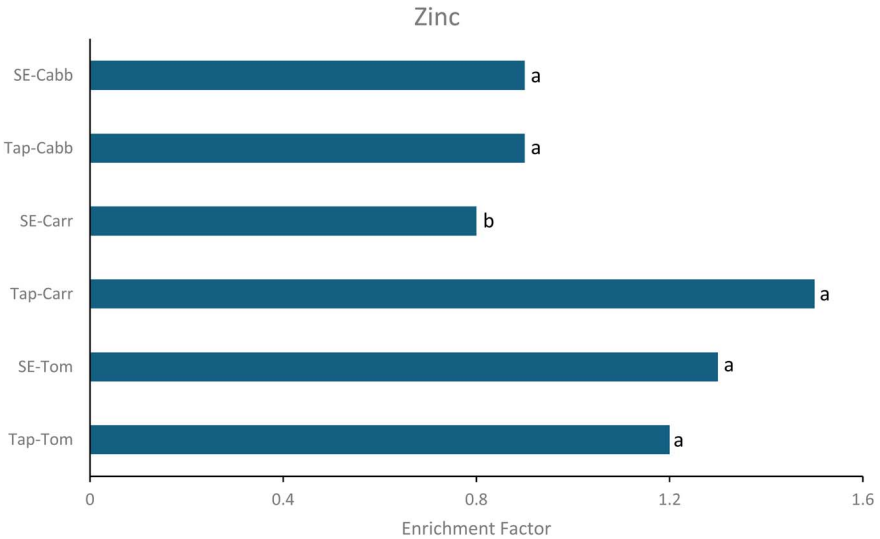

Fig. 1 Accumulation of zinc in the tap water and TWW irrigated soils at the end of the irrigation exercise. Significant accumulation was observed for Tap-Tom, Tap-Carr and SE-Tom irrigated soils (Student's *t*-test; $p < 0.05$). Different alphabets represent significant differences.

3.3 Health risk exposure assessment

The result of the non-carcinogenic health risk is presented under two scenarios: (1) risk *via* ingestion of raw or uncooked vegetables/crops (Table 4, a) and (2) risk *via* ingestion of cooked vegetables/crops (Table 4, b). Under scenario 1, the hazard quotient (HQ) of Cu in adults ranged from 0.25 to 0.47 for tap-water irrigated crops and 0.19 to 0.39 for TWW-irrigated crops. In children, the HQ for tap-water irrigated crops ranged from 1.20 to 2.92 and 0.90 to 1.83 for TWW irrigated crops (Table 4, a). The latter had a lower hazard quotient than tap-water irrigated crops. A similar trend was observed for Zn. The HQ of Zn in adults ranged from 0.10 to 0.48 and 0.08 to 0.45 for crops irrigated with tap water and TWW, respectively. In children, the HQ ranged from 0.51 to 2.24 for tap water irrigated crops and 0.39 to 2.10 for TWW irrigated crops. Among the TWW irrigated crops, tomatoes recorded the highest quotient in both adults and children for Cu and Zn. In tap water and TWW irrigated crops, the HQ for children was higher than for adults, which is consistent with existing literature.[14,16] Rezapour *et al.* reported HQ values of 0.060 (Zn) and 0.168 (Cu) for adults and 0.132 (Zn) and 0.375 (Cu) for children for wheat grains produced by TWW irrigation.[16]

The outcome of the assessment of the potential risk posed by a single contaminant suggested that the consumption of the crops irrigated by the TWW does not raise health concerns in adults. The hazard quotient was significantly lower than the threshold limit (1). Adults consuming tomatoes, cabbage and carrots are at no risk of Cu or Zn toxicity.[17] However, in children, there is a potential health risk with the consumption of tomatoes. For Cu and Zn, the quotient was greater than 1, implying a health risk concern in children. The study highlights the disparity in the risk of exposure between adults and children to crops irrigated by TWW. Differences in the consumption rate and body weight account for this disparity. A significant outcome of the study is the need to always evaluate the risk of exposure of consumers to agricultural products produced under TWW irrigation by considering the age groups of consumers (adults and children) since their susceptibility to the perceived risk is different.

Assessment of the overall risk of exposure (hazard index) to Cu and Zn through the ingestion pathway revealed no risk of non-carcinogenic health effects (HI < 1) in adults. A contrary outcome was obtained for children. In all the treatments, the health hazard index for children was greater than 1, implying concern for a potential health risk. Therefore, the consumption of the crops irrigated (raw or uncooked) in this study is only safe for adult consumption. It is safer to consume the carrots and cabbage than the tomatoes. The order of risk in adults and children among the different crops was the same: Tap-Tom > SE-Tom > Tap-Cabb > Tap-Carr > SE-Carr > SE-Cabb.

Under scenario 2, the trend of the hazard quotient among the different treatments was very similar to that of scenario 1. The hazard quotient of crops irrigated with tap water ranged from 0.42 to 1.16 for Cu in adults and 2.00 to 5.41 for Cu in children (Table 4, b). In TWW irrigated crops, the HQ of Cu ranged from 0.32 to 0.41 in adults and 1.50 to 1.91 in children (Table 4, b). The HQ of Zn in adults was below 1.0 for all crops under tap and TWW irrigation. Similar results have been published by Kim *et al.* and Rezapour *et al.*[16,48] Unlike Cu, the HQs of Zn for Tap-Cabb and SE-Cabb in children were below 1.0 (0.94 and 0.72, respectively), while the rest were above the 1.0 safe limit. The results of the assessment of the potential risk posed by a single contaminant indicated that it is safe for adults to consume the edible parts (cooked) of the crops irrigated in this study. However, the consumption of the crops by children is unsafe.

Evaluation of the combined risk of Cu and Zn revealed that the HI of all the treatments for adults was below the threshold limit of 1, except for Tap-Cabb. In contrast, none of the irrigated crops fulfilled the <1 HI safe limit in the case of children. The consumption of the cooked form of all the TWW irrigated crops by adults poses no threat of Cu or Zn toxicity or health risk.[17,35]

Table 4 Hazard quotient and hazard index of oral consumption of raw/uncooked and cooked vegetables by adults and children

Element	Tap-Tom	Tap-Carr	Tap-Cabb	SE-Tom	SE-Carr	SE-Cabb
(a) Raw vegetables						
As	HQ	HQ	HQ	HQ	HQ	HQ
Adult	nd	nd	nd	nd	nd	nd
Child	nd	nd	nd	nd	nd	nd
Cd	HQ	HQ	HQ	HQ	HQ	HQ
Adult	nd	nd	nd	nd	nd	nd
Child	nd	nd	nd	nd	nd	nd
Cu	HQ	HQ	HQ	HQ	HQ	HQ
Adult	0.47143	0.25714	0.62679	0.39286	0.19286	0.19286
Child	2.20000	1.20000	2.92500	1.83333	0.90000	0.90000
Pb	HQ	HQ	HQ	HQ	HQ	HQ
Adult	nd	nd	nd	nd	nd	nd
Child	nd	nd	nd	nd	nd	nd
Zn	HQ	HQ	HQ	HQ	HQ	HQ
Adult	0.48190	0.17143	0.10929	0.45048	0.14143	0.08357
Child	2.24889	0.80000	0.51000	2.10222	0.66000	0.39000
Exposure	**HI**	**HI**	**HI**	**HI**	**HI**	**HI**
Adult	0.95333	0.42857	0.73607	0.84333	0.33429	0.27643
Child	4.44889	2.00000	3.43500	3.93556	1.56000	1.29000
(b) Cooked vegetables						
As	HQ	HQ	HQ	HQ	HQ	HQ
Adult	nd	nd	nd	nd	nd	nd
Child	nd	nd	nd	nd	nd	nd
Cd	HQ	HQ	HQ	HQ	HQ	HQ
Adult	nd	nd	nd	nd	nd	nd
Child	nd	nd	nd	nd	nd	nd
Cu	HQ	HQ	HQ	HQ	HQ	HQ
Adult	0.49286	0.42857	1.16071	0.41071	0.32143	0.35714
Child	2.30000	2.00000	5.41667	1.91667	1.50000	1.66667
Pb	HQ	HQ	HQ	HQ	HQ	HQ
Adult	nd	nd	nd	nd	nd	nd
Child	nd	nd	nd	nd	nd	nd
Zn	HQ	HQ	HQ	HQ	HQ	HQ
Adult	0.50381	0.28571	0.20238	0.47095	0.23571	0.15476
Child	2.35111	1.33333	0.94444	2.19778	1.10000	0.72222
Exposure	**HI**	**HI**	**HI**	**HI**	**HI**	**HI**
Adult	0.99667	0.71429	1.36310	0.88167	0.55714	0.51190
Child	4.65111	3.33333	6.36111	4.11444	2.60000	2.38889

Our results in both scenarios imply that the consumption of raw and cooked tomatoes, carrots and cabbage from the current study poses no non-carcinogenic health risk for adults but is unsafe for children. Comparatively, the HQ and HI of consuming raw tomatoes, carrots and cabbage were lower than those of consuming the cooked counterpart due to the relatively high consumption rate associated with cooked food. These findings are crucial and beneficial as the European Union directives on TWW for irrigation came into effect this year. Member countries can utilize this study's outcome in assessing the suitability of TWW irrigation from a health risk standpoint. Since irrigating crops with TWW is a global phenomenon, the outcome of our study could be replicated in other regions of the globe outside the European Union.

3.4 Risk of exposure to pathogens

The evaluation of the risk of exposure to pathogens through ingestion of tomato fruits revealed no potential risk to consumers, even though the level of microbial loads of the treated effluent was far above the safety limits stipulated in the EU and WHO directives and guidelines. In Table 5 and Fig. 2, none of the targeted indicator microorganisms were detected on the tomato fruits. This observation is similar to the findings of similar studies reported in the literature.[21–23] The type of irrigation system employed in the current study may account for this outcome. The tomato plants were irrigated directly at the base of the plant (analogous to drip irrigation), so there was no direct contact of the aboveground part of the plant with the irrigation water. Since there was no direct contact of the fruits with the water, the transfer of pathogens from the irrigation water to the fruits did not occur. This provides significant knowledge in understanding the pathogenic contamination risk associated with TWW irrigation and elucidates the non-spontaneous microbial contamination risk associated with consuming TWW irrigated crops. The results indicate the possibility of eliminating microbial contamination of TWW irrigated crops by using appropriate irrigation methods. Gatta *et al.* reported the absence of *E. coli* and *Salmonella* spp. on artichoke heads after irrigation with TWW. The authors partly attributed their findings to the drip irrigation system used which prevented fruits or plants from having direct contact with irrigation water.[21]

The observed outcome in the present study would have been different if an irrigation method that allows direct contact of fruits with irrigation water had been employed. For instance, in the case of the sprinkler irrigation system, there could have been a possible transfer of pathogens from the treated effluent or wastewater to the tomato fruits since the water could have had direct contact with the tomato fruits. This would have created a risk of pathogen exposure through the ingestion pathway. The assertion is corroborated by existing literature on the microbial risk of TWW irrigation. Makkaew *et al.* assessed the risk of *E. coli* contamination in TWW irrigated lettuce under different irrigation configurations and found that the spray type of irrigation system led to contamination of lettuce with *E. coli*, while the drip type of irrigation did not.[60]

The high levels of microbial loads in the treated effluent or wastewater (Table 3) pose potential health risks to farmers and farm workers through inhalation and ingestion. There is an

Table 5 Microbial water quality characteristics of water collected after rinsing the tomato fruits

	Coliform bacteria [CFU 100 mL^{-1}]	Thermotolerant coliform bacteria [CFU 100 mL^{-1}]	*Escherichia coli* [CFU 100 mL^{-1}]	*Clostridium perfringens* [CFU 100 mL^{-1}]
Tap	nd	nd	nd	nd
SE	nd	nd	nd	nd

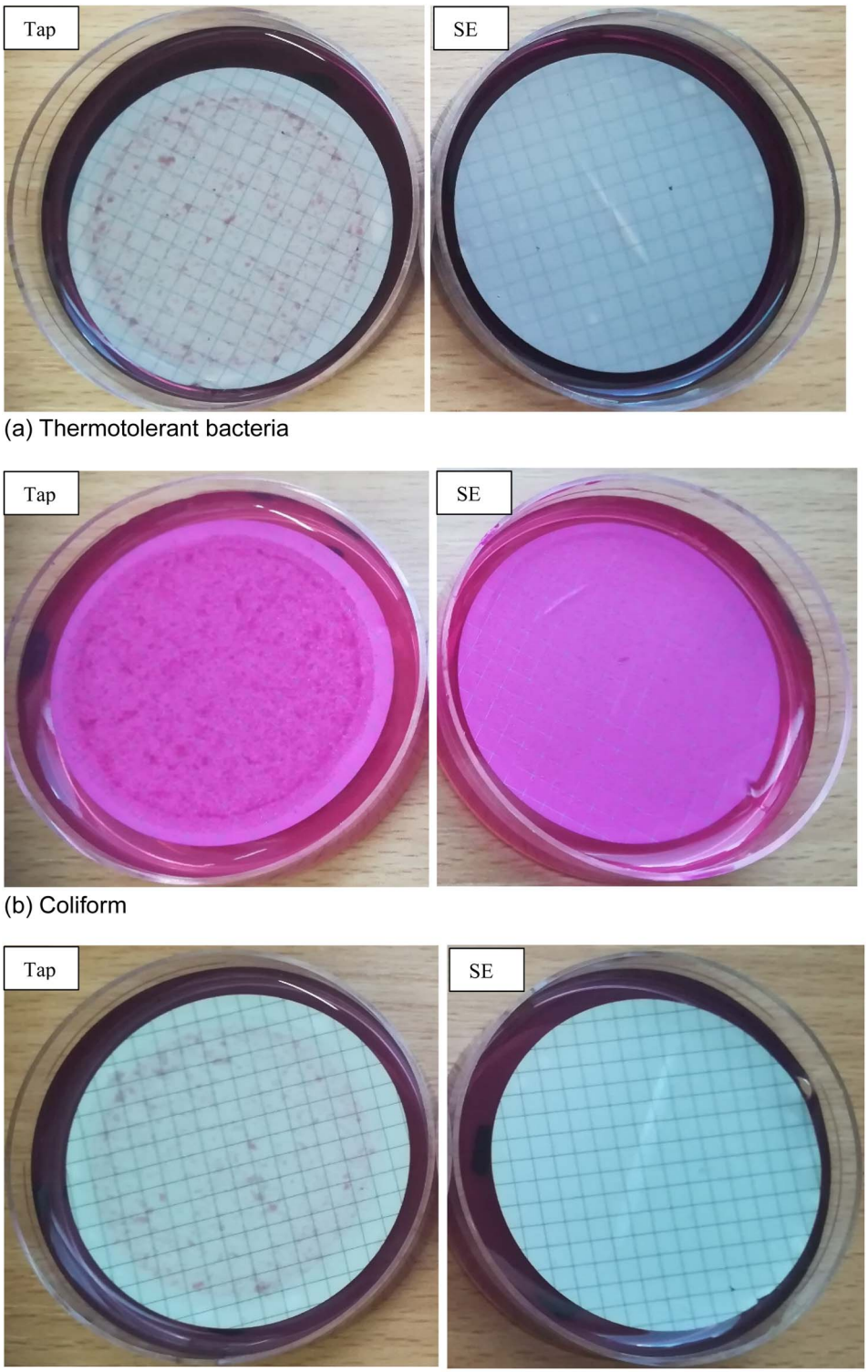

Fig. 2 Qualitative and quantitative determination of thermotolerant coliform, coliform bacteria and *C. perfringens* in water collected after being used for rinsing tomato fruits. "Tap" is tap water irrigated tomato and "SE" is TWW irrigated tomato, respectively. None of the indicator microorganisms was detected. Substances found on top of tap water irrigated tomato filter paper are tomato fruit particles.

occupational exposure pathway since these persons may have direct contact with the irrigation water.[61] The microbiological quality of the treated effluent does not fulfil the threshold limits established by EU Regulation 2020/741 and WHO guidelines; therefore, farmers and farm workers working with such irrigation water need to wear appropriate protective gear such as nose

masks and gloves to prevent direct contact and transfer of pathogens from the irrigation water.[62,63] Considering that the pathogen loads are in the order of 10^3 and greater, the TWW must be disinfected to reduce the microbial content to an acceptable or safe limit. The application of disinfection processes like ozonation and UV disinfection is efficient in reducing the microbial load to safe limits.[9,42] Even though in this study the potential risk of exposure to pathogens *via* the consumption of tomato fruits did not exist, disinfection of the effluent prior to crop irrigation is strongly recommended. Also, irrigation practices that avoid direct contact of water with edible parts of crops are strongly recommended for wastewater irrigation. This is to ensure maximum consumer safety. These recommendations are not only applicable in the European Union where the study was conducted but also applicable to other geographical locations, especially arid and semi-arid regions where TWW irrigation is relatively predominant.

3.5 Uptake and bioaccumulation of micropollutants/pharmaceuticals

Table 6 presents the bioaccumulation of micropollutants/pharmaceuticals in the edible tissues of the tomato plant. No difference was observed in the amount of micropollutants bioaccumulated in the tap water irrigated tomato fruits and that of the treated effluent irrigated tomato fruits except for gabapentin. The amount of gabapentin accumulated in the latter was 3 μg kg^{-1} compared to <1.0 μg kg^{-1} of the former. This might be due to gabapentin's apparent concentration in the soil solution, which was influenced by the quality of the irrigation water, as was also reported by a previous study.[64] All the other pharmaceuticals were not quantifiable even though some were present in the TWW at relatively high levels. The concentration of naproxen, diclofenac, carbamazepine, gabapentin, tramadol, sulfamethoxazole, trimethoprim and saccharin in the effluent was 113.75 ± 18.87 ng L^{-1}, 1950 ± 100 ng L^{-1}, 460 ± 14.14 ng L^{-1}, 1900 ± 81.65 ng L^{-1}, 2625 ± 170.78 ng L^{-1}, 770 ± 112.25 ng L^{-1}, 675 ± 64.55 ng L^{-1}, and 645 ± 242.83 ng L^{-1} and that of tap water was <LOD, <LOD, 11.25 ± 2.47 ng L^{-1}, 79 ± 1.41 ng L^{-1}, 0.41 ± 0.01 ng L^{-1}, 0.39 ± 0.10 ng L^{-1}, <LOD and <LOD, respectively (Table 3). Similar concentrations of naproxen, diclofenac, carbamazepine, gabapentin and trimethoprim in treated effluent have been reported by Diaz-Sosa *et al.*[28]

Table 6 Micropollutants/pharmaceuticals in the biomass of the tomato fruits

Parameter	Tap water	Secondary effluent
Ibuprofen (μg kg^{-1})	<10.0	<10.0
Naproxen (μg kg^{-1})	<10.0	<10.0
Diclofenac (μg kg^{-1})	<10.0	<10.0
Paracetamol (μg kg^{-1})	<2.0	<2.0
Carbamazepine (μg kg^{-1})	<1.0	<1.0
Gabapentin (μg kg^{-1})	<1.0	3.0
Tramadol (μg kg^{-1})	<1.0	<1.0
Caffeine (μg kg^{-1})	<2.0	<2.0
Erythromycin (μg kg^{-1})	<10.0	<10.0
Triclosan (μg kg^{-1})	<10.0	<10.0
Sulfamethoxazole (μg kg^{-1})	<1.0	<1.0
Trimethoprim (μg kg^{-1})	<10.0	<10.0
Saccharin (μg kg^{-1})	<10.0	<10.0
Warfarin (μg kg^{-1})	<1.0	<1.0

Generally, the amount of micropollutants accumulated in the tomato fruit did not correlate with the levels in the treated effluent. The irrigation water quality did not significantly influence the concentration of micropollutants bioaccumulated in the fruits (except for gabapentin), which contrasts with findings reported in some studies. Mordechay *et al.* concluded that the plant uptake of pharmaceuticals partly depends on the concentration and occurrence of these substances in the irrigation water after the authors found that high-quality irrigation water (low concentration of micropollutants) resulted in crops containing fewer and relatively low concentrations of these pollutants.[64] The uptake of pharmaceuticals is not solely dependent on the characteristics of irrigation water but also on environmental factors, plant physiology, nutrients, and soil properties.[64,65] This suggests that other factors may have critically affected the uptake of pharmaceuticals in our study, rather than the quality of the irrigation water. The high organic matter content of the soil (5.6 ± 0.08%) may have immobilized the micropollutants, thereby affecting their uptake by the tomato plants. This is achieved by controlling the bioavailability of the pharmaceuticals by strongly binding them to the soil particles and reducing their uptake potential by the roots of the tomato plants.[66,67] Existing studies have shown the contribution of soil organic matter in facilitating the lower uptake of these substances by plants.[64,68] Generally, significant quantities of micropollutants have been found in the biomass of crops grown on soil containing lower organic matter than soils having higher organic matter. The significance of the results is the support of the assertion that the bioavailability and bioaccumulation of micropollutants under TWW irrigation are not exclusively dependent on the irrigation water characteristics. Therefore, the evaluation of health risks arising from micropollutant accumulation should not be limited to the pharmaceutical characteristics of the TWW alone, but also consider the soil characteristics, since the soil properties strongly influence their uptake by plants.

The very low concentration of micropollutants in the fruits suggests that they may be less bioaccessible to the tomato fruits. As already mentioned, plant physiology also plays an important role in the uptake and translocation of pollutants. The aboveground parts of plants have a lower accumulation tendency compared to the roots, possibly due to low translocation potential. Among the aboveground parts, higher bioaccumulation or distribution occurs in the leaves than in the fruits due to translocation by the transpiration stream.[67,69,70] Stomata, which play an important role in transpiration, are absent in the tomato fruit. Therefore, the fruits were unable to transpire water through the stomata to create a driving potential for water and solutes (pharmaceuticals) to flow into them in significant quantities.[67] Our assertions are supported by previous studies, which found that fruits with stomata (cucumber) accumulated more micropollutants than fruits without stomata (tomato) and had higher levels of bioaccumulation in stems and leaves than the fruits.[67,71] Since the objective of the study focuses on only the edible parts of crops, no evaluation of

bioaccumulation of micropollutants in other parts of the tomato plants (roots, stems, leaves, and flowers) or soil was done.

No major or significant difference was observed in the risk of exposure to pharmaceuticals in consuming tomatoes irrigated by the two irrigation water streams. This result is similar to the uptake and bioaccumulation of heavy metals or PTEs in the tomato fruits as noted in the previous section. We are of the view that the bioavailability, uptake and bioaccumulation of these pollutants are strongly influenced by the soil properties, plant physiology and physicochemical properties of the micropollutants rather than the quality of the irrigation water. Therefore, in any TWW irrigation project, the effort to eliminate or reduce the risk of consumers being exposed to micropollutants should not only focus on the water quality but also on soil properties that could enhance the uptake of micropollutants. Such an approach is applicable on a global scale and not limited to a particular region.

3.6 Risk of exposure to ARGs

Zhang *et al.* ascribe that edible parts of plants represent an important means through which resistance genes are spread to humans.[72] To evaluate the direct risk of ARG exposure *via* food ingestion, we performed PCR analyses of the DNA that was extracted from the edible part of the cabbage plant (leaves). Although the extracted DNA samples did not all test positive for all the ARGs tested, they all tested positive for the amplification of the 16S rRNA gene target. The PCR results revealed that five of the target genes (*ermB*, *sul2*, bla_{TEM}, *tetA* and *qnrS*) were present in both the tap water irrigated cabbage and TWW irrigated cabbage (Table 7, Appendix). These genes were ubiquitous in the tap water, the treated effluent and the soil except *ermB,* which was absent in tap water and soil.

Two (2) out of the nine (9) targeted ARGs were not detected in any of the sample matrices (irrigation water, soil, and crops/plant biomass) and these were *tetW* and *blaZ*. The *ermB*, bla_{TEM} and *tetA* genes were present in all the treated effluent samples (5 samples), *sul1* and *sul2* were detected in four (4) samples, and *qnrS* in three (3) of the effluent samples. The distribution and abundance of most of these ARGs in TWW have already been reported in the literature. Marano *et al.* documented the presence of bla_{TEM} and *qnrS* together with other ARGs in TWW effluent from different WWTPs in Israel.[6] Teixeira *et al.* have also reported the detection of *sul1* and *ermB* in the effluent of five WWTPs across Europe.[29] However, a different distribution of ARGs was observed for the tap water. *sul2* was ubiquitous in all the tap water samples (3 samples) while *tetA* and *qnrS* were detected in two of the samples. bla_{TEM} was present in only one sample of the tap water while the other remaining ARGs were absent. The low prevalence of the target ARGs in tap water compared to the TWW is due to the highly efficient and rigorous treatment processes employed in the production of tap water due to public safety.

The soil environment harbours a large number of antibiotics and ARGs from irrigation water. Under the pressure of antibiotic selection, the prevalence of ARB could occur making the soil environment a repository of ARGs.[73] However, in this study, a contrary observation was made regarding the irrigated soils. Except for *sul2* which was present in TWW irrigated soil, all the remaining targeted ARGs were not detected in both tap water and TWW irrigated soils. However, this finding could also be consistent with the fact that the relative amount of DNA carrying ARG genes can be underrepresented due to soil biodiversity and fall below the detection limit of the used technique. Indeed, studies have shown that a change in the microbial community can affect the distribution and abundance and, therefore, detection of ARGs in soil.[72,74]

In contrast, the results suggest a bioaccumulation effect of ARGs in the cabbage leaves that might originate from the irrigation water and soil. In a similar study, Cerqueira *et al.* reported the presence of bla_{TEM} and *qnrS1* genes in lettuce irrigated with water comprising 90% TWW. They asserted that the soil was the main driver for ARG uptake into the lettuce and the quality of the irrigation water played a limited role.[32] The presence of bla_{TEM}, *tetA* and *qnrS* in the cabbage plant could be attributed to the irrigation water and the soil media since these ARGs were present in the soil prior to the irrigation exercise and were present in the irrigation water too. The *sul2* gene was not initially detected in the soil prior to the irrigation exercise but was present in the tap water and TWW; therefore, the irrigation water is ascribed as the source of *sul2* in the cabbage. The *ermB* gene was absent in the soil matrix and tap water but present in the TWW indicating that its presence in the effluent irrigated cabbage was probably due to the irrigation. The presence of *ermB* in the tap water irrigated cabbage is suspected to have been caused by contamination during sample preparation since the gene was not found in the soil or the tap water and is also xenobiotic to the cabbage plant. Qualitatively, no difference was observed in the bioaccumulation of the different ARGs in the tap-water irrigated cabbage and TWW-irrigated cabbage. In both cases, there was uptake of ARGs from the soil and irrigation water into the phyllosphere of the cabbage. ARGs may have accumulated in the rhizosphere soil after irrigation and then transferred to the root system where they were uptake and migrated to the leaves of the cabbage.[72] In the leaf zone, endophytes may serve as hosts for the ARGs and subsequently confer resistance creating a potential risk of antibiotic resistance dissemination. Theoretically, the consumption of this cabbage has the potential for ARG dissemination to humans since the genes could be hosted by pathogenic bacteria and confer resistance in infective human diseases. The studies of Duan *et al.* and Song *et al.* have identified that human pathogenic bacteria can serve as hosts for ARGs.[75,76] The potential risk of ARG dissemination or exposure in the present study is associated with the use of not only the TWW/effluent but also the tap water. The abundance of the targeted ARGs in the different sample matrices is outside the scope of this study and therefore not discussed. The results indicate that both tap water and TWW contain ARGs and that soil seems to possess the capability of making their abundance underrepresented and thus not detectable (Table 7, Appendix A). In contrast, plant biomass (possibly the plant microbiome) acts as an ARG bioaccumulator (Table 7, Appendix). To further elucidate this key aspect, additional quantitative studies are needed.

4 Conclusion

The study investigated and evaluated the health risks associated with the use of TWW for crop irrigation using tomatoes, carrots

and cabbage as test crops. The comprehensive assessment of the potential risk arising from pathogens, heavy metals/PTEs, micropollutants or pharmaceuticals and ARGs in the irrigation water led to the following findings:

(i) The use of the TWW did not result in the contamination of tomato fruits with pathogens.

(ii) Bioaccumulation and bioaccessibility of As, Cd and Pb in the test crops were insignificant.

(iii) Health quotient and health hazard assessment of Cu and Zn indicated that the irrigated crops are safe for adult consumption but may be unsafe for children.

(iv) Except for gabapentin (3 μg kg^{-1}), the levels of bioaccumulated pharmaceuticals in the irrigated crops were below the detection limit.

(v)*tetA*, *ermB*, bla_{TEM}, *sul2*, *sul3*, and *qnrS* were taken up and accumulated in TWW-irrigated cabbage plants as well as the control plants (tap water-irrigated cabbage). No difference was observed in the presence and distribution of the ARGs between the TWW and the tap water-irrigated cabbage biomasses.

The results of the study suggest that TWW could be a suitable source of water for irrigation; however, risk management strategies should be implemented to protect consumers and the environment. The health risk associated with the use of treated effluent or TWW for crop irrigation is pollutant specific and therefore to ensure safe use of TWW, different treatment processes aimed at removing the different contaminants should be employed to protect public health. The focus should not be solely dependent on the quality of the TWW but also on other factors such as soil characteristics which may contribute to the risk of exposure.

Data availability

The data supporting this article have been included in the manuscript. Any additional data will be submitted upon request.

Author contributions

Solomon Ofori: conceptualization, methodology, investigation, formal analysis, writing – original draft, and writing – review and editing. Ylenia Di Leto: methodology, formal analysis, and writing – review and editing. Štěpánka Smrčková: methodology, formal analysis, and writing – review and editing. Marco Antonio Lopez Marin: methodology, formal analysis, visualization, and writing – review and editing. Giuseppe Gallo: methodology, writing – review and editing, supervision, and resources. Iveta Růžičková: methodology, writing – review and editing, supervision, resources, and funding acquisition. Jiří Wanner: methodology, writing – review and editing, supervision, resources, and funding acquisition.

Conflicts of interest

There are no conflicts of interest to declare.

Appendix

Table 7 Positive hits of targeted ARGs in water, soil and plant biomass samples[a]

	Erythromycin	Sulfonamide			Beta-lactamase		Tetracycline		
Sample description	*erm(B)*	*sul1*	*sul2*	*sul3*	bla_{TEM}	*blaZ*	*tet(A)*	*tet(W)*	*qnrS*
Biomass_tap	x		x	x	x		x		x
Biomass_SE	x		x	x	x		x		x
Soil_tap 1									
Soil_tap 2									
Soil_tap 3									
Soil_tap 4									
Soil_SE 1									
Soil_SE 2			x						
Soil_SE 3									
Soil_SE 4			x						
Soil_BF					x		x		x
Tap water 1			x		x		x		x
Tap water 2			x				x		x
Tap water 3			x						
Secondary effluent 1	x	x			x		x		x
Secondary effluent 2	x	x	x		x		x		x
Secondary effluent 3	x	x	x		x		x		
Secondary effluent 4	x		x		x		x		x
Secondary effluent 5	x	x	x		x		x		

[a] Biomass_Tap: tap water irrigated cabbage; Biomass_SE: secondary effluent irrigated cabbage; Soil_Tap (1, 2, 3, and 4): tap water irrigated soil samples; Soil_SE (1, 2, 3, and 4): secondary effluent irrigated soil samples; Soil_BF: soil sample taken before the start of the irrigation exercise; Tap water (1, 2, and 3): samples of tap water used for irrigating the cabbage crops; Secondary effluent (1, 2, and 3): samples of secondary effluent used for irrigating the cabbage crops.

Table 8 QQQ parameters

Compounds name	Precursor ion	Product ion	Fragmentor voltage	Collision energy	Accelerator voltage	Ret. time	Polarity
2-Hydroxy-IB	221.1	159.1	78	16	4	6.77	Negative
2-Hydroxy-IB	221.1	177	78	4	4	6.77	Negative
3-Hydroxy-CBZ	253.1	209.9	113	16	4	7.15	Positive
3-Hydroxy-CBZ	253.1	207.9	113	20	4	7.15	Positive
Paracetamol	152	110.1	122	16	4	3.91	Positive
Paracetamol	152	93	122	24	4	3.91	Positive
Paracetamol IS	156	114	122	16	4	3.89	Positive
Paracetamol IS	156	97	122	24	4	3.89	Positive
ACS-K	162	82	89	12	4	2.26	Negative
ACS-K	162	78	89	40	4	2.26	Negative
Aspartame	295.1	235	129	8	4	5.95	Positive
Aspartame	295.1	179.9	129	8	4	5.95	Positive
Caffeine	195.5	138.1	209	20	4	5.17	Positive
Caffeine	195.5	110	209	24	4	5.17	Positive
Caffeine IS	198	140.1	209	24	4	5.17	Positive
Caffeine IS	198	112	209	28	4	5.17	Positive
Carbamazepine	237	194.1	143	16	4	7.76	Positive
Carbamazepine	237	193.1	143	36	4	7.76	Positive
Carbamazepine IS	243	200	143	20	4	7.76	Positive
Carbamazepine IS	243	199	143	36	4	7.76	Positive
CBZ-epo	253	210.1	107	8	4	6.92	Positive
CBZ-epo	253	180.1	107	28	4	6.92	Positive
Chloramphenicol	325	277	107	16	4	6.48	Positive
Chloramphenicol	325	275	107	16	4	6.48	Positive
Diclofenac	296	250	130	12	4	8.34	Positive
Diclofenac	296	213	130	36	4	8.34	Positive
Diclofenac IS	302	256	120	8	4	8.34	Positive
Diclofenac IS	302	220	120	40	4	8.34	Positive
E1	269.1	145	134	36	4	8.64	Negative
E1	269.1	143	134	60	4	8.64	Negative
E1 IS	272.1	148	134	44	4	8.64	Negative
E1 IS	272.1	146	134	68	4	8.64	Negative
E3	287.17	171	167	40	4	7.27	Negative
E3	287.17	145	167	44	4	7.27	Negative
E3 IS	290.15	174	167	40	4	7.27	Negative
E3 IS	290.15	148	167	44	4	7.27	Negative
EE2	295.2	269	194	32	4	8.62	Negative
EE2	295.2	145	194	56	4	8.62	Negative
EE2 IS	297.2	269	194	24	4	8.62	Negative
EE2 IS	297.2	145	194	44	4	8.62	Negative
Fluoxetine	310.1	148.1	101	0	4	7.94	Positive
Fluoxetine	310.1	44	101	8	4	7.94	Positive
Gabapentin	172.1	154	101	12	4	4.11	Positive
Gabapentin	172.1	137	101	16	4	4.11	Positive
Gemfibrozil	249.2	127	98	8	4	9.43	Negative
Gemfibrozil	249.2	121	98	12	4	9.43	Negative
Ibuprofen	205.12	161.1	83	0	4	8.79	Negative
Ibuprofen	205.1	159.1	83	0	4	8.79	Negative
Ibuprofen IS	211.1	167.1	83	0	4	8.79	Negative
Naproxen	229.1	185.1	90	4	4	7.59	Negative
Naproxen	229.1	170,7	90	28	4	7.59	Negative
NHDC	611.2	303	200	36	4	7.18	Negative
NHDC	611.2	125	200	56	4	7.18	Negative
Neotame	379.2	172.1	140	20	4	8.41	Positive
Neotame	379.2	85.2	140	40	4	8.41	Positive
Nimesulide	307	229.05	134	12	4	8.15	Negative
Nimesulide	307	122	134	44	4	8.15	Negative
Saccharin	182	106	149	16	4	3.55	Negative
Saccharin	182	62	149	32	4	3.55	Negative
Salicylic acid	137	93	83	16	4	4.10	Negative
Salicylic acid	137	65	83	36	4	4.10	Negative
Salicylic acid IS	143	99	83	16	4	4.10	Negative

Table 8 (*Contd.*)

Compounds name	Precursor ion	Product ion	Fragmentor voltage	Collision energy	Accelerator voltage	Ret. time	Polarity
Salicylic acid IS	143	71	83	36	4	4.10	Negative
Sucralose	397	361	161	8	4	5.61	Negative
Sucralose	397	359	161	8	4	5.61	Negative
Sulfamethoxazole	254	156	113	12	4	5.31	Positive
Sulfamethoxazole	254	92	113	24	4	5.31	Positive
Sulfamethoxazole IS	260.1	98.1	110	28	4	5.31	Positive
Sulfamethoxazole IS	260.1	70.1	110	56	4	5.31	Positive
Testosterone	289.2	108.9	134	24	4	8.80	Positive
Testosterone	289.2	97	134	32	4	8.80	Positive
Tramadol	264.2	58.1	110	12	4	5.71	Positive
Tramadol	364.2	30.1	110	64	4	5.71	Positive
Trimethoprim	291	261	170	24	4	5.26	Positive
Trimethoprim	291	230	170	20	4	5.26	Positive
Warfarin	309.1	251	107	16	4	7.81	Positive
Warfarin	309.1	163	107	8	4	7.81	Positive

2-Hydroxy-IB = 2-hydroxyibuprofen, 3-hydroxy-CBZ = 3-hydroxycarbamazepine, 17β-E2 = 17β-estradiol, ACS-K = acesulfame potassium, CBZ-epo = carbamazepine-10,11-epoxide, E1 = estron, E3 = estriol, and EE2 = 17α-ethinylestradiol.

Acknowledgements

This work was funded by the Horizon 2020 project: Achieving wider uptake of water-smart solutions (H2020-SC5-2019-2) (Grant Agreement ID: 869283) and supported by the University of Chemistry and Technology Internal Grant, grant number: 217 88 2205. This research was also supported by: the European Commission – NextGenerationEU, Project SUS-MIRRI.IT "Strengthening the MIRRI Italian Research Infrastructure for Sustainable Bioscience and Bioeconomy", code no. IR0000005PO, CUP D13C22001390001; the European Commission – NextGenerationEU, National Recovery and Resilience Plan (NRRP), Mission 4, Component 2, Investment 1.4 "Strengthening of research structures and creation of R&D 'national champions' on some Key Enabling Technologies"- Call for tender no. 3138 of 16 December 2021, rectified by Decree no. 3175 of 18 December 2021 of Italian Ministry of University – CN5 "National Biodiversity Future Center", NBFC – code no. CN00000033, CUP B73C22000790001. The support of the Prague Wastewater Treatment Company (PVS and PVK) is highly acknowledged.

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