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Integrated assessment of biostimulants with water and fertilizer saving techniques: implications for environmental contamination and health hazard in tomato crops across Europe

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Abstract

Background Tomato (*Solanum lycopersicum* L.) production faces increasing challenges related to water scarcity, intensive input use, and environmental contamination. In this context, biostimulants combined with water- and fertilizer-saving strategies have been proposed to enhance crop performance while reducing environmental impacts. However, their integrated effects on soil contamination and potential health hazards remain insufficiently understood. This study, conducted within the Horizon 2020 TOMRES project, provides a multidisciplinary evaluation of these aspects across different European agroecosystems.

Results Results showed that pseudo-total potentially toxic elements (PTE) concentrations varied among sites, occasionally exceeding reference thresholds. However, bioaccessible PTEs generally remained low and below toxicity levels. Biostimulant application and reduced water and nutrient inputs had limited effects on PTE bioaccessibility, although localized increases were observed in soils with higher baseline contamination. Microbiological analyses revealed high variability across sites, with total plate counts (TPCs) influenced more by environmental conditions than by agronomic treatments. Faecal indicators were generally low, and *Salmonella* spp. was detected only by molecular methods, without clear association with other microbial parameters. Ecotoxicological assays indicated low to moderate toxicity overall, with algae showing the highest sensitivity; biostimulants occasionally mitigated toxic effects, particularly under well-watered conditions. Multivariate analysis confirmed that site-specific factors, including soil properties and baseline conditions, were the primary determinants of chemical, microbiological, and ecotoxicological variability, exceeding the influence of agronomic treatments.

Conclusions Despite some methodological limitations inherent in this multidisciplinary study, the results suggest that the integration of biostimulants with water- and fertilizer-saving practices did not result in significant environmental or health hazards under the studied conditions. However, the outcomes highlight the importance of site-specific assessments, particularly in soils with elevated contaminant levels. These findings support the potential of sustainable agronomic strategies in tomato production while emphasizing the need for context-dependent management and further research on long-term impacts and food safety implications. Future research on this topic should be expanded by developing integrated risk assessment frameworks that combine chemical, microbiological, and ecotoxicological factors to support sustainable agriculture and protect public health.

Keywords Tomato cultivation, Public health, Biostimulants, Potential toxic elements, Agronomic techniques, Microbiological contamination, Ecotoxicity, Water scarcity

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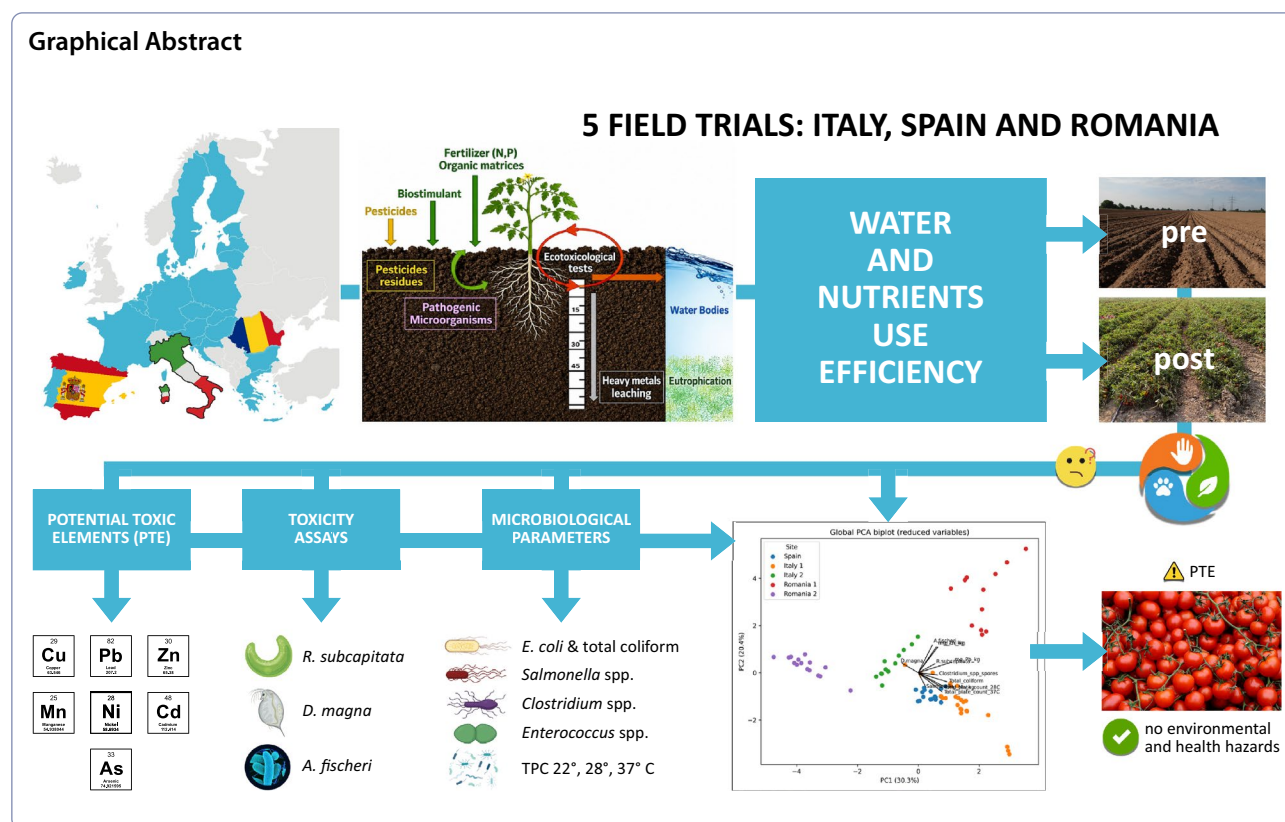
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Graphical Abstract



Background

Tomato (*Solanum lycopersicum* L., 1753) is among the most widely cultivated crops globally and represents a key agricultural product in the European Union, grown both in open fields and greenhouses. It plays an important role in global food security and trade, being consumed both fresh and as a raw material for processed products such as sauces, purees, and canned goods. Tomatoes are rich in vitamins (A, C, and E), essential micronutrients, and bioactive compounds such as lycopene, which contribute to their nutritional and health-promoting value [1, 2]. Globally, fresh tomato production reached 188.5 million tons in 2024 [3]. The main tomato producers in the world are China (61.7 million tons), India (21.3 million tons), Turkey (14.6), USA (10.7), Egypt (7.5) and Italy, with an annual output of 6.0 million tons. In Europe, tomato production reached 22.5 million tons [3]. The sustainability of tomato production is increasingly challenged by the dual pressures of climate change and resource depletion. Agriculture consumes approximately 70% of global freshwater and contributes significantly to soil degradation due to agricultural pollutants (i.e. pesticides, fertilizers, heavy metals) and nutrient loss [4, 5]. The water footprint applied to tomato production systems has variable impacts based on the cultivation method, the area, its physical and climatic characteristics, in addition to

the local water supply situation [6–8]. Improving water-use efficiency and nutrient management is therefore crucial for the long-term sustainability of agricultural systems [9]. Excessive fertilizer use has historically sustained high crop yields, but has also caused imbalances in soil micronutrients, increased heavy metal accumulation, and nutrient leaching into water bodies, contributing to eutrophication and pollution [10]. In response, plant biostimulants have emerged as promising tools to improve nutrient uptake, plant resilience, and soil health, acting through biological mechanisms rather than direct nutrient addition [11–13]. These substances include inorganic compounds, chitosan derivatives, seaweed extracts, humic acids, proteins and protein hydrolysates, bacteria and fungi that stimulate physiological processes in plants [14, 15]. Despite their agronomic benefits, the environmental and public health implications of biostimulant use remain poorly understood. In fact, their influence on soil contaminant dynamics, microbiological safety, and potential toxic element (PTE) mobility is largely unexplored. Moreover, the combination of biostimulants with water- and fertilizer-saving techniques may introduce complex interactions that affect both productivity and environmental quality. Additionally, agricultural practices, such as using contaminated irrigation water [16], reusing wastewater [17, 18], and applying organic

fertilizers can spread pathogenic and antibiotic-resistant microorganisms, increasing the risk of faecal–oral disease transmission [19].

The present study, conducted under the Horizon 2020 TOMRES project (Grant Agreement No. 727929), investigates how integrated sustainable agronomic practices influence soil contamination and health hazard in tomato cultivation. Specifically, it evaluates the impacts of biostimulant application and optimized irrigation–fertilization regimes on PTE, microbiological contamination parameters, and ecotoxicological responses in soil samples collected in experimental sites located in Spain, Italy, and Romania. This multidisciplinary approach aims to link agronomic innovation with environmental protection and public health within a One Health framework.

Methods

The study adopted a multidisciplinary approach combining chemical, microbiological, and ecotoxicological analyses to provide complementary lines of evidence on soil quality and potential environmental and health implications.

The study was conducted over two cropping seasons (April–October). The health hazards related to the environmental pollution associated with various cultivation techniques were assessed using soil samples collected both before (*pre-cultivation*) and after (*post-cultivation*) tomato growth at each site. Comparisons were also made among *post-cultivation* samples subjected to different combinations of water and nutrient treatments.

Experimental trials

Five experimental trials were established in Spain, Italy (Italy 1, Italy 2), and Romania (Romania 1, Romania 2) using comparable cultivation techniques. The soils ranged from low-fertility arid soils in Spain to highly fertile volcanic soils in Italy and very fertile chernozemic soils in Romania. They differed primarily in texture (ranging from sandy silt to loamy). The pH was around neutrality in the Italian Andisol, whereas the other soils were alkaline. The Total Organic Carbon (TOC) content varied from 1.98 to 23.4 g kg⁻¹, but TOC/TN (Total Nitrogen) was always below 10, indicating a consistent mineralization of organic matter, except for the Romanian Mollisol, which showed a value of 14.3. Cation exchange capacity varied from 8.5 to 31.3 cmol₍₊₎ kg⁻¹. A detailed description of the sites, soil types and main soil properties is reported in Table 1.

Selected tomato genotypes were cultivated under varying water and nutrient regimes, both with and without the application of biostimulants. The description of each experimental design is reported in Table 1. Pesticides and herbicides were applied following crop needs (Table 1).

Experimental design and site-specific treatments

The experimental conditions adopted at each site were defined on the basis of the results obtained from previous trials conducted within the TOMRES project, particularly in the framework of four complementary Work Packages, which identified the most relevant agronomic practices, stress conditions, and biostimulant strategies to be validated under field conditions.

Spain

The Spanish trial was conducted under greenhouse conditions using TOMRES (T) tomato genotypes under well-watered (WW) and combined stress (CS) conditions, with and without biostimulants (e.g., TWW, TWWB, TCS, TCSB). Two commercial biostimulants produced by Edypro (Coupé Regeneración and Procuaje Radicular) were applied in combination via irrigation or fertigation. Coupé Regeneración, based on amino acids derived from enzymatic hydrolysis and fermentation processes, was applied at transplanting (80 L ha⁻¹) and after 14 days, whereas Procuaje Radicular, containing micronutrients and fertilization precursors, was applied at the onset of flowering (15 L ha⁻¹) and weekly thereafter. No pesticides were applied during the experimental period.

Italy

The Italian trials (Italy 1 and Italy 2) were carried out under open-field conditions. In Italy 1, a factorial design combined TOMRES genotypes (T) with optimal (WW) and suboptimal (CS) fertigation regimes and biostimulant treatments (e.g., TWW, TWWB1, TWWB2, TCS, TCSB1, TCSB2). Biostimulants included Black-Jack+HP400 (B1), applied at 2 L ha⁻¹ and 100 g ha⁻¹ respectively, and Redcal (B2, 4 L ha⁻¹), applied as foliar sprays every 15 days starting at 33 days after transplant.

In Italy 2, a different biostimulant (B3, Strigolab, Torino, Italy) was applied as foliar treatment. The product was prepared by dilution in water and applied by spraying 15–20 mL per plant at two growth stages (seedling stage and flowering stage). This treatment was associated with earlier fruit ripening and potential yield improvements under both optimal (WW) and reduced (CS) fertigation conditions.

Romania

Two trials (Romania 1 and Romania 2) were conducted under greenhouse and open-field conditions using commercial tomato varieties (C). Treatments included combined stress conditions (CCS), with and without biostimulants (e.g., CCSA, CCSS, CCSTh). Biostimulants included *Trichoderma harzianum* T22 (Th), *Azotobacter vinelandii* (A), and shungite (S).

Table 1 Experimental trials locations, soil classification and properties, such as texture, pH, total organic carbon (TOC), total nitrogen (TN), and cation exchange capacity (CEC). Agronomic practices include the tomato genotype selection and application of water/nutrient regimen, pesticides and herbicides and biostimulants

Site	Soil classification and properties					Genotypes	Water/nutrient regimen	Pesticides and herbicides	Biostimulant
	Soil type	Texture	pH	TOC g kg ⁻¹	TN g kg ⁻¹	CEC cmol ₍₊₎ kg ⁻¹			
Valencia (Spain) 36°53'22.6"N 2°22'25.9"W Greenhouse 1300 m ²	Fluvaquent	Sandy loam	8.6	1.98	0.20	8.5	WW = 100% CS = 70%	Not used	-B, PROCUAJE RADICU-LAR + (liquid mixture of micronutrients, Boron, total and water soluble, Molibdenum total and water soluble, Zinc total and water soluble) COUPÉ REGENERACIÓN (free amino acid + total nitrogen + nitric nitrogen + organic nitrogen + ammoniacal nitrogen) (Edypro)
	Andisol	Sandy silt	6.5	14.8	1.70	31.3	-Commercial variety (scion) + rootstock TOM-RES BIL6191 (T)	Metaxyl, Cymoxanil, Chlorantraniliprole, Emamectin Benzoate, Enalaxil, Cipermetrina, Abamectina, Indoxacarb, Dimetomorf, Foseetil aluminium, Fluzinam, Lambda-cialotrina, Hexythiazox, Azoxystrobin, Boscalid, Pyraclostrobin	B1, a soil drench treatment with BLACKJACK® (Sipcam) with HP 400 (Sipcam) -B2 an acidified calcium-based product + mannitol REDCAL (Sipcam)- -B3, Strigolactones -10 mg L ⁻¹ (STRIGOLAB)
Villa Littero (Italy 2) 41°00'48.1"N 14°04'08.4"E Open field 2750 m ²	Andisol	Sandy silt	6.5	14.8	1.70	31.3	-TOMRES T149 (T) - Pietra rossa F1 (Hybrid) (C)		
	Andisol	Sandy silt	6.5	14.8	1.70	31.3			
Poiana, Constanta (Romania 1) 44°00'36"N 28°27'00"E Greenhouse 1300 m ²	Mollisol	Loamy	8.8	23.4	1.63	26.1	-Rootstock: Emperador F1 a Rijk Zwaan commercial hybrid -Scion: Abellus RZ F1 (C)	Mancozeb, Thiophant-methyl, Propamocarb hydrochloride, Mefenoxam, Deltamethrin, Alpha-cypermethrin, Abamectin	-S, treated with Shungite -A, treated with <i>Azotobacter vinelandii</i> -Th, treated with <i>Trichoderma harzianum</i> T22
	Mollisol	Loamy	7.8	9.3	0.91	19.3	-Rootstock: Emperador F1 a Rijk Zwaan commercial hybrid -Scion: Abellus RZ F1 (C)	Mancozeb, Thiophant-methyl, Propamocarb hydrochloride, Mefenoxam, Deltamethrin, Alpha-cypermethrin, Abamectin	-S, treated with Shungite -A, treated with <i>Azotobacter vinelandii</i> -Th, treated with <i>Trichoderma harzianum</i> T22

Azotobacter vinelandii ($1\text{--}2\text{ L ha}^{-1}$) is a free-living nitrogen-fixing bacterium that enhances soil nitrogen availability and microbial activity. *Trichoderma harzianum* T22 was used as a microbial biostimulant to promote plant growth under stress conditions. Shungite, a mineral amendment rich in macro- and micronutrients, was applied as granules ($300\text{--}600\text{ g m}^{-2}$) to improve soil fertility, water retention, and plant tolerance to abiotic stress. Reduced treatments involved a decrease in irrigation (-20%) and fertilization (-30%) compared to standard practices. Treatments were arranged in randomized blocks with three replicates.

Experimental design considerations and limitations

Due to the multi-site nature of the study and the need to adapt experimental conditions to local agronomic practices, the experimental design was not fully factorial or completely balanced across all sites. Consequently, treatment comparisons are primarily interpreted within each site and agronomic system, and caution is required when comparing results across different locations or management conditions. The study should therefore be considered as a multidisciplinary and comparative evaluation of agronomic strategies under real-field conditions, rather than a strictly controlled experimental design aimed at isolating individual factors.

Soil sampling and preparation

Since one of the project's main objectives was to maximise water use efficiency (WUE), and given the challenges associated with collecting irrigation water samples using lysimeters, the evaluation of health hazards related to environmental pollution from the agronomic techniques tested in the experimental trials was conducted through soil analysis. Chemical analyses of the soil were performed to detect the presence of pesticides applied during the trials and to assess the impact of the adopted practices on the bioaccessible fraction of naturally occurring or anthropogenically introduced heavy metals. Additionally, a series of ecotoxicological tests (using *Aliivibrio fischeri*, *Daphnia magna*, and *Raphidocelis subcapitata*) were carried out on soil samples to evaluate the overall environmental impact of the TOMRES agronomic practices. From a One Health perspective, microbiological analyses were also conducted to assess the potential spread of microbial parameters and human pathogens. These included total coliforms, *Escherichia coli*, Enterococci, total plate counts (TPC) at $22\text{ }^{\circ}\text{C}$, $28\text{ }^{\circ}\text{C}$, and $37\text{ }^{\circ}\text{C}$, *Clostridium* spp. spores, and *Salmonella* spp.

Soil samples collected before and after tomato cultivation were analysed to assess potential health hazards associated with the agronomic techniques proposed

within the TOMRES project. Sampling was carried out in triplicate for each treatment plot using a randomized split-plot design. Within each plot, multiple subsamples were collected at regular intervals (Figure S1), pooled, and homogenized to reduce spatial variability. Soil samples for the analysis of pseudo-total and bioaccessible potentially toxic elements (PTEs), pesticide residues, microbiological parameters, and ecotoxicological assays were collected at a depth of $5\text{--}30\text{ cm}$, stored at $+4\text{ }^{\circ}\text{C}$, and transported to the laboratory in accordance with Iso 18400–206:2018 [20]. Upon arrival at the laboratory, samples were divided into five aliquots for dry weight determination, toxicological, microbiological, and chemical analyses. Before analysis, samples were sieved through a 2 mm mesh and air-dried for 12 h at room temperature, following the preparation method outlined in Italian Decree No. 010175/2002 [21]. Dry weight (DW) was determined by drying 20 g of each soil sample at $105\text{ }^{\circ}\text{C}$ for 24 h , according to Iso 11465:1993 [22].

Analysis of potential toxic elements (Cu, Mn, Zn, Ni, Cd, Pb, As)

Pseudo-total concentrations of potential toxic elements (PTEs) were determined in *pre-cultivation* soil samples collected at depths of $0\text{--}5\text{ cm}$, $5\text{--}15\text{ cm}$, and $15\text{--}30\text{ cm}$, using extraction with concentrated HCl and HNO_3 ($3:1\text{ v/v}$) in accordance with Iso 11466:1995 [23]. The extraction was carried out by heating 1 g of soil sieved at 0.5 mm and 8 mL of “aqua regia” at $300\text{ }^{\circ}\text{C}$ until digestion was complete. The concentration of all pseudo-total heavy metals was determined using inductively coupled plasma–mass spectrometry (ICP–MS; PerkinElmer NexION 350X), except for arsenic, which was analysed by flame atomic absorption spectrometry [24].

All *pre-* and *post-cultivation* soils were analysed for the determination of pH in water ($1:2.5$, soil:water). Based on pH, the bioaccessible PTE pool was determined in *post-cultivation* soil samples at all three depths by extraction with sodium ethylenediamine tetraacetate (Na EDTA) by shaking 20 g of air-dried 2 mm sieved soil samples with 40 mL of Na EDTA solution adjusted to pH 7.3 for 2 h . Arsenic was extracted using the P-Olsen method with 0.5 N NaHCO_3 solution adjusted to pH 8.5 [25]. The amount of bioaccessible PTE was determined on the extracts as described above.

Analysis of pesticides/herbicides

Pesticide analyses were carried out by a certified laboratory (Tentamus Agriparadigma S.r.l., Ravenna, Italy) accredited according to ISO/IEC 17025:2017 [26]. Analyses employed LC/MS/MS, GC/MS/MS, and FPD on eluates from soil samples used as proxies for leaching water

[27, 28]. The pesticides used in the different trials are reported in Table 1.

Microbiological analyses

Soil samples (30 g) were placed in stomacher bags containing 270 mL of sterile sodium pyrophosphate solution (1 g L^{-1} , pH 7). The samples were shaken at 100 rpm for 2 min. The resulting suspensions were then subjected to serial tenfold dilutions (from 10^{-1} to 10^{-7}), and each dilution was tested in triplicate according to EN Iso 17155:2020 [29].

Total Plate Counts (TPCs) were determined by incorporating 1 mL of the sample into Soil Extract Agar (HiMedia Laboratories, Einhausen, Germany) supplemented with sterile cycloheximide solution (1%, VWR International Srl, Milan, Italy). Petri dishes were incubated at 22 °C, 28 °C, and 37 °C for 10, 30, and 7 days, respectively, in accordance with Italian Decree 010175/2002 [21]. Results were expressed as Log CFU g^{-1} DW.

Total coliforms, *Escherichia coli*, and Enterococci were quantified using Colilert 18™ and Enterolert™ (Quantitray/2000, IDEXX Water, Milan, Italy) following the manufacturer's instructions. Results were expressed as Log MPN g^{-1} DW [30].

Clostridium spp. spores were quantified by heat-treating liquid samples at 80 °C for 15 min to eliminate vegetative cells. Subsequently, 1 mL of each dilution was plated on TSC Agar supplemented with MUP and D-cycloserine (Biolife, Milan, Italy). The plates were incubated anaerobically at 44 °C for 24 h. Anaerobic conditions were achieved using anaerobic jars with gas-generating systems, as recommended by Iso 14189:2013 [31]. Black, phosphorescent colonies were further confirmed on Nutrient Agar (Merck, Milan, Italy). Results were expressed as Log CFU g^{-1} DW.

The detection of *Salmonella* spp. was performed using a combination of molecular PCR and culture-based methods. Briefly, 25 g of soil were pre-enriched in sterile buffered peptone water (APT, Oxoid, Thermo Scientific, Rodano, MI, Italy) and incubated at 37 °C for 24 h. Subsequently, 100 μL of the pre-enriched sample was transferred into 10 mL of Rappaport Vassiliadis (RV) broth (Biogenetics Diagnostics, Ponte San Nicolò, PD, Italy) and incubated at 42 °C for 24 h. After incubation, 10 μL of the RV culture were plated onto XLD agar (Sigma-Aldrich, Milan, Italy) and incubated at 37 °C for 24 h. Colonies exhibiting a red color with black or green centers were subjected to biochemical confirmation using the API 20E system (BioMérieux Italia S.p.A., Bagno a Ripoli, FI, Italy).

For molecular detection, DNA was extracted from 1.8 mL of the APT enrichment broth using the PowerSoil DNA Isolation Kit (Qiagen Italia, Milan, Italy), followed by PCR analysis as described by Bonetta et al. (2021) [30].

Ecotoxicological analyses

Soil samples (250 g) were extracted with 750 mL of demineralised ultrapure water (1:4 v/v) according to APAT (2002) [27] and Iso 21268–2:2019 [28], by shaking at room temperature for 24 h. Elutriates were separated by overnight settling followed by centrifugation at 3750 g. The pH of the elutriates was measured prior to ecotoxicological testing with *Aliivibrio fischeri*, *Daphnia magna*, and *Raphidocelis subcapitata*.

The acute toxicity test with *A. fischeri*, based on the reduction of the bacterium's natural bioluminescence, was performed using the Microtox Model 500 (Azur Environmental, Carlsbad, California, USA) following UNI EN Iso 11348–3:2019 [32]. Results were expressed as bioluminescence inhibition percentage (I%).

The acute toxicity test with *D. magna*, based on the immobilization of crustaceans after 24 h of exposure, was conducted using the DaphToxKit F™ magna (Ecotox LDS, Milan, Italy) in accordance with Iso 6341:2012 [33]. Results were expressed as immobilization percentage (I%).

The toxicity test with *R. subcapitata*, based on growth inhibition after 72 h of exposure, was conducted using the AlgalToxKit F™ (Ecotox LDS, Milan, Italy) in accordance with Iso 8692:2012 [34]. Results were expressed as growth inhibition percentage (GI%) that derived from growth rate comparisons with controls. Quality control tests were performed for all ecotoxicological assays in accordance with the relevant ISO standards [32–34], using potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) as a reference toxicant (positive control) to verify the sensitivity and health status of the test organisms. All tests met the acceptability criteria specified by the respective standards. It should be noted that the ecotoxicological assays were performed on soil elutriates and therefore primarily reflect the soluble fraction of contaminants and biostimulants, representing a partial estimation of overall soil toxicity. In addition, sample preparation procedures (sieving to 2 mm and air-drying at room temperature) may reduce the contribution of labile and volatile organic compounds, potentially leading to an underestimation of actual ecotoxicological effects. Consequently, the results should be interpreted with these methodological limitations, particularly with respect to hydrophobic contaminants associated with the solid soil matrix.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics v26.0 (IBM Corp., Armonk, NY, USA). Differences between *pre-* and *post-cultivation* conditions were evaluated using paired t-tests. The effects of agronomic treatments were assessed by paired t-test and one-way ANOVA followed by Tukey's post-hoc test. Binary logistic regression was used to examine the relationship between microbial counts and the presence of *Salmonella* spp. Multivariate patterns were explored using Principal Component Analysis (PCA) on standardized variables, after removal of highly correlated variables (Pearson $r > 0.9$). The suitability of the dataset for PCA was confirmed for the global dataset (KMO=0.68; Bartlett's test $p < 0.001$).

Results and discussion

Pseudo-total PTE content in the soils used for tomato cultivation

Pseudo-total concentrations of potentially toxic elements (PTEs) in the soils selected for tomato cultivation showed substantial variability, reflecting differences in lithology, pedogenesis, and anthropogenic inputs associated with agricultural practices (Table 2). Although many heavy metals occur naturally in soils, there are no specific regulatory thresholds for their natural background levels. Existing EU directives primarily address limits for heavy metals introduced through sewage sludge or other

anthropogenic sources [35, 42]. In this regard, a proposal for a European Directive on a Soil Monitoring Law was put forward in 2023 and is currently under review [43]. Moreover, at low concentrations, heavy metals such as Cu, Ni, and Zn act as essential trace elements for plants and represent important nutraceutical components in foods [44]. However, their accumulation and/or excessive bioaccessibility can adversely affect soil and plant health, as agronomic practices and soil management may alter their speciation and increase the bioaccessible fraction beyond toxicity thresholds [45].

To evaluate the potential toxicity of soils selected for tomato cultivation, PTE concentrations were compared with the limit values for heavy metals established by Directive 86/278/EEC [35], which regulates the use of sewage sludge in agriculture providing ranges of permissible concentrations in soils; within these ranges, Member States may set specific limit values, taking into account soil characteristics such as pH, with stricter limits generally applied to more sensitive (e.g. acidic) soils. In addition, recent studies based on the LUCAS top-soil database have proposed EU-wide reference ranges for heavy metals (EU lower and upper limits, EU_LL and EU_UL), derived from soil properties such as pH and texture [42, 46, 47]. The comparison was further extended to the relevant national implementing regulations in the countries where the agronomic trials were conducted [36–41]. Collectively, these regulations aim

Table 2 Pseudo-total concentrations of PTEs (mg kg⁻¹ dry weight) in *pre-cultivation* soil samples, compared with the limit values set by Directive 86/278/EEC for the agricultural use of sewage sludge and by the corresponding national implementing regulations

Site	Cu	Pb	Zn	Mn	Ni	Cd	As
Spain	21.67 ± 6.25	57.88 ± 3.47	58.92 ± 14.56	752.34 ± 47.05	18.11 ± 1.78	0.21 ± 0.03	25.79 ± 2.35
Italy 1 and 2	32.55 ± 0.48	72.66 ± 2.43	47.72 ± 2.57	3619.79 ± 303.68	19.10 ± 0.45	0.42 ± 0.01	36.15 ± 0.80
România 1	33.39 ± 2.57	34.65 ± 2.18	69.44 ± 9.89	767.86 ± 34.46	44.16 ± 1.49	0.33 ± 0.03	11.62 ± 0.40
România 2	161.47 ± 0.85	44.35 ± 11.67	161.47 ± 5.10	1088.26 ± 24.37	39.77 ± 5.47	0.67 ± 0.01	8.60 ± 1.99
Limit values							
Regulations	Cu	Pb	Zn	Mn	Ni	Cd	As
Directive 86/278/EEC [35] [§]	50–140	50–300	150–300	–	30–75	1–3	–
Royal Decree 1310/1990 (Spain) [36]	50–210	50–300	150–450	–	30–112	1–3	–
Junta de Andalucía 1999 (Spain) [37]	–	–	–	–	–	–	20–50
Decree n. 99 of January 27, 1992 (Italy) [38]	100	100	300	–	75	1.5	–
Decree n. 46 of March 1, 2019 (Italy) [39]	–	–	–	–	–	–	30
Ordinance n. 344/708 of August 16, 2004 (Romania) [40]	100	50	300	–	50	3	–
Order n. 16/2004 of August 16, 2004 (Romania) [41]	–	–	–	–	–	–	10

[§] The “range” of limits defined in Directive 86/278/EEC refers to the minimum and maximum permissible concentrations of heavy metals in sludge and soil, as well as to the permitted annual accumulation rates

The Directive allows Member States, depending on soil sensitivity, to establish either a single limit or both lower and upper limits, and to link sludge application rates to soil pH classes

to prevent excessive heavy metal accumulation in soils, thereby reducing environmental and human health risks, particularly those associated with the consumption of food crops grown on contaminated land. By these comparisons Cu, Zn, and Ni concentrations were generally below the lower limits (LL) referred to the EU Directive and national regulations, except at the Romanian sites, where exceedances were observed. At the Romania 2 site, Cu concentrations exceeded both the lower and upper limits established by the EU Directive, as well as the national threshold for soils eligible to receive sewage sludge. At the same site, Zn concentrations were above the EU Directive lower limit but remained within the Romanian national limit. Both Romanian sites (Romania 1 and Romania 2) exhibited Ni concentrations exceeding the EU lower limit, although values were still below the national regulatory threshold. In contrast, Pb concentrations at the Romanian sites complied with both EU and national limits. Conversely, the Spanish site showed Pb levels exceeding the lower limit set by both the EU Directive and national legislation, while the Italian site exceeded the EU lower limit but remained within the national threshold. Cadmium concentrations were below both EU and national limits at all sites. Copper, Zn, and Ni may derive from the parent material of the soil; however, anthropogenic activities—such as the application of fertilizers and pesticides, the use of sewage sludge, industrial emissions, and irrigation with contaminated water—can substantially increase their concentrations. In addition, specific agricultural practices, particularly the use of Cu-based fungicides, contribute to metal accumulation in soils [48]. Relatively high Cu and Zn concentrations at the Romania 2 site and elevated Ni levels at the Romania 1 site, compared with optimal values for plant growth, indicate that these areas should be managed with caution. Although Cu and Zn have been reported to preferentially accumulate in tomato roots rather than being efficiently translocated to fruits [49, 50], their potential accumulation in edible tissues cannot be excluded and may pose a risk to fruit quality and food safety [51, 52]. Because As is not regulated under EU Directive 86/278/EEC [35] or its national counterparts [36, 38, 40], its concentrations were compared with national reference limits available for agricultural soils [37, 39, 41]. At the Spanish, Italian, and Romania 1 sites, As concentrations slightly exceeded the threshold values established by the respective national regulations, whereas at the Romania 2 site they remained below national reference limits. Manganese (Mn) concentrations were consistent with average levels typically reported for agricultural soils across the EU, ranging from 600 to 900 mg kg⁻¹ dry weight (DW) in Spain and from 900 to 1000 mg kg⁻¹ DW in Italy [53]. The comparatively higher Mn levels observed at the Italian

site are likely related to the volcanic origin of the soil [54]. As previously observed for Cu, Zn, and Ni, also Pb, Cd, and As may derive from natural sources, and their concentrations can be substantially enhanced by anthropogenic inputs. Unlike essential elements, these PTEs are not required for plant metabolism and may be phytotoxic to tomato plants, as well as hazardous to human health due to their potential bioaccumulation in edible fruits [55]. In this context, the relatively elevated Pb and As concentrations detected at the Italian and Spanish sites warrant particular attention. With respect to Mn, elevated concentrations generally do not indicate soil contamination and rarely pose a significant risk to plants; instead, Mn deficiency is typically of greater agronomic concern [45].

While total metal content provides an overview of soil contamination potential, bioaccessible fractions better represent environmental and health hazards. Therefore, subsequent analyses focused on the bioaccessible pool of PTEs in *post-cultivation* soils.

Bioaccessible PTEs in soils after tomato cultivation

Based on this background, we assessed whether the use of biostimulants under conditions of nutrient and water stress could influence the bioaccessible pool of PTEs and their potential toxicity. The soil bioaccessible PTEs are indeed more indicative than the soil pseudototal content to assess the potential toxicity risk and may help to define environmental and health impacts [56]; however, it should be noted that regulatory limit values are currently not established for bioaccessible metals. Referring to the concentrations of the soil bioaccessible fraction of PTEs measured in *post-cultivation* soil samples (Table 3) Cu and Zn were particularly low at the Spain site (Cu 0.4–0.7; Zn 1.0–2.5 mg kg⁻¹ DW), while the highest values were found in Romania 2 (Cu 35.1–47.8; Zn 20.2–22.4 mg kg⁻¹ DW). Similarly, the concentration of bioaccessible Mn was lowest at the Spain site (0.9–1.2 mg kg⁻¹ DW), while the highest concentration was detected in Italy 2 (21.0–26.2 mg kg⁻¹ DW). The concentration of bioaccessible Ni was low and comparable at the Spain and Italy sites (0.02–0.07 mg kg⁻¹ DW), while higher values, by an order of magnitude, were observed at both Romanian sites (0.51–0.67 mg kg⁻¹ DW).

Similarly, for toxic metals, the Spain site had the lowest values of the mobilizable portion of Pb (1.5–1.7 mg kg⁻¹ DW), Cd (0.003–0.006 mg kg⁻¹ DW), and As (0.003–0.006 mg kg⁻¹ DW). In contrast, Italy 1 and 2 (3.5–4.6 mg kg⁻¹ DW) and Romania 2 (5.3–7.4 mg kg⁻¹ DW) exhibited the highest concentrations of bioaccessible Pb. The highest concentration of Cd was found in Romania 2 (0.23–0.27 mg kg⁻¹ DW), while the concentration of As was similar across all sites (0.10–0.25 mg kg⁻¹ DW),

Table 3 Bioaccessible PTEs (mg kg⁻¹ DW) in post-cultivation soil samples

Site	Variety	Treatment	Biostimulant	Cu	Pb	Zn	Mn	Ni	Cd	As
Spain	Commercial variety (scion) + rootstock TOMRES BIL6191 (T)	WW	+B	0.434±0.216	1.638±0.229	1.080±0.256	0.652±0.149	0.024±0.008	0.003±0.002	0.042±0.017
		CS	+B	0.708±0.518	1.592±0.354	2.344±0.610	1.197±0.666	0.040±0.019	0.006±0.006	0.039±0.026
		WW	–	0.448±0.283	1.646±0.059	1.808±0.184	0.933±0.262	0.029±0.008	0.003±0.002	0.053±0.008
		CS	–	0.578±0.329	1.714±0.193	2.583±1.418	1.105±0.325	0.041±0.012	0.005±0.004	0.052±0.032
Italy 1	TC162 control (M82) (T)	WW	+B1	4.692±0.149	4.672±0.415 ^c	4.379±0.538	16.378±4.872	0.062±0.007	0.100±0.009	0.197±0.034
		CS	+B1	4.908±0.151	3.655±0.156	4.923±0.202	10.470±0.918	0.057±0.003	0.090±0.003	0.219±0.004
		WW	+B2	4.376±0.295	4.136±0.499	4.240±0.389	13.661±1.910	0.075±0.005 ^d	0.094±0.007	0.165±0.012
		CS	+B2	4.610±0.300	3.655±0.353	4.575±0.380	9.898±1.335	0.066±0.005 ^e	0.082±0.005	0.218±0.012
Italy 2	TOMRES T149 (T)	WW	–	4.761±0.194	3.536±0.604 ^c	3.948±0.420 ^a	10.443±0.721	0.058±0.002 ^d	0.086±0.004	0.197±0.008 ^b
		CS	–	4.666±0.097	3.567±0.135	4.573±0.587 ^a	10.152±1.004	0.055±0.000 ^e	0.087±0.002	0.234±0.016 ^b
		WW	+B3	5.815±0.736	4.462±0.193	5.016±0.539	23.094±2.612	0.070±0.005	0.092±0.006	0.205±0.018
		CS	+B3	5.192±0.470	4.106±0.100	4.865±0.336	21.011±2.462	0.068±0.006	0.090±0.006	0.200±0.017
Romania 1	Pietra rossa F1 (Hybrid) (C)	WW	–	5.517±0.404	4.534±0.036	5.251±0.201	24.123±1.538	0.060±0.006	0.088±0.007	0.195±0.020
		CS	–	5.277±0.016	4.114±0.166	4.411±0.498	22.062±1.860	0.065±0.004	0.085±0.005	0.210±0.015
		WW	+B3	6.031±0.552	4.546±0.118	4.985±0.195	22.868±3.415	0.071±0.006	0.095±0.007	0.215±0.019
		CS	+B3	5.011±0.698	4.671±0.445	5.190±0.891	26.266±7.960	0.064±0.006	0.091±0.006	0.208±0.017
Romania 2	Rootstock: Emperor F1 a Rijk Zwaan -Scion: Abellus RZ F1 (C)	WW	–	5.749±0.692	4.230±0.302	4.957±0.630	21.845±2.225	0.062±0.005	0.087±0.006	0.198±0.016
		CS	–	5.955±0.494	4.650±0.451	5.368±0.791	23.861±0.440	0.067±0.005	0.089±0.006	0.202±0.018
		CS	+S	7.627±3.605	2.959±0.117	9.072±6.493	8.054±1.863	0.627±0.029	0.092±0.007	0.238±0.020
		CS	+A	6.466±1.628	3.136±0.126	12.493±14.542	10.259±3.289	0.635±0.079	0.107±0.010	0.192±0.044
Romania 2	Rootstock: Emperor F1 a Rijk Zwaan -Scion: Abellus RZ F1 (C)	CS	+Th	3.716±1.456	2.853±0.040	7.538±6.506	7.991±1.673	0.673±0.066	0.087±0.013	0.253±0.053
		CS	–	9.091±4.077	3.013±0.067	12.444±9.240	8.517±2.214	0.611±0.126	0.102±0.009	0.233±0.096
		CS	–	40.966±0.402 ^f	5.540±0.015 ⁱ	22.381±0.714	10.131±0.465 g ^h	0.520±0.051 h ^m	0.47±0.006	0.150±0.008 ⁿ
		CS	+S	35.100±0.707	7.463±0.569 ⁱ	21.670±0.649	8.661±0.462	0.511±0.013	0.230±0.005	0.130±0.006
Romania 2	Rootstock: Emperor F1 a Rijk Zwaan -Scion: Abellus RZ F1 (C)	CS	+A	38.387±1.209	5.378±0.089	20.260±1.177	12.583±0.504 ⁱ	0.659±0.039 ^m	0.279±0.028	0.106±0.001 ⁿ
		CS	+Th	47.867±5.509 ^f	5.399±0.077	22.467±0.231	12.400±0.759 g	0.631±0.043 h	0.249±0.006	0.111±0.007 ⁿ

WW = well water and fertilised; CS = combined stress; B, B1, B2, B3, S, A, Th = biostimulants

t-test: a = p < 0.05; b = p < 0.001

one way-ANOVA: c = < 0.05; d = < 0.005; e = < 0.001; f = p < 0.05; g = p < 0.05; h = p < 0.001; i = < 0.01; m = < 0.01; n = p < 0.01

except in Spain, where the concentration was on average an order of magnitude lower ($0.008\text{--}0.053\text{ mg kg}^{-1}\text{ DW}$).

Overall, considering the results across the different sites, the bioaccessible PTEs were generally below toxicity thresholds and ensured an adequate nutritional supply [57]. In the Spanish trial, comparisons among *post-cultivation* treatments revealed no significant differences in the bioaccessible fractions of most PTEs. In the Italian trial (Italy 1), PTE bioaccessibility was unaffected by water and fertilizer saving techniques except for a significant increase for Zn (+15.8%) ($p < 0.05$) and As (+18.8%) ($p < 0.001$) likely due to competition between phosphorus and As for adsorption sites. The drier conditions should have increased phosphate adsorption and precipitation making As more mobile [58]. The application of biostimulant B1 significantly increased the bioaccessibility of Pb (+32.1%) ($p < 0.05$), and also of Zn (+10.9%) and Mn (+56.8%), although the difference is not statistically significant, in the WW plots, probably by enhancing biological activity in the rhizospheric zone due to the synergic effect of water availability and biostimulants. The application of biostimulant B2 highlights a reduction in Ni concentration under both optimal irrigation conditions (WW) (−22.7%) ($p < 0.005$) and with water and fertilizer saving techniques (CS) (−16.7%) ($p < 0.001$). Nevertheless, in all other cases, when water and nutrient supply was reduced to 50% of crop needs, the bioaccessibility of PTEs remained almost unaltered with or without biostimulants. In the other Italian trial (Italy 2) the effect of water and nutrient saving techniques although was more marked did not lead to significant differences in the content of Cu and Zn between WW and CS treated plots, independently of the application of the biostimulant with the tomato variety TOMRES T149. With the other tomato genotype the behaviour was variable and with no significant differences. Mn varied among plots with no clear effect. Toxic elements such as Ni, Cd and As were not affected by both water and nutrient saving techniques nor the use of biostimulants. In the first trial conducted in Romania (Romania 1), no significant differences in the concentration of bioaccessible PTEs were associated with the different agronomic treatments applied. In general, *Azotobacter vinelnadaii* showed a tendency to reduce Cu, Zn and Pb content, whereas *Trichoderma harzianum* T22 caused an increase of the bioaccessible pool. In this site, the high level of soil organic matter likely buffered the effects due to the different treatments and immobilised the soluble forms in the solid phase reducing the risk of contamination and migration to the other environmental compartments.

In the second trial of Romania (Romania 2) in which the concentration of PTEs in *pre-cultivation* samples was generally higher than the levels found in Romania 1, the

application of the three biostimulants caused the increase of different PTE bioaccessibility in soil: *Trichoderma harzianum* T22 was associated to an increase ($p < 0.05$) of bioaccessible concentration of Cu (+16.4%), Mn (+22.4%) and Ni (+21.3%); shungite was associated with an increase of bioavailability of Pb (+34.7%) ($p < 0.001$), and *Azotobacter vinelnadaii* resulted associated with a greater bioavailability of Ni (+26.7%) and Mn (+24.2%) ($p < 0.01$). Conversely, the three biostimulants likely reduced the bioavailability of As, especially with *Azotobacter vinelnadaii* (−29.3%) and *Trichoderma harzianum* T22 (−26.0%) ($p < 0.01$).

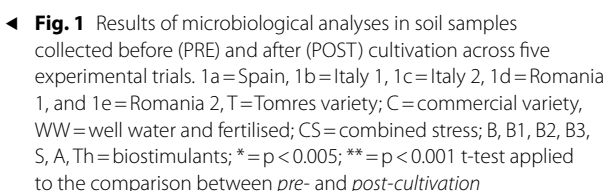
The use of biostimulants can be a promising solution to overcome crop mineral deficiency, however, an excessive biostimulant effect could have negative consequences, particularly in metal-enriched or acidic soils, where it may enhance PTE mobility into other environmental compartments, posing potential ecological and health hazards. This could therefore represent a risk for human health due to the possible accumulation of PTEs in plants and fruits. Other authors reported the accumulation of PTEs in horticultural products in relation to their concentration in soil and irrigation water [51, 52]. PTEs, such as As, Cd and Pb, always represent a risk for plants and the environment. In this context, biostimulants could threaten food health due to their capacity to mobilize cations and increase their mobility and availability to organisms. On the other hand, reduction of water and nutrients decreased in general PTE bioaccessibility, due to a decrease of water circulation and microbial activity [59].

Beyond chemical parameters, microbiological parameters were also analysed to evaluate whether different agronomic strategies influence the hygienic and sanitary quality of soils.

Soil microbiological contamination before and after tomato cultivation

Figure 1 summarizes mean values and standard deviations for microbiological parameters measured in *pre*- and *post-cultivation* soil samples across all experimental sites.

In the Spain trial (Fig. 1a), TPCs at 22, 28 and 37 °C significantly increased by ~1 Log after cultivation (t-test $p < 0.001$), rising from 6.1–6.3 to 6.7–7.1 Log CFU $\text{g}^{-1}\text{ DW}$. Enterococci and *Clostridium* spp. spores also rose significantly *post-cultivation* (t-test $p < 0.005$ and $p < 0.001$, respectively; Table S1), while *E. coli* remained undetected, except in TWWB with a very low concentration (0.02 Log MPN $\text{g}^{-1}\text{ DW}$). This increase likely reflects enhanced microbial activity associated with plant growth and rhizosphere processes, as commonly reported in agricultural soils [60, 61]. No significant differences



At Italy 2 (Fig. 1c), TPCs were generally higher (up to 9 Log CFU g⁻¹ DW) than the other sites. Moreover TPCs were significantly higher in plots cultivated in WW condition compared to CS (t-test $p < 0.01$ – 0.05 ; Table S6). These results suggest that water availability promotes microbial growth. Biostimulant application (B3) significantly increased TPCs ($p < 0.01$ – 0.05 ; Table S7), suggesting stimulation of microbial activity. Similar increases in soil microbial concentration and activity following organic or biostimulant inputs have been reported in previous studies [60, 61]. In Romania 1 (Fig. 1d), TPCs and coliforms significantly decreased *post-cultivation* (t-test, $p < 0.001$ – $p < 0.005$, Table S8), while Enterococci and *Clostridium* spp. spore increased (t-test, $p < 0.001$, Table S8). *E. coli* was detected only in *pre-cultivation* samples and was below the detection limit in *post-cultivation* samples, confirming its low persistence in soil environments compared to other faecal indicators [62]. Significant differences among treatments were observed (Table S9), indicating that *A. vinelnadii*, *T. harzianum* T22 and shungite can influence microbial communities.

However, responses were treatment-specific and not consistent across parameters.

In the Romania 2 trial (Fig. 1e), conducted under open-field conditions, no statistically significant difference was observed between the *pre*- and *post-cultivation* samples, while treatment effects were significant (Table S10). The TPCs concentrations varied depending on the applied biostimulant, while total coliform significantly decreased in samples treated with biostimulants compared to the control (CCS) (ANOVA, $p < 0.05$). *Clostridium* spp. spores showed different responses depending on treatment applied.

In summary, across all sites, microbial parameters showed high variability related to local soil characteristics and agronomic practices. TPCs values ranged between 6 and 9 Log CFU g⁻¹ DW, and are aligned with those found in two studies on soil samples from lettuce and tomato crops [63, 64]. Faecal indicators (total coliforms, *E. coli*, Enterococci, *Clostridium* spp. spores) showed heterogeneous patterns. *E. coli*, the most typical indicator of faecal contamination, was rarely recovered in *pre*- or *post-cultivation* soils across all trials and showed no clear relationship with treatments. When present, it was found in low concentrations (0.01–3.50 Log MPN g⁻¹ DW). This finding is consistent with results obtained by Gatta et al. (2015) [63] in a study evaluating the safety of various irrigation sources for tomato crops. It is important to note that *E. coli* exhibits poor persistence in soil and the environment compared to other faecal bacterial indicators such as Enterococci and Clostridia. Therefore, the absence or low counts of *E. coli* may be attributed to its limited survival and persistence under environmental soil conditions, as previously reported by other researchers. [62]. In contrast, Enterococci and *Clostridium* spp. spores were more persistent, although not uniformly affected by agronomic practices [65, 66]. The values of Enterococci reported across all trials ranged between 3–4 Log MPN g⁻¹ DW, with concentrations comparable to those found in the literature for soil from tomato crops [67]. Clostridia are resilient, sporogenic bacteria, which

are commonly found in soil and are considered a reliable indicator of (past) faecal contamination.

Overall, no consistent or generalizable effect of biostimulants on microbial parameters was observed across all sites. Instead, responses were strongly influenced by site-specific conditions, confirming the importance of environmental context in soil microbiology [60, 61]

Most studies evaluating the environmental and health impact of agricultural practices have focused on researching human pathogens and faecal indicators directly in fertilizers (such as manure, reclaimed wastewater, compost, etc.), or in water used for irrigation, or directly in plants and fruits. In contrast, this project aimed to evaluate environmental and sanitary safety aspects related to different agronomic techniques by monitoring several soil microbiological parameters of contamination. Therefore, this new approach means that the microbiological results obtained in the individual trials, whether in the field or greenhouse, cannot always be directly compared to equivalent data in the literature and must be interpreted in the context of the agronomic practices.

Salmonella spp. was never detected by the culture method in either *pre*- or *post*- soil samples in all trials. On the other hand, the molecular method pointed out the presence of *Salmonella* in some *pre*- and in several *post-cultivation* samples in all the trials considered (Table 4).

The discrepancy in contamination between *pre*- and *post-cultivation* could be attributed to the variability of the soil conditions and to potential sources of contamination, such as the influence of previous agricultural practices or the water used for irrigation (e.g. Italy 1). When *Salmonella* is found in both *pre*- and *post-cultivation* soil samples, as in Italy 2, it is challenging to pinpoint which historical treatment might have contributed to the contamination. Possible sources include direct historical contamination of the soil, poor quality of irrigation water, or impacts from runoff and the presence of wildlife. These findings are consistent with previous studies showing that irrigation water represents a major pathway

Table 4 *Salmonella* spp. positive soil samples detected by molecular methods in *pre*- and *post-cultivation* samples from the experimental trials

Site	<i>pre-cultivation</i> number of positive samples/number of analysed soil samples	<i>post-cultivation</i> number of positive samples/number of analysed soil samples
Spain	1/15	2/15
Italy 1	0/6	6/6
Italy 2	8/8	3/8
Romania 1	0/4	1/4
Romania 2	0/4	1/4

for the dissemination of foodborne pathogens in agricultural systems, particularly when surface water sources are used [68]. In such systems, pathogens can be transported through irrigation networks and persist in soil and plant environments. Similarly, agricultural practices such as the use of organic amendments or contaminated water have been identified as key factors influencing the occurrence and persistence of *Salmonella* in tomato production systems [69]. The discrepancy between culture-based and molecular detection is consistent with previous findings showing higher sensitivity of PCR-based methods for environmental samples [30] particularly for microorganisms occurring at low abundance in soils, which may be underestimated by culture-based methods alone. Moreover, several studies have demonstrated that *Salmonella* can persist in soil environments in a viable but non-culturable (VBNC) state or under sublethal stress conditions, which may explain the detection of *Salmonella* DNA in the absence of culturable cells [69]. The presence of *Salmonella* DNA without culturability may reflect low viability or environmental stress conditions.

No association between *Salmonella* occurrence and other microbiological parameters was observed in any trials (binary logistic regression, n.s.). This finding suggests that commonly used microbiological parameters of faecal contamination are not reliable indicators of *Salmonella* spp. presence, aligning with conclusions from other studies [30, 70, 71].

Despite site-specific variability, the microbiological profiles observed in this study did not indicate conditions associated with significant sanitary risk. However, the results highlight the importance of site-specific evaluation and the limitations of relying on single microbial parameters to assess environmental and health hazards.

To complement microbiological assessments, ecotoxicological tests were performed to evaluate the broader environmental impact of the different agronomic treatments.

Soil ecotoxicity before and after tomato cultivation

This study provides one of the first ecotoxicological evaluations of soils used for tomato cultivation under combined biostimulant and water-saving treatments. In general, considering all the trials analysed, *A. fischeri* and *D. magna* revealed very low toxic effect (<20%) or any effect both in *pre-cultivation* samples and in *post-cultivation* samples as reported in Fig. 2, and therefore no statistical analyses were performed. The alga *Raphidocelis subcapitata* seems to be the most sensitive organism as reported in other studies [72, 73]. The alga reveals toxic effects >20% in several soil samples coming from all trials, with the exception for the trial in Spain. This trial was conducted in greenhouses under strictly controlled

conditions and without the use of pesticides/herbicides, factors that contributed to the lack of toxicity. This fact can lead to the deduction that the Edypro biostimulants (Procuaje Radicular and Coupé Regeneración Plus) whose composition is reported in Table 1, used in this trial as well as the other agronomic practices tested do not determine a toxic impact on the water obtained from the soil samples washes. However, results varied considerably among sites, as illustrated in Fig. 2.

Regarding the two trials carried out in Italy, although they were conducted with different varieties and with different biostimulants, in both trials an increase of toxicity in the *post-cultivation* soil samples was detected with the alga, probably due to the use of pesticides even if they were not detected in soil elutriates [74]. The growth inhibition (GI%) ranged between 0 and 59% in the first trial and between 0 and 54% in the second trial. Statistical significant differences were shown in Fig. 2.

The application of biostimulants influenced the ecotoxicological response of the alga, as evidenced by a significant reduction in growth inhibition ($p < 0.001$ – 0.05) in both Italian trials. In Italy 1, this effect was observed with B1 and B2 treatments, whereas in Italy 2 it was associated with the application of the strigolactone-based biostimulant (B3), with a reduction in growth inhibition under both well-watered (WW) and combined stress (CS) conditions (Fig. 2). The magnitude of this effect was generally more pronounced under well-watered conditions compared to combined stress, suggesting that the effectiveness of biostimulants in mitigating ecotoxicological responses may depend on water availability and related changes in soil solution dynamics and contaminant bioavailability.

Finally, in the two experimental sites conducted in Romania, opposite results were obtained: a higher toxicity was recorded in the *pre-cultivation* samples at the Romania 1 site (Fig. 2) with a growth inhibition of $49\% \pm 2\%$ (t-test $p < 0.001$) than in the *post-cultivation* samples (t-test $p < 0.001$) at the Romania 2 site (Fig. 2) where the GI% ranged between 6 and 53%. Since the same tests were carried out in the two sites with the same experimental conditions, this difference could be attributable to the different type of soil that characterises the two sites and to the different concentrations of PTEs found. As reported in a previous paragraph, in Romania 2 higher concentrations of bioaccessible PTEs was found. Regarding the biostimulants used in these trials, in Romania 1, where the bioaccessible PTEs were lower, the application of the biostimulants determined a statistically significant difference in the GI% respect to the non treated control (CCS, ANOVA, $p < 0.001$). In particular, *Azotobacter* determined the lower GI% ($13\% \pm 1\%$), while the use of *Trichoderma* determined

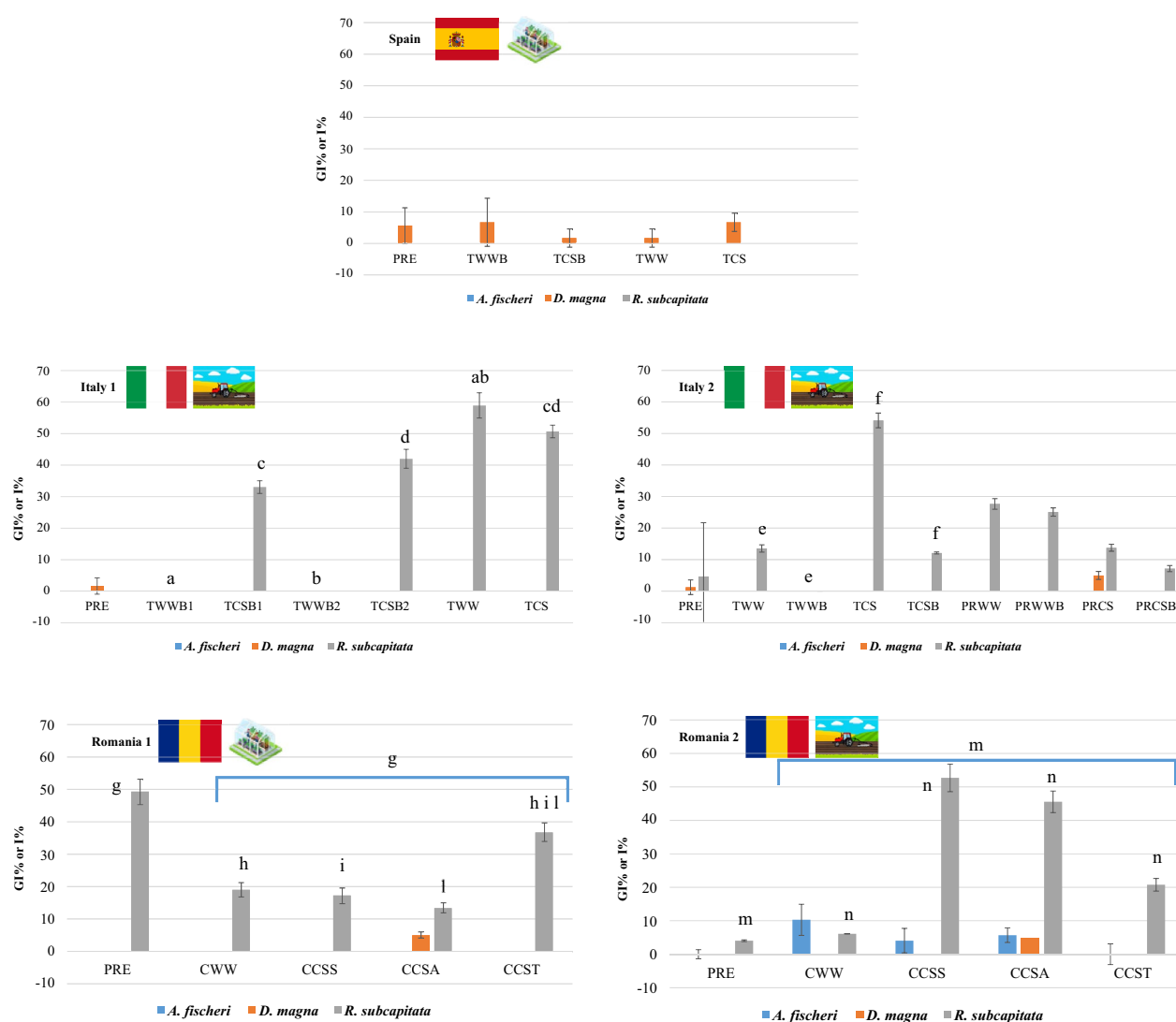


Fig. 2 Results of ecotoxicological tests across the experimental trials. PRE = pre-cultivation; T = Tomres variety; C = commercial variety; WW = well-watered and fertilised; CS = combined stress; B, B1, B2, B3, S, A, Th = biostimulants; I% = inhibition and immobilization percentage. Statistically significant differences refer to the algal response (growth inhibition = GI%) among treatments are indicated as follows: g, m = $p < 0.05$; a, b, c, d, e, f, h, i, l, n = $p < 0.001$

the higher GI% ($37\% \pm 1\%$), probably because *Azotobacter* helps the metabolization of metals as reported by Sumbul et al. (2020) [75] even if we didn't find a statistically significant difference due to the application of the various biostimulants. In Romania 2 where the concentration of bioaccessible PTEs was higher, the use of the biostimulant increased the GI% and a statistically significant difference with respect to the control (CWW) (ANOVA, $p < 0.001$) was found. This difference of the biologic biostimulants effect compared to the greenhouse trial (Romania 1) could be associated with a higher concentration of some PTEs (Cu, Zn and Mn) measured in

the soil of the field trial as reported above. The high concentration of bioaccessible PTEs may cause a toxic effect on the alga, despite the presence of nutrients associated with the treatments applied. These findings underscore that biostimulant application should be evaluated not only for its agronomic benefits but also in the context of soil properties that may modulate toxicity, with potential implications for fruit quality and consumer health. The interpretation of the ecotoxicological results should take into account some methodological limitations of the approach adopted. The bioassays were performed on soil elutriates and therefore mainly reflect the water-soluble

fraction of contaminants and biostimulants. As a consequence, potential effects associated with hydrophobic compounds, such as several pesticides or strongly sorbed substances, may not be fully captured. Moreover, it should be noted that the pesticides applied in the experimental trials were analytically investigated and were not detected in the soil elutriates, suggesting a limited contribution of these compounds to the observed ecotoxicological responses under the tested conditions. In addition, soil pre-treatment procedures, including sieving and air-drying, may have reduced the contribution of labile and volatile organic compounds, potentially leading to an underestimation of the actual ecotoxicological response of fresh soils. Future studies could integrate solid-phase ecotoxicological assays to provide a more comprehensive assessment of soil toxicity.

Multivariate statistical analysis (PCA)

All of the data resulting from the different analyses were further studied through Principal Component Analysis (PCA). The aim was to combine these data in different

ways to explore patterns of variability among sampling sites (Spain, Italy 1, Italy 2, Romania 1 and Romania 2) and treatment conditions: fertigation regimen (well watered WW and combined stress CS), biostimulant applications (presence/absence) and biostimulant type (Absence, B, B1, B2, B3, S, A, Th).

The correlation matrix shows coherent relationships among microbial, chemical, and ecotoxicological variables, supporting the multivariate structure of the dataset (Figure S2).

The matrix highlights the relationships among microbiological parameters, PTEs concentrations, and ecotoxicological assays. Strong positive correlations are observed among TPCs at different temperatures and among PTEs concentrations (Cu, Zn, Pb, Mn), indicating coherent environmental gradients. Since co-linearity was identified in the correlation matrix among TPC at 22, 28, and 37 °C, the variable TPC at 22 °C was removed prior to performing the PCA.

Only the PCA that yielded a satisfactory clustering solution is presented (Fig. 3).

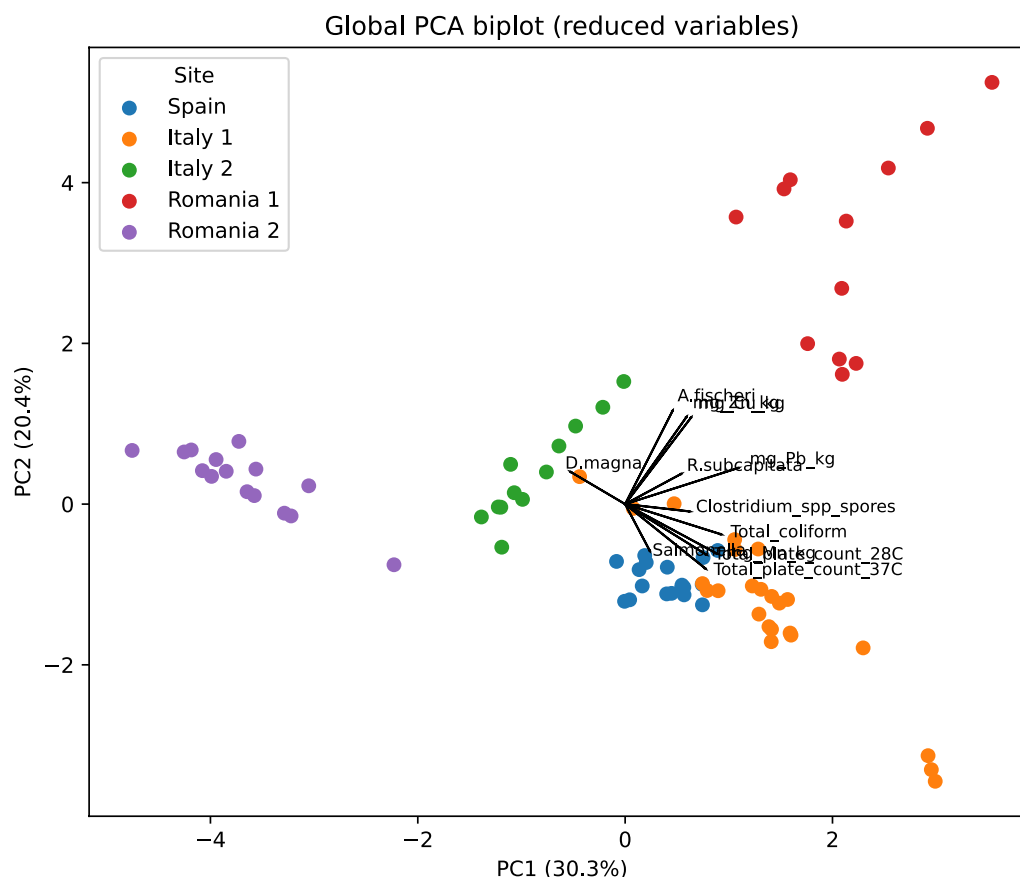


Fig. 3 PCA biplot based on standardized variables after removal of highly correlated parameters (Pearson $r > 0.9$). Samples are grouped by site (Spain, Italy 1, Italy 2, Romania 1, Romania 2). Arrows represent the variables contributing most strongly to sample separation, including microbial parameters, PTEs concentrations, and ecotoxicological assays (to enhance visualization, the figure was generated using Python version 3.X)

The PCA showed that the first two components explained 51.5% of the total variance (PC1 31.5%; PC2 20.1%). The KMO measure of sampling adequacy was 0.63, and Bartlett's test of sphericity was significant ($p < 0.001$). The scores plot showed a clear separation of samples according to geographical origin, with distinct clustering of Spain, Italy 1, Italy 2, Romania 1, and Romania 2. The PCA results indicated that PC1 is mainly composed by gradients in PTEs concentrations (Cu, Zn, Pb, Mn), and PC2 by microbial parameters (TPCs at different temperatures), and ecotoxicological responses (e.g. *A. fischeri* and *R. subcapitata*). The PCA biplot suggests that these variables contributed most strongly to site differentiation. The multivariate analyses indicate that site identity was the main source of variation in the dataset, exceeding the effects of irrigation water source and biostimulant treatments. This interpretation is consistent with a broad soil–microbiome literature showing that soil physicochemical context, hydrology, and local habitat properties strongly structure microbial assemblages and their functions [76].

Conclusions

This study provides a comprehensive, multidisciplinary assessment of the environmental and potential health implications associated with the integration of biostimulants and water- and fertilizer-saving strategies in tomato cultivation across diverse European agroecosystems. By combining chemical, microbiological, ecotoxicological, and multivariate analyses, the results contribute to a more holistic understanding of how sustainable agronomic practices could influence soil quality and contamination dynamics within a One Health framework.

Overall, the findings indicate that the tested agronomic strategies did not lead to widespread or systematic increases in environmental or health hazard. Bioaccessible fractions of potentially toxic elements (PTEs) generally remained below critical thresholds, microbiological parameters did not reveal significant health hazards, and ecotoxicological responses were mostly low to moderate. These results support the potential of integrated resource-saving and biostimulant-based approaches as viable tools for improving sustainability in tomato production.

However, some limitations of the study must be explicitly acknowledged. First, the multi-site experimental design, while representative of real agricultural conditions, was not fully balanced or factorial, limiting the ability to isolate the individual effects of biostimulants, irrigation, and fertilization across all sites. Second, the relatively short duration of the trials (two cropping seasons) does not allow for the assessment of long-term accumulation processes, particularly for

PTEs and persistent contaminants. Third, the ecotoxicological analyses based on soil elutriates and the soil sample pretreatment may have underestimated actual toxicity by excluding poorly soluble, hydrophobic, or volatile contaminants.

From a microbiological perspective, the reliance on soil-based parameters, rather than direct assessment of contamination in irrigation water, plants, or fruits, limits the direct inference of food safety risks. Another important limitation is the absence of regulatory thresholds for bioaccessible PTEs, which constrains the interpretation of potential health implications.

Beyond these methodological constraints, the results clearly demonstrate that site-specific factors—including soil properties, baseline contamination levels, and local agronomic conditions—represent the dominant drivers of variability, often outweighing the effects of the tested treatments. In particular, in soils with higher background PTE concentrations, biostimulants may increase PTE bioaccessibility, suggesting a potential hazard that requires careful management.

In conclusion, the integration of biostimulants with water- and nutrient-saving practices represents a promising pathway toward more sustainable tomato production systems in Europe. Nevertheless, their safe and effective implementation requires site-specific evaluation and cautious management, especially under conditions of existing soil contamination. Future research should prioritize long-term studies, the assessment of contaminant transfer to edible plant parts, and the development of integrated risk assessment frameworks that incorporate chemical, microbiological, and ecotoxicological indicators to better support agricultural sustainability and public health protection.

Abbreviations

A	<i>Azotobacter vinelandii</i>
B	Biostimulant
B1	Biostimulant 1
B2	Biostimulant 2
B3	Biostimulant 3
C	Commercial tomato variety
CS	Combined stress
PTE	Potential toxic element
S	Shungite
T	Tomres tomato variety
Th	<i>Trichoderma harzianum</i> T22
TOC	Total organic carbon
TPC	Total plate count
WUE	Water use efficiency
WW	Well watered and fertilised

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12302-026-01446-0>.

Supplementary Material 1.

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Author contributions

C.P., E.C., S.B., L.C. and G.G. designed experiments. L.C. performed the analysis of the PTEs. C.P., E.G. and V.P. performed the microbiological and ecotoxicological analysis. C.P., E.C., S.B., E.F. and L.C. interpreted the results. C.P. and E.C. wrote the manuscript. S.B., L.C. and E.F. revised the manuscript. S.F. performed and interpreted the multivariate analysis. All authors read and approved the final manuscript.

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Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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