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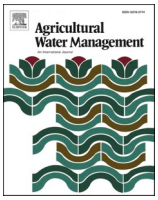

Managing microbial risks in informal wastewater-irrigated agriculture through irrigation water substitution

Luis Fernando Perez-Mercado [a,b,*], Cecilia Lalander [a], Abraham Joel [c], Jakob Ottoson [d], Mercedes Iriarte [b], Björn Vinnerås [a]

[a] Department of Energy and Technology, Swedish University of Agricultural Sciences, Box 7032, 75007 Uppsala, Sweden
[b] Center for Water and Environmental Sanitation (Centro de Aguas y Saneamiento Ambiental, CASA), Universidad Mayor de San Simon, Calle Sucre y Parque Latorre, Cochabamba, Bolivia
[c] Department of Soil and Environment, Swedish University of Agricultural Sciences, Box 7014, 75007 Uppsala, Sweden
[d] Department of Risk and Benefit Assessment, Swedish Food Agency, Box 622, 75126 Uppsala, Sweden

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ABSTRACT

On-farm measures can be used in multi-barrier schemes to manage microbial risks from consumption of wastewater-irrigated vegetables, especially where informality of the practice determines minimal external support for farmers. Evidence indicates that cessation of irrigation greatly reduces microbial contamination on leafy vegetables, but at the expense of produce quality. Replacing wastewater with higher-quality irrigation water during the last days of cultivation is an alternative to cessation of irrigation that does not compromise produce quality. This study evaluated the effect of wastewater substitution under on-farm conditions on different indicators of microbial contamination of lettuce. Lettuce was cultivated in experimental plots and irrigated with three water sources: spring water, water from a wastewater-polluted river and effluent from a primary wastewater treatment plant, but with the river water replaced by spring water in half the plots about two weeks before harvest. The experiment was repeated four times in different seasons. Irrigation water samples collected during cultivation and lettuce samples collected at harvest were analysed for helminth eggs, *Escherichia coli* and coliphages. Variables characterizing the irrigation practices and environmental conditions were recorded. There were no significant differences in helminth egg or *E. coli* concentrations on lettuce (medians ranged from −0.7 to −0.1 $\log_{10}$ eggs g^{-1} and 0.6–1.4 $\log_{10}$ cfu g^{-1}, respectively) between any of the treatments involving wastewater irrigation; no statistical analysis was possible for coliphages because concentrations on lettuce were mostly at or below the detection limit (94% of samples). Variables associated with temperature and soil explained helminth egg and *E. coli* concentrations on lettuce, while number of days of irrigation with spring water (representing wastewater substitution) was significant only for *E. coli*. It was concluded that the experimental conditions were suboptimal for successful implementation of wastewater substitution for on-farm microbial risk management, but key variables for successful implementation were identified.

1. Introduction

Reusing wastewater for crop irrigation is an effective way to recirculate plant nutrients and water, thereby contributing to achievement of a circular economy (Chojnacka et al., 2020; Mahjoub et al., 2018). However, using wastewater for irrigation poses risks to human health by introducing pathogens into agricultural production systems. These risks are higher in low- and middle-income countries, where coverage by wastewater treatment services is generally low (Qadir et al., 2010), existing treatment plants commonly face sustainability problems that hamper their performance (Cossio et al., 2019) and irrigation with wastewater is mostly informal (Scott et al., 2009) (*i.e.* unplanned, unregulated and with minimal external support for farmers, Huibers et al., 2009). The risks posed by wastewater irrigation to human health and the environment derive from variety of pollutants (WWAP, 2017), but pathogens are the major concern in low- and low-middle-income countries (Prüss-Ustün et al., 2019). In order to address the risks from pathogens, a multi-barrier approach in which different measures act as

* Corresponding author at: Department of Energy and Technology, Swedish University of Agricultural Sciences, Box 7032, 75007 Uppsala, Sweden.
E-mail addresses: fernando.perez@slu.se, ferpermer@yahoo.com (L.F. Perez-Mercado).

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barriers along the farm-to-fork pathway has been proposed, to complement and even replace treatment plants (WHO, 2006). Some examples of such barriers are rinsing the produce with clean water right after harvest and disinfecting the vegetables before consumption. There are various measures available at farm level to reduce microbial contamination of produce, *e.g.* allowing irrigation water to settle and modifying irrigation technique and regime (Keraita et al., 2014). In informal contexts -where farmers lack external support and wastewater treatment is not necessarily a feasible alternative-, on-farm measures are among the few realistic alternatives to manage microbial risks posed by wastewater irrigation (Keraita et al., 2010).

One suggested on-farm measure is cessation of irrigation several days prior to harvest, to allow time for natural pathogen die-off on the crop (Qadir et al., 2010). The actual level of die-off will depend on environmental factors such as temperature, humidity and exposure to sunlight (Sánchez and Bosch, 2016). According to the WHO (2006), die-off of bacteria and viruses on crop surfaces is 0.5 $\log_{10}$ day^{-1} in cool, wet weather and reduced exposure to direct sunlight and 2 $\log_{10}$ d^{-1} in hot, dry weather. Based on this, cessation of irrigation is considered a reliable barrier (Keraita et al., 2010). However, many field studies have shown much higher variability and complexity in die-off rates than estimated by the WHO (2006), *e.g.* reduction rates from ~2 $\log_{10}$ within 2 h to 1.7 $\log_{10}$ in 7 days have been reported for the pathogenic bacterium *E. coli* O157:H7 on leafy crops (Gutiérrez-Rodríguez et al., 2012; Moyne et al., 2020). Other studies have found that bacterial die-off on vegetables can be biphasic (*i.e.* two decay rates instead of only one), due to the existence of persistent subpopulations of bacteria that can decay as slowly as 0.01 $\log_{10}$ day^{-1} (Belias et al., 2020) after several hours or days following an initial rapid decay of the less-persistent subpopulation (Seidu et al., 2013). Studies by Belias et al. (2020) and Chase et al., (2019, 2017) even found periods of *E. coli* growth on vegetable crop surfaces. As regards variability, similar findings have been made for enteric viruses. For example, Li and Uyttendaele (2018) found a reduction of > 5.5 $\log_{10}$ in 3 days for murine norovirus (MNV-1) on basil leaves, while an earlier study found rates of 0.01 and 0.12 $\log_{10}$ d^{-1} for human adenovirus (HAV) on cantaloupe and lettuce, respectively (Stine et al., 2005). A biphasic decay rate has also been reported for the bacteriophage virus *Bacteroides fragilis* on lettuce (Petterson et al., 2001).

The unexpected variability and complexity in literature findings suggested that the dynamics of microbe die-off on vegetables under field conditions are insufficiently well understood. Some studies examining multiple factors have been conducted to better understand the dynamics of bacterial die-off (Belias et al., 2020; Castro-Ibáñez et al., 2015; Park et al., 2015). Those studies identified some key explanatory factors (*e.g.* bacterial species, crop species, relative ambient humidity, temperature and precipitation), but the findings appeared to be specific to the context of the study. For example, relative humidity was found to affect the survival of *E. coli* in the study by Belias et al. (2020), while it did not show any apparent effect in the study by Castro-Ibáñez et al. (2015). Therefore, there is a continuing need for multi-factor studies of microbe die-off dynamics under different field conditions. Multi-factor studies are also needed on other pathogens that are present in wastewater and persist longer than bacteria in the environment, such as viruses and helminths (Aw, 2018). For instance, the bacteria *Salmonella* spp. can survive for up to 70 days in soil and 30 days on crops, while enteroviruses survive 30 days longer in both surfaces, and the eggs of the helminth *Ascaris lumbricoides* survive many months in soil and up to 60 days on crops (Ilic et al., 2009). Investigating die-off of multiple types of pathogens is of critical importance in relation to cessation of irrigation as part of the multi-barrier approach, as differences in their persistence can determine the need for additional measures. Thus, more information about die-off of different types of foodborne pathogens under field conditions is necessary.

A major challenge with cessation of irrigation is its effect on the physical quality and yield of vegetable crops, which has been found to reduce acceptance of the measure among farmers (Amoah et al., 2011; Mayilla et al., 2016). Replacing wastewater with higher-quality irrigation water, instead of ceasing irrigation, could be a viable option to extend the die-off period without affecting produce quality. While alternative higher-quality water sources are most likely unavailable in regions where wastewater irrigation is performed, it is possible to envision a system in which higher-quality effluent from the same wastewater treatment source is produced and used for irrigation. In such a system, wastewater would be used for bulk irrigation during the initial stages of crop growth, while at the same time small wastewater volumes would be diverted to on-farm water treatment units and then stored. Various on-farm treatments, such as on-farm ponds (Keraita et al., 2014) and filtration systems (Kaetzl et al., 2019; Perez-Mercado et al., 2019), have been shown to reduce the concentrations of pathogens in wastewater. While these treatments cannot handle the large flows needed for irrigation, they can probably efficiently treat smaller flows (Perez-Mercado et al., 2019), such as untreated wastewater volumes diverted during bulk irrigation. The treated wastewater would be accumulated on-farm until two weeks prior to harvest and used then as the sole source for crop irrigation, replacing untreated wastewater before harvest and thus extending the pathogen die-off period.

To our knowledge, no previous study has examined extended die-off following irrigation water substitution. The aim of this study was therefore to test the core mechanism of the envisioned extended die-off system. This was done by assessing the impact of replacing wastewater with a higher-quality water source on the microbial hygiene status of wastewater-irrigated lettuce in field conditions, using Cochabamba, Bolivia, as an example of a semi-arid agricultural region where informal wastewater irrigation is practiced. Lettuce was chosen because it is the crop for raw consumption with the largest area (~2 800 ha in 2018) harvested in Bolivia (INIAF et al., 2019) and it has been found to be the main crop in several production systems that use wastewater for irrigation (Perez-Mercado et al., 2018). Specific objectives were to: i) assess the concentration of viral, bacterial and pathogenic helminth indicators on lettuce samples in plots where wastewater was replaced with a cleaner irrigation water source for two weeks before harvesting; and ii) analyse the combined effects of water quality, irrigation regime and environmental factors on microbial contamination of lettuce.

2. Methodology

2.1. Experimental set-up

The effect of wastewater substitution in experimental field lettuce plots supplied by furrow irrigation was investigated by determining the concentrations of viral, bacterial and helminth indicators (Fig. 1). In total, six treatments were evaluated during four different crop cycles in different seasons. Four treatments were defined based on combining two factors: i) frequency of irrigation events throughout the crop cycle, and ii) replacement of a wastewater-polluted river water as the source for irrigation. Two irrigation frequencies (3 and 2 irrigations per week, representing intervals of 2.3 and 3.5 days, respectively) throughout the cycle were defined, based on previous work showing these to be common practice in lettuce cultivation in the study region (Perez-Mercado et al., 2018). For substitution of the wastewater-polluted source, two alternatives were assessed: a) no substitution (*i.e.* irrigating with wastewater-polluted river water for the entire crop cycle), and b) substitution with cleaner water from a nearby spring during the last two weeks before harvest (the spring water had lower levels of pathogens -see Table S2 in Supplementary Material- and was used as a proxy for wastewater treatment on-farm). Combining the two irrigation frequencies with the two substitution alternatives resulted in four treatments: Three irrigations per week only with wastewater-polluted source (River 3/week); two irrigations per week only with wastewater-polluted river water (River 2/week); three irrigations per week with substitution of wastewater-polluted water (Riv&Spring 3/week); and two irrigations

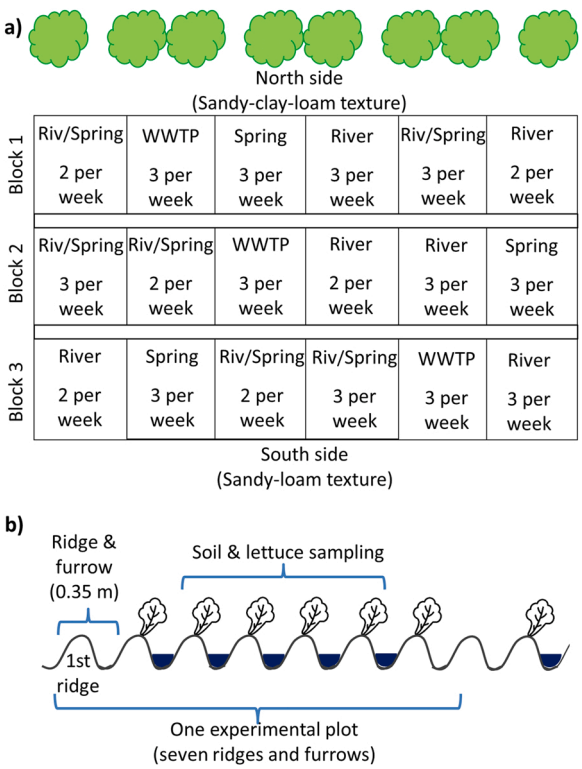

Fig. 1. a) Location of the experimental plots relative to a nearby forest, and block and plot layout. b) Cross-sectional view of an experimental plot showing ridges and adjacent furrows and planted, irrigated and sampled.
Each treatment was defined by water source used for irrigation (river water, spring water, primary effluent from a wastewater treatment plant (WWTP) and river water replaced by spring water several days before harvest (Riv/Spring)) and irrigation frequency during cultivation (2 and 3 times per week).

per week with substitution of wastewater-polluted source (Riv&Spring 2/week). Two control treatments were also included: irrigation with spring water three times per week (Spring 3/week), representing a best case scenario; and irrigation with effluent from primary treatment (upflow anaerobic sludge blanket (UASB) reactor) at a nearby wastewater treatment plant (WWTP) three times per week (WWTP 3/week), representing a worst case scenario.

2.2. Lettuce plots and irrigation system

The experiment was conducted at San Pedro Magisterio Treatment Plant, Sacaba municipality, Cochabamba, Bolivia, a semi-arid region with mean annual temperature 16–18 °C, mean relative ambient humidity 45% and mean annual rainfall 400–500 mm, of which less than 40 mm falls between April and September (Gossweiler et al., 2019; Salini Calderón and Medina Mitma, 2017). The soil at the site was found to transition from sandy-clay loam texture at the north side of the field to sandy loam at the south side, with moderately alkaline pH (7.9–8.1), 6.7 dS m^{-1} salinity, 1.2–2.6% of total organic matter and bulk density 1.4–1.5 g cm^{-3} (see Table S1 in Supplementary Material). The soil had not been used for agricultural production for at least seven years prior to the experiment, but had occasionally received raw wastewater diverted from the nearby WWTP during intense rainfall events and maintenance tasks.

In order to account for the effect of differences in soil texture, shade from nearby trees and slope (north side), the field site was divided into three blocks of 15 m x 5 m each, separated by a 0.7 m wide footpath to avoid cross-contamination (Fig. 1a). Each block was divided into six plots (2.5 m wide x 4 m long) and the experiment was laid out in a randomised complete block design, with which each of the six treatments randomly assigned to a plot within each block. Seven ridges measuring 0.35 m width (including their respective furrow) and 4.5 m long were created in each plot. The first ridge in each plot was considered the plot edge and was therefore not planted, and the adjacent furrows were not irrigated (Figure1b). All six remaining ridges were planted with lettuce, but only the four central ridges were used for soil and lettuce sampling. The distance between lettuce plants along the ridges was ~15 cm.

All treatments (see Fig. 1a) were applied to the same plots during four crop cycles of lettuce (*Lactuca sativa* var. *crispa*). Agricultural practices were kept the same for all plots during the four crop cycles. Cycles one and two were in spring 2016 (September-December) and cycles three and four were in autumn-winter 2017 (April-August), lasting 47–50, 42–45, 60–61 and 53–55 days, respectively. The agricultural routines followed were as prescribed by an experienced lettuce farmer, who decided the dates for applying fertilisers and pest control, and the irrigation times and amounts (*i.e.* the water volume) for each irrigation event. The harvest dates in each cycle were also defined in agreement with that farmer, but harvesting was carried out on different days for each block due to some constraints in laboratory capacity (see full dataset in Table S3 in Supplementary Material).

Following the experienced farmer's instructions, in the first and third crop cycles dried cow manure was applied at ~15 ton ha^{-1} one week before lettuce transplanting. One week after transplanting in all four cycles, a complete foliar fertiliser (NPK) was applied to the soil at ~0.5 ton ha^{-1} (*i.e.* ~0.1, ~0.1 and ~0.03 ton ha^{-1} for N, P_2O_5 and K_20, respectively) and the plantlets were sprayed with an insecticide-fungicide solution diluted in tap water at the dosage recommended on the product label. One month after transplanting, urea was added in pellet form to the soil at a rate of ~30 kg ha^{-1} and the plants were given a second spraying with the insecticide-fungicide solution.

The irrigation method used was furrow irrigation and the water was transported by means of a water pump and assembled PVC pipelines. Irrigation was always performed between 08.00 and 11.00 h or 16.00–18.00 h. When irrigation from different water sources had to be performed on the same day, the sequence started with spring water, followed by river water and finally primary effluent (*i.e.* from cleanest to dirtiest), as the same pipes were used for all water sources. The duration (and therefore the volume) of irrigation for every event and plot was defined by the experienced farmer. The amount of irrigation water was measured on two occasions each during the first and third cycles, and on three occasions during the second cycle, using the volumetric method (no measurements were performed during the fourth cycle). Irrigation runoff seldom occurred and therefore was considered negligible. During the first weeks after transplanting, each plot was irrigated with its corresponding water source (four plots with river water, one plot with spring water and one plot with primary effluent, all randomly assigned per block). About two weeks (11–15 days) before harvest (estimated by the experienced farmer based on crop development), the river water was replaced by spring water in two plots per block.

2.3. Irrigation water sources

2.3.1. River water

Due to unplanned urban expansion in Sacaba municipality, the nearby river Rocha receives wastewater discharges from many sewage systems with no or poor treatment along the valley (Gossweiler et al., 2019). As a result, the river is heavily polluted with domestic wastewater and it is believed that its flow mostly comprises wastewater in the driest months (Huibers et al., 2009). Despite being heavily polluted, the river is still a major source of irrigation water in surrounding periurban and rural zones in Cochabamba (Verbyla et al., 2016), especially during the dry season (March-November) (Perez-Mercado et al., 2018). The river was located around 45 m of the experimental plots, to where river water was pumped.

2.3.2. Effluent from primary wastewater treatment

The San Pedro Magisterio WWTP receives wastewater from 235 households. The incoming wastewater undergoes a preliminary treatment comprised of screening and a rectangular grit chamber. The effluent then passes through a UASB reactor and finally to a horizontal sub-surface flow constructed wetland. The effluent water used for irrigation of the experimental plots was pumped from the inspection chamber located between the UASB reactor and the wetland. This source represented a worst-case scenario in which farmers irrigated their vegetables with undiluted, settled wastewater.

2.3.3. Spring water

The spring water source was located approximately 25 m away from the experimental plots. The emerging water flowed to a dug pond of ~30 m^3 and the overflow was conducted to the river. The water used for irrigation of experimental plots was pumped from the dug pond. No information was available about the source of the spring water, but a slow reduction in water flow was observed during the experimental year, which suggests that the water may infiltrate into the soil in the high part of the basin during the rainy season.

2.4. Analysis of water samples

2.4.1. Sampling

Samples from the three water sources were collected between 08.00 and 11.00 h on 2–3 occasions during each crop cycle (at 2 and 4 weeks after transplantation for the first crop cycle, and at 2, 4 and 5 weeks for the second to fourth crop cycles; see Table S2 in Supplementary Material). In brief, 400 mL from each water source were collected in sterile plastic bottles and transported on ice to the laboratory for analysis of *Escherichia coli* and coliphages. In addition, 10 L of each water type were collected for helminth egg analysis. At the laboratory, all samples were stored at 4 °C. The samples used for *E. coli* and coliphages analysis were processed within 2–4 h after collection, while those for helminth egg analysis were processed within 1–4 weeks. A total of 33 water samples were collected during the four crop cycles.

2.4.2. Processing

The 400 mL water samples were analysed according to method EPA 1603 (U.S.EPA, 2014) and modified EPA 1602 (U.S.EPA, 2001) for *E. coli* and coliphages, respectively. For determination of *E. coli*, 100 mL of water sample were filtered through a membrane onto modified mTEC agar medium, and then incubated at 44 °C for 24 h. Red/magenta colonies were counted at the end of the period. For determination of coliphages, log-phase host bacteria (*E. coli* C3000, ATCC #15597, which is susceptible to somatic and F-specific coliphages) and 100 mL of double-strength molten tryptic soy agar were added to 100 mL of water sample, and the mixture was poured into five Petri plates. After 16–20 h of incubation at 36 °C, circular lysis zones were enumerated. Helminth eggs were extracted following the procedure described in the Mexican norm NMX-AA-113-SCFI- (2012, 2012). In brief, the 10 L samples of the different irrigation waters were allowed to sediment, sieved at 150–170 μm, re-suspended and centrifuged at 400 rpm for 5 min. The sediment was collected and immersed in magnesium sulphate. Any material floating was collected and centrifuged, and the pellet was immersed in ether-ethyl/0.1 N sulphuric acid solution (35/65% v/v). Subsamples of the resulting concentrated material were placed in Neubauer Improved counting chambers with immersion oil and enumerated by microscopy at 100x magnification.

2.5. Analysis of soil and manure samples

2.5.1. Sampling

Soils from the experimental plots were sampled at transplanting (*i.e.* right before transplantation) in each crop cycle. Each plot was sampled by collecting soil from the top 7 cm at six points located at the intersections of a grid formed by the third and sixth ridges widthways and three transverse lines, at 1, 2 and 3 m, along both ridges. The six samples per plot were then mixed to give one composite sample of about 1.5 kg. Manure samples for analysis were collected from three points on the manure pile one week before manure was added to soil in the first crop cycle (see Section 2.2). The manure samples and composite soil samples were placed in plastic bags and transported to the laboratory, where they were analysed on the same day for *E. coli* and coliphages and then stored at 4 °C until helminth analysis. In total, three manure samples and 72 soil samples (6 treatments x 3 blocks x 4 transplantations) were collected during the four crop cycles.

2.5.2. Processing

The same analytical methods as used for the water samples were applied to the soil and manure samples, but modified to suit solid samples according to the procedure described by Verbyla et al. (2016) for *E. coli* and coliphages, and complemented with the Tulane method for helminth eggs (Bowman et al., 2003). For analysis of *E. coli* and coliphages, 25 g of soil sample were added to 225 mL sterile water and shaken vigorously for 1 m, and the mixture was then processed as described for water samples (see Section 2.4.2). For extraction of helminth eggs, 20 g of soil were immersed and homogenised in 200 mL of sterile water and blended. The mixture was diluted to 900 mL with 1% aqueous solution of "7X" (Limbro) (v/v) and allowed to settle overnight, after which the sediment was collected, re-suspended with 300 mL of sterile water and blended again. The homogenised mixture was then passed through a 50-mesh sieve, diluted to 900 mL with "7X" and allowed to settle for 2 h. The sediment was collected and centrifuged, the supernatant was discarded and the pellet was immersed in magnesium sulphate. After that, the same procedure as for water samples was followed (Section 2.4.2).

2.6. Analysis of lettuce samples

2.6.1. Sampling

A composite lettuce sample was collected from each plot at harvest in each crop cycle. To obtain a representative sample, four plants were randomly selected from the four central ridges of each plot (see Section 2.2). Outer leaves whose appearance would make selling of the lettuce difficult were discarded (following the experienced farmer's instructions) using an aseptic technique. Six leaves from each selected plant were cut from the stem and placed in a plastic bag to give one composite sample of 24 leaves (6 leaves x 4 selected plants). The composite samples were transported to the laboratory, where they were analysed on the same day for *E. coli* and coliphages and stored at 4 °C until helminth analysis (storage time varied between 1 and 7 weeks). Helminth eggs were analysed only for the first two crop cycles. Therefore, 36 composite samples (6 treatments x 3 blocks x 2 harvests) were collected and analysed for helminth eggs, while 72 composite samples (6 treatments x 3 blocks x 4 harvests) were analysed for *E.coli* and coliphages.

2.6.2. Processing

The lettuce leaf samples were processed following the same procedure used for soil samples (see Section 2.5.2).

2.7. Statistical analysis

The concentrations of the different indicator microbes on lettuce samples cultivated in the six treatments (see Section 2.1) were tested for significant differences ($\alpha = 0.05$) using one-way analysis of variance (ANOVA) in randomised blocks, after verifying normality in the model residuals and homoscedasticity by means of Shapiro-Wilk and Bartlett tests, respectively. When ANOVA indicated a significant difference, Tukey *post hoc* tests were performed to compare the means of the six treatments ($\alpha = 0.05$). Concentrations below the detection limit were

replaced with a value between zero and the detection limit in the statistical analysis (Section 3.1), following common practice (Wood et al., 2011).

Principal component analysis (PCA) was used to analyse the influence of irrigation practices (irrigation frequency, and time between the last irrigation with wastewater-polluted river water and harvest) and environmental factors (concentrations of microbial indicators in soil at transplantation, number of days until harvest and block) on the concentrations of each of the indicator microorganisms on lettuce leaves. The analysis was based on the relationships between microbial concentrations on lettuce and the target variables when the two principal components (PC) with the highest proportions of explained variance were plotted.

It should be noted that the time between the last irrigation with wastewater-polluted river water and harvest (Substitution.days) included the treatments where no water substitution was performed. For instance, since wastewater-polluted river water was not used for irrigation in the control treatment with spring water (Section 2.1), the value for Substitution.days was the number of days from transplantation until harvest. For the treatment where wastewater-polluted river water was the sole irrigation source, the value for Substitution.days was the number of days between the last irrigation event and harvest. The analysis of graphical relationships in PCA plots was complemented with multiple linear regressions between microbial concentrations on lettuce as response variable and the remaining variables as predictors. Statistical analyses and graphical plotting were carried out with R software (R Foundation for Statistical Computing; Vienna, Austria). Input data for the statistical analysis and the R code applied are shown in Table S3 (sections S5, S6 and S7 in Supplementary Material).

3. Results and discussion

3.1. Concentrations of indicator microorganisms on lettuce

The proportion of lettuce samples with microbial concentrations below the detection limit was different for each indicator microorganism. Helminth eggs and *E. coli* were detected in 56% and 93% of samples, respectively. Values below the detection limit were replaced with − 0.7 $\log_{10}$ for helminth eggs and − 0.05 $\log_{10}$ for *E. coli* (Section 2.7, Table S3). Most samples of lettuce (75%) had coliphage concentrations below the detection limit and consequently it was not possible to perform any statistical analyses on these (Fig. S8).

The median concentration of helminth eggs on lettuce irrigated with spring water (−0.06 $\log_{10}$ eggs g^{-1}) was higher than that in the treatments involving wastewater (*i.e.* ranging from −0.70 to −0.26 $\log_{10}$ eggs g^{-1}, Fig. 2). This was unexpected, since spring water had lower concentrations of faecal microorganisms throughout the experiment (Table S2). As the same pipes were used for all the water sources, plots watered with spring water were always the first to be irrigated during a given day in order to avoid cross-contamination (Section 2.2). However, pathogens remaining in the pipes from the previous day were likely flushed with the first daily irrigation (*i.e.* plots irrigated only with spring water), cross-contaminating the irrigation water and therefore the lettuce. Such cross-contamination has been shown previously for bacteria (Blaustein et al., 2016) and likely affected all the indicator microorganisms, but its effect more evident for helminth eggs because they persist longer in the environment (*i.e.* the pipes), leading to greater accumulation than seen for less persistent organisms over time (Ilic et al., 2009). Therefore we excluded data for lettuce irrigated with spring water in the statistical analysis for helminth eggs. Moreover, during the statistical analysis, we found noticeable differences between the data collected in spring and autumn-winter for *E. coli*. Multiple regressions performed with two separate datasets for *E. coli* yielded higher R-squared values (R^2 =0.27 for spring dataset and 0.22 for autumn-winter dataset) than one dataset (R^2 =0.13) (see Tables S9 and S10 in Supplementary Material). Therefore, *E. coli* concentrations on lettuce were analysed separately for spring and autumn-winter, and the respective results are referred to as spring lettuce or spring *E. coli* and autumn-winter lettuce or autumn-winter *E. coli*.

Median microbe concentration on lettuce irrigated only with wastewater or wastewater-polluted river water ranged from − 0.7 to − 0.1 $\log_{10}$ eggs g^{-1} for helminth eggs, 1.1–1.4 $\log_{10}$ cfu g^{-1} for spring *E.* coli and 0.6–1.2 $\log_{10}$ cfu g^{-1} for autumn-winter *E. coli* (Fig. 2). These values are higher than those reported by Woldetsadik et al. (2017) and Amahmid et al. (2021) (1.6 and 0.7 $\log_{10}$ eggs kg^{-1}, respectively) for helminth eggs on lettuce and coriander furrow-irrigated with

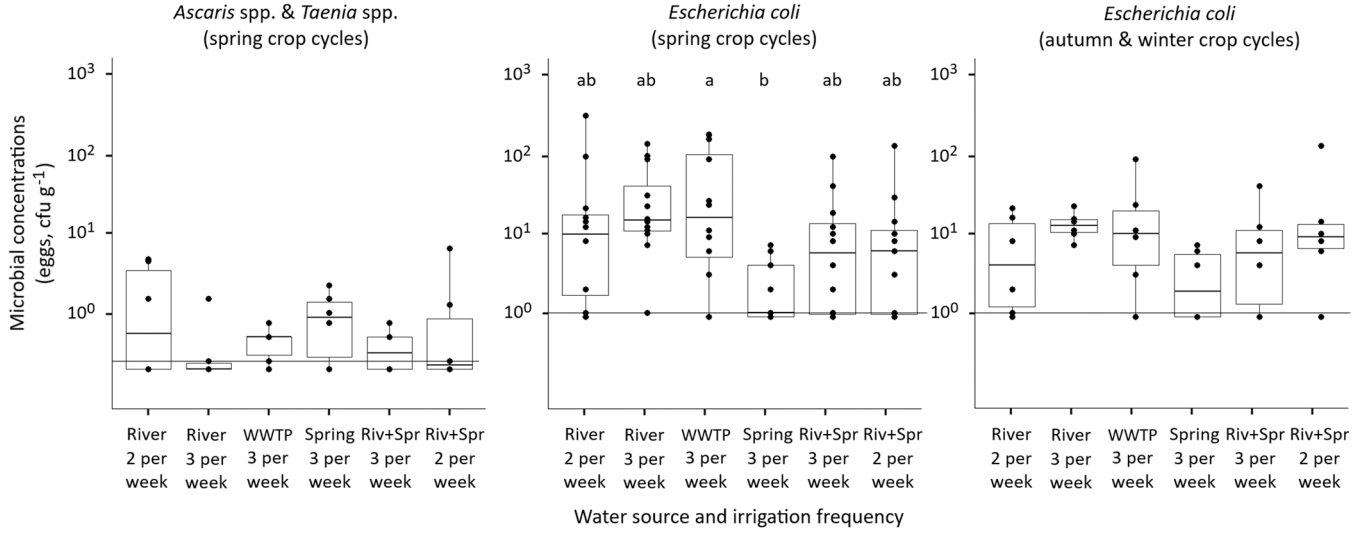

Fig. 2. Concentration of helminth eggs (*Ascaris* spp. and *Taenia* spp.) and *Escherichia coli* on lettuce samples according to season of crop cycle, water source used for irrigation and irrigation frequency during cultivation. Results from Tukey HSD tests performed to compare concentrations after one-way analysis of variance with randomised blocks are also shown. The sources were river water, spring water, effluent from a primary treatment at a wastewater treatment plant (WWTP), and river water replaced by spring water several days before harvest (Riv+Spr). The irrigation frequencies were 2 and 3 times per week. The solid lines indicate the detection limits. Letters on top of each boxplot indicate results from the Tukey test: different letters signify significant difference ($p > 0.05$) in microbial indicator concentration within the season when crop cycles were performed; no letters indicate that no significant differences were found. Data for helminth eggs on lettuce irrigated only with spring water were not included in the Tukey test because of likely cross-contamination.

wastewater, respectively. Since average concentration of helminth eggs in wastewater found in this study (0.37 $\log_{10}$ eggs kg^{-1}) was similar to both Woldetsadik et al. (2017) and Amahmid et al. (2021) (0.25–0.49 and 0.43–0.86 $\log_{10}$ eggs kg^{-1}, respectively), there can be several other reasons for the discrepancy in concentration of helminth eggs on produce between studies. First, irrigation frequency was higher in the present study, with 2–3 weekly irrigations compared with 1–2 in Woldetsadik et al. (2017) and Amahmid et al. (2021). Second, larger volumes of water per irrigation event were used in this study (50–100 L m^{-2}, based on local practice), while Amahmid et al. (2021) applied only 12 L m^{-2}. Third, the leaves of the lettuce cultivated in this study may grow closer to the soil (and, therefore, to the irrigation water) compared with the variety (*i.e.* not specified) cultivated in Woldetsadik et al. (2017) and with the coriander in Amahmid et al. (2021). On the other hand, helminth and *E. coli* concentrations on lettuce irrigated only with wastewater and river water in this study were lower than in our previous study, where we found 1.6 $\log_{10}$ eggs g^{-1} and ~3 $\log_{10}$ cfu g^{-1} for lettuce irrigated with water from the same river (Perez-Mercado et al., 2018). This can be explained by differences in sampling technique, as outer leaves were included in the previous study, while they were discarded before sampling for this study (see Section 2.6.1). Since outer leaves are more exposed to contamination than inner leaves, discarding them presumably reduced the concentrations of microorganisms to the levels observed in this study.

The concentrations of helminth eggs (*Ascaris* spp. and *Taenia* spp.) and *E. coli* on lettuce were not significantly different between any of the treatments involving wastewater for irrigation (Fig. 2). This contradicted our hypothesis that late irrigation of lettuce with higher-quality water would reduce microbial contamination of the lettuce. Considering the favourable conditions for die-off of microorganisms, with the semi-arid climate in Cochabamba and sunlight exposure during 11–15 days (Ottoson et al., 2011; Stine et al., 2005), the high *E. coli* concentration found on lettuce is particularly surprising. Although not significantly different, the median concentrations of *E. coli* on spring lettuce with irrigation water substitution were consistently numerically lower (by around 0.4–0.6 $\log_{10}$) than on spring lettuce irrigated only with river water. In contrast, the median concentration of helminth eggs on spring lettuce with irrigation water substitution were numerically similar to or lower than the median concentrations on lettuce irrigated only with river water. The median concentrations of autumn-winter *E. coli* with irrigation water substitution also varied from lower to higher than those on lettuce irrigated only with river (Fig. 2). Assuming a constant decay rate since the last irrigation with river water, the reduction in helminth eggs (0–0.4 $\log_{10}$ in 11–15 days) was lower than that reported by (Amahmid et al., 1999) on coriander (0.9 $\log_{10}$ in 7 days) and by Amahmid et al. (2021) on lettuce (0.5 $\log_{10}$ in 7 days). Likewise, the reductions in *E. coli* throughout the four crop cycles ranged from 0.6 $\log_{10}$ in 11–15 days in spring to "negative reduction" (likely due to bacterial growth) in autumn-winter, which were lower than the lowest reduction determined by Belias et al. (2020) for *E. coli* on spinach (0.07 $\log_{10}$ day^{-1}) and much lower than the minimum reference value (~0.5 $\log_{10}$ day^{-1}) set by the WHO (2006). However, the reductions in our study were similar to those found for persistent subpopulations on lettuce of the helminth *Ascaris suum* (~0.2 $\log_{10}$ in 25 days) by Seidu et al. (2013) and for *E. coli* (some persistent subpopulations increased their concentrations) by Belias et al. (2020), in modelling studies assuming biphasic decay patterns in die-off. This suggests that die-off is biphasic for both organisms, and indicates that their persistent subpopulations can have a strong influence on concentrations on lettuce, limiting the efficiency of extended die-off from irrigation water substitution.

3.2. Effect of multiple factors on microbial contamination of lettuce

3.2.1. Factors affecting concentrations of helminth eggs on lettuce

The PCA and linear regression results showed that the concentrations of helminth eggs on lettuce had a significant direct relationship with the length of the crop cycle (time between transplantation and harvest) (Fig. 3 and Table S9). There are two possible reasons for this, the first being the difference in temperature between crop cycles. The length of a crop cycle is determined by the time it takes lettuce to reach commercial size and thus depends on the ambient conditions. A longer crop cycle and higher concentrations of helminth eggs on lettuce were both seen at lower minimum daily temperatures (11.7 °C) than in the shorter crop cycle (14.6 °C) (Table S11 in Supplementary Material). This is in agreement with conclusions drawn in recent reviews on *Ascaris* spp. (Asaolu and Ofoezie, 2019) and *Taenia* spp. (Jansen et al., 2021) that the persistence of the eggs decreases with increasing ambient temperature. However, those conclusions were based on investigations comparing survival at constant temperatures with larger differences than seen in this study (8–18 °C for *Ascaris* spp., 5–20 °C for *Taenia* sp.). Thus it is uncertain whether the difference in temperature between crop cycles in this study was enough to have a significant impact on helminth egg prevalence. The second possible reason for the relationship between helminth egg concentrations and crop cycle length was the higher number of irrigation events during the longer crop cycle, since every additional irrigation event represents an additional opportunity for lettuce contamination due to wastewater splash, resulting in higher concentrations of persistent microorganisms, such as helminth eggs, on the crop (see Section 3.1).

Time between irrigation events had a significant inverse relationship with concentration of helminth eggs on lettuce, meaning that concentrations of helminth eggs on lettuce with two irrigations per week tended to be higher than with three irrigations per week (Fig. 3, Table S9). This was unexpected, because a longer interval between irrigation events implies longer exposure of microorganisms to drier conditions, sunlight and low relative humidity (RH 35–36%, see Table S11), which is known to hamper environmental survival of helminth eggs (RH ≥35% for *Taenia* spp. (Jansen et al., 2021) and ≥ 40% for *Ascaris* spp. (Asaolu and Ofoezie, 2019)). A possible explanation is variation in turgor of lettuce leaves, with the longer irrigation interval likely causing greater loss of turgor than the shorter interval (Kirkham, 2014). As turgor loss causes temporary wilting of leaves, it can be speculated that contact between leaves and soil or irrigation water increased, favouring contamination.

The concentration of helminth eggs in soil before transplantation had a direct relationship with concentrations on lettuce (Fig. 3, Table S9).

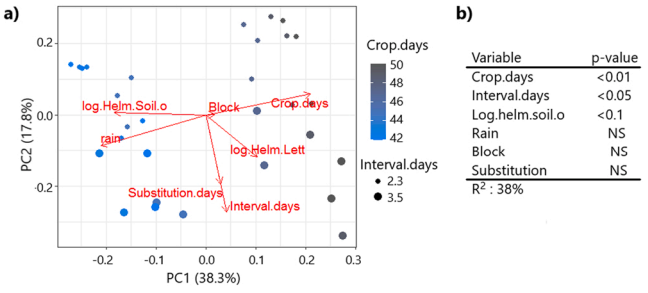

Fig. 3. a) Graphical representation of relationships between variables influencing the concentration of helminth eggs on lettuce irrigated with wastewater according to principal component analysis (PCA), and b) coefficient of determination (R^2) and significance level of each variable in the multiple linear regression model in helminth eggs on lettuce (log.Helm.Lett) and the variables in the PCA. The percentage of variance explained by the two main principal components (PC1 and PC2) is also shown in a). Results of the multiple linear regressions are available in Table S9 in Supplementary Material. *Log.Helm.Lett* = logarithm of the concentrations of helminth eggs on lettuce, *Substitution.days* = time between last irrigation with wastewater-polluted river water and harvest, *Log.helm.soil.o* = logarithm of the concentrations of helminth eggs in soil before transplantation of lettuce, *Crop.days* = time between transplantation and harvest, *Block* = block where lettuce was cultivated, *Interval.days* = time between each irrigation event and *Rain* = occurrence of rain during the cultivation. NS = not significant.

This is in line with Jimenez et al. (2006), who found that concentrations of helminth eggs in spinach leaves increased with increasing concentrations of helminth eggs in soils fertilised with ECOSAN (a dry, source-separation latrine sludge). Helminth eggs in soil before transplantation likely originated from manure addition to soil for the first cycle (Table S4). However, the higher concentrations before transplantation for the second crop cycle compared with the first (Table S3) suggest that the relative contribution of helminth eggs from manure would decrease after several crop cycles due to continuous addition of helminth eggs from wastewater-polluted river water. As there is no evidence of helminth egg internalisation in crops through the roots, transference of helminth eggs to crops likely occurred *via* splashing of contaminated soil onto aboveground plant parts (Alegbeleye et al., 2018). The cropping system in the present study included soil hoeing two or three times per crop cycle in order to loosen the soil, which involves translocation and suspension of soil particles (Ziegler et al., 2007) and potentially contamination of the crop. Furthermore, as helminth eggs survive longer in soil than on vegetables (Stien and Schwartzbrod, 1990), contamination of irrigation water with helminth eggs from soil (*i.e.* re-suspension of helminth eggs from soil) followed by water splashing to lettuce is plausible. Such a mechanism could counteract the effect of wastewater substitution, by contaminating the cleaner irrigation water. To our knowledge, none of these routes of contamination (splashing of contaminated soil or re-contaminated water applied by furrow) from soil to produce has been thoroughly studied to date, and thus further investigations are needed.

3.2.2. *Escherichia coli*

As previously mentioned (Section 3.1), the data on *E. coli* concentrations on lettuce in the spring and autumn-winter crop cycles were analysed separately, because of marked differences between these seasons. Overall, median *E. coli* concentrations on lettuce were lower with irrigation water substitution in spring, but not in autumn-winter (Fig. 2). Furthermore, in autumn-winter, median *E. coli* concentrations on lettuce irrigated only with spring water were statistically similar to those on lettuce irrigated only with wastewater. Both findings indicate lower die-off of *E. coli* during autumn-winter compared with spring, which can be explained by the different temperature regimes in these seasons (Table S11, Fig. S12). Minimum daily temperatures in spring were mostly above 12 °C and tended to increase (to 15 °C) by harvest, while temperatures in autumn-winter were mostly below 12 °C (up to 5 °C) and tended to decrease by harvest (Fig. S12). Higher die-off of *E. coli* with increasing temperatures has been reported in previous studies, *e.g.* Ottoson et al. (2011) found significantly higher concentrations of *E. coli* on lettuce leaves exposed to light at 11 °C than at 18 and 25 °C, while McEvoy et al. (2009) observed no loss of viability in *E. coli* populations on lettuce at 5 °C. Apart from the linear relationship with *E. coli* concentrations on lettuce, seasonal temperatures (*i.e.* spring and autumn-winter) also affected the relationships between *E. coli* on lettuce and other variables. For instance, the time between irrigation events was significant for *E.* coli in spring, but not in autumn-winter (Fig. 4). This means that temperature regime, and perhaps other seasonal weather factors, conditioned the effect of these variables.

Time between the last irrigation with wastewater-polluted river water and harvest was the only variable with a significant (inverse) relationship with *E. coli* concentrations on lettuce in the spring and autumn-winter datasets (Fig. 4, Table S10). This suggests that concentrations of *E. coli* on lettuce with irrigation water substitution were lower than with no substitution, especially in spring cycles (Fig. 2, Section 3.1), although the difference was not sufficiently large to be statistically significant. Cross-contamination of the spring water could partly explain this lack of significance, with spring water continuing to contaminate the lettuce during river water substitution, as *E. coli* and even helminth eggs were found in spring water samples at similar/higher concentrations (Table S2) to the threshold values for unrestricted irrigation of 10^4 cfu of faecal bacteria and 1 helminth egg L^{-1} (WHO, 2006). The degree

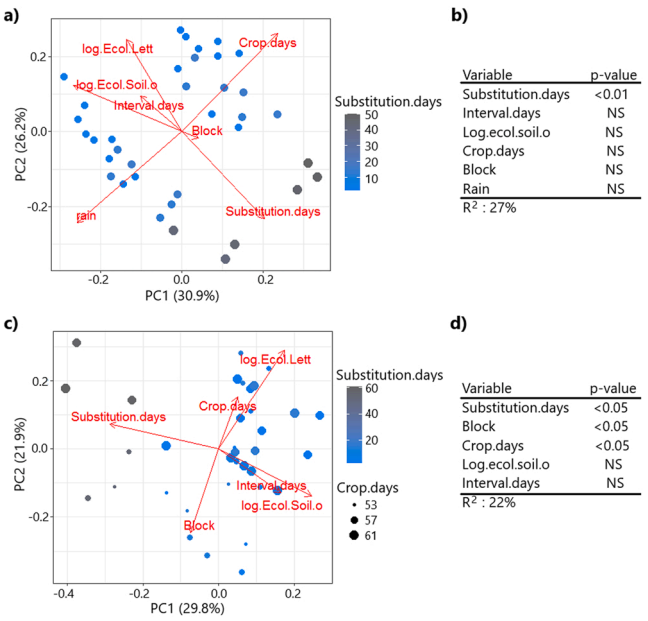

Fig. 4. Graphical representation of relationships between variables according to principal component analysis (PCA) and percentage of variance explained by the two main principal components (PC1 and PC2) for concentration of *E. coli* on lettuce (log.Ecol.Lett) irrigated with wastewater in a) two crop cycles in spring, and c) two autumn-winter crop cycles. The coefficient of determination (R^2) and significance level of each variable from multiple linear regression models between *E. coli* on lettuce (log.Ecol.Lett) and the variables in the respective PCAs are shown in b) for both spring cycles and d) for autumn-winter cycles. Results of the multiple linear regressions are shown in Table S10 in Supplementary Material. *Log.ecol.lett* = logarithm of the concentrations of *E. coli* on lettuce, *Substitution.days* = time between last irrigation with wastewater-polluted source and harvest, *Block* = block where lettuce was cultivated, *Crop.days* = time between transplantation and harvest, *Log.ecol.soil.o* = concentration of *E. coli* in soil before transplantation of lettuce, *Interval.days* = time between each irrigation event, and *Rain* = occurrence of rain during the cultivation. NS= not significant.

of cross-contamination will depend heavily on the microbial quality of the cleaner source, but the mechanism is plausible and should be considered when planning water substitution.

For autumn-winter lettuce, time between transplantation and harvest and blocks were the only two variables significantly related to *E. coli* concentration on lettuce (Fig. 4). Concentrations of *E. coli* on lettuce tended to be higher with longer time between transplantation and harvest (Table S10). This is in line with our finding for helminth eggs (Section 3.2.1), *i.e.* a longer period between transplantation and harvest is required at lower temperatures, which are in turn favourable for microbial survival, and a longer cropping period also requires more irrigation events, creating more opportunities for contamination and cross-contamination. Experimental block showed an inverse relationship with *E. coli* on lettuce (Table S10), with plots located closer to the shaded and slightly more clayey north side of the field (see Section 2.2 and Fig. 1) having higher *E. coli* concentrations. This influence of blocks on the *E. coli* concentration was likely due to increasing shade from the trees during autumn-winter, due to Bolivia's latitude. Having fewer hours of sunlight appeared to increase survival of bacteria, and likely also caused a gradient of lower temperatures in blocks towards the north side, which may have been favourable for *E. coli* survival on vegetable surfaces (McEvoy et al., 2009; Ottoson et al., 2011). The shade likely also improved bacterial survival in the soil by reducing evapotranspiration, resulting in higher soil moisture levels. Higher clay content probably also increased soil moisture retention. Survival of *E. coli* is widely reported to be higher in soils under saturated/flooded conditions (see review by Alegbeleye et al., 2018). Higher *E. coli* survival in the soil may

have increased internalisation of bacteria from soil into the lettuce (Wright et al., 2017), and may also have acted as a reservoir for cross-contamination of irrigation water.

3.2.3. Implications of irrigation water substitution as an on-farm measure

Under the experimental conditions in this study, substitution of irrigation water did not significantly reduce the concentrations of helminth eggs or *E. coli* on lettuce, and explained little of the variation in *E. coli* concentrations on lettuce. However, most variables found to be significant in explaining concentrations of the indicator microorganisms on lettuce were related, directly or indirectly, to two major factors: temperature and soil acting as a reservoir of faecal microorganisms. The impact of temperature was linked to three significant variables: i) time between transplantation and harvest (helminths and autumn-winter *E. coli*), ii) different concentrations/interactions of *E. coli* on spring and autumn-winter lettuce, and iii) shade effect from nearby trees (autumn-winter *E. coli*). The impact of soil as pathogen reservoir was linked to i) concentrations of microorganisms during transplantation (helminth eggs), ii) time between transplantation and harvest, and iii) shade effect from nearby trees. Based on both major factors (temperature and soil), it is possible to make some recommendations on use of irrigation water substitution as an on-farm measure to reduce microbial risk.

Regarding temperature, our results indicated that irrigation water substitution begins to reduce *E. coli* at minimum daily temperatures between 12 and 15 °C and that concentrations of helminth eggs on lettuce are lower with increasing temperature. Therefore, greater reductions in *E. coli* and some reduction in helminth eggs can be expected if irrigation water substitution is carried out in hot countries. This is in line with previous findings of higher reduction rates on lettuce after irrigation cessation in locations warmer than Cochabamba (>1.5 $\log_{10}$ day^{-1} for *E. coli* in Ghana (Seidu et al., 2013) and ~ 0.5 $\log_{10}$ day^{-1} for helminth eggs in Morocco (Amahmid et al., 2021)). Thus, irrigation water substitution as an on-farm measure could significantly reduce faecal microorganisms in regions where minimum daily temperature is at least 15 °C.

Soil acting as a reservoir of helminth eggs and *E. coli* (when temperature and humidity are favourable for *E. coli* survival) can likely counteract other mechanisms that decrease the *E. coli* concentration, including irrigation water substitution. Thus lower concentrations of *E. coli* and helminth eggs following irrigation water substitution could be expected when concentrations in soil and mechanisms for their transference to crop are limited. This might be achieved by reducing the volume of water applied, as indicated when comparing the water volumes and microorganism concentrations applied in this and previous studies (as discussed in Section 3.1). There is very often scope for reducing irrigation water volume, as water consumption efficiency by the crop with furrow irrigation varies between 50% and 70% (Brouwer et al., 1989). The efficiency can be improved by simple measures such as decreasing the length of furrows or applying irrigation water to the furrows in pulses in order to reduce the losses due to soil infiltration (Brouwer et al., 1989; van Opstal et al., 2021). Implementing drip irrigation could be also an alternative, as it reduces water volume and splash compared with furrow irrigation (Song et al., 2006), although its affordability in informal irrigation contexts should be investigated. Therefore, in other contexts where lower irrigation volumes or other irrigation techniques are applied, water substitution might be more efficient than found in this study. Caution is needed, however, as highly persistent organisms such as helminths or protozoa could accumulate in soil during many crop cycles despite lower irrigation volumes, likely resulting in increasing risks of crop contamination over time (Amoah et al., 2018).

Much of the variation in faecal microorganism concentrations remains unexplained by the variables considered in this study. The linear regression models for concentration of helminth eggs and *E. coli* on lettuce accounted for 38% and 22–27% of variance, respectively. Any die-off from irrigation water substitution could thus have been counteracted by other factors not considered or monitored in this study. For instance, Paez-Rubio et al. (2005) identified wind as a transporter of *E. coli* at a flooded wastewater irrigation site. The large variability in microbial concentrations on lettuce between the different treatments in this study should also be highlighted (*e.g. E. coli* ranged from 0 to >2 $\log_{10}$ g^{-1}; Fig. 2). This variability made it difficult to determine the reduction achieved by irrigation water substitution, as some microbial concentrations on lettuce were similar or even higher with water substitution than in the control (Fig. 2). Further research is required to determine whether this variability can be reduced and how it affects the health risks from consumption of lettuce following irrigation water substitution.

From a more practical perspective, contamination from irrigation pipes should be considered if irrigation water substitution is implemented. We found this effect to be especially significant for highly persistent pathogens, as using dirty pipes when irrigating with spring water resulted in concentrations of helminth eggs similar to those on lettuce irrigated with wastewater-polluted river water (Section 3.1). Although we omitted these data to avoid misleading results, they imply that cross-contamination *via* pipes could counteract the effect of irrigation water substitution in any context where irrigation devices and infrastructure may be shared, as in Andean zones of Bolivia (del Callejo-Veracc, 2019). This issue could be overcome by simply flushing the pipes with cleaner water before irrigation, a practice that should be emphasised as an essential part of irrigation water substitution.

4. Conclusions

Water substitution as an on-farm barrier did not significantly reduce the concentrations of helminth eggs and *E. coli* on lettuce mainly irrigated with wastewater. Temperature and soil acting as a reservoir were the main factors explaining the concentrations of both microbial indicators on lettuce, while water substitution explained the concentrations of *E. coli* to a smaller extent. Water substitution reduced bacteria concentrations on lettuce, but the effect was largely counteracted by low temperatures in autumn-winter and cross-contamination from soil facilitated by the low temperatures, shade from trees, soil texture and irrigation regime. Thus, we concluded that the conditions in which the study was undertaken were suboptimal for implementing water substitution as on-farm measure for risk management of wastewater irrigation. Further investigations -especially on influence of temperature regime on microbial survival on crops and microbial transference from soils, both in field studies are required to determine whether water substitution can be optimised as a microbial barrier under the studied conditions or whether it is a more appropriate on-farm measure for warmer regions.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agwat.2022.107733.

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