Agricultural Water Management 301 (2024) 108966

Contents lists available at ScienceDirect

Agricultural Water Management

journal homepage: www.elsevier.com/locate/agwat

Wastewater as a nutrient source for hydroponic production of lettuce: Summer and winter growth

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ARTICLE INFO

Handling Editor - Dr. B.E. Clothier

Keywords:
Hydroponics
Wastewater reuse
Lettuce *(var. crispa L.)*
Water quality
Plant growth

ABSTRACT

Solutions combining soilless cropping systems with wastewater reuse can offer benefits in the agriculture sector, reducing pressure on water resources, promoting sustainable production, and reclaiming wastewater. However, assessing the sanitary risks associated with wastewater reuse is of utmost importance. This study aimed to investigate the hydroponic growth of lettuce (*Lactuca sativa var. crispa L.)* in wastewater from an urban treatment plant with different levels of treatment and evaluate potential sanitary risks. Crop growth took place in a greenhouse, during summer and winter periods, using wastewater after primary (PTW) or secondary (STW) treatment, and a nutrient solution (NS), as control. Physical and chemical water quality parameters, morphological crop growth parameters, and environmental conditions inside the greenhouse were monitored. Toxicity analyses were carried out through cell viability assays with the Caco-2 cell line and total coliforms and *Escherichia coli* (*E. coli)* were determined. Wastewater-grown plants achieved acceptable growth, even though presenting lower fresh weight than NS-plants. STW-plants' growth was limited essentially by nutrient deficiency, and PTW-plants were affected by nutrient deficiency, pH values, solid load, and N-NO_2 concentration. Higher temperatures in summer led to faster crop growth, and lower temperatures in winter allowed better nutrient uptake by the crop. Wastewater-grown plants did not evidence toxicity in leaf extracts up to 1 % w/v. Coliform enumeration data indicated an accumulation in plant roots, with high removal from the wastewater. *E. coli* was not detected on plants' leaves and total coliforms were within acceptable limits. Furthermore, the results point to an improvement in the wastewater quality, with minimum removal values of 75.2 % BOD_5, 83.1 % COD, 43.4 % P, 44.9 % N, and 90.4 % K. The results demonstrated the viability of wastewater reuse for hydroponic production allowing a better understanding of its processes and contributing to mitigating water scarcity for food production, and the impacts of treated wastewater discharge in freshwater courses, particularly those associated with nutrient delivery to aquatic systems.

1. Introduction

Agriculture causes a wide range of negative environmental impacts, including high pressure on water resources, accounting for 69 % of global freshwater (United Nations, 2021). It also leads to fast-depleting land and soil pollution (Sharma et al., 2018). Therefore it is a priority to search for more efficient technologies that aim for sustainable production, reducing water consumption and waste generation, inducing wastewater reuse, and soil conservation (Tomasi et al., 2014).

Soilless cropping systems are an alternative to conventional agriculture due to known benefits such as shorter cultivation cycles, high densities of cultivated plants, absence of weeds, and avoidance of crop rotations. Additionally, these systems reduce risks of soil infection, pollution, and impoverishment (Tomasi et al., 2014). Hydroponics is a technique for growing plants in nutrient solutions. These systems are gaining popularity among the various solutions because of their efficient resource management, food production, and environmental benefits (Buckseth et al., 2016; Sharma et al., 2018).

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https://doi.org/10.1016/j.agwat.2024.108966
Received 8 January 2024; Received in revised form 31 May 2024; Accepted 20 July 2024

Reducing freshwater usage and the environmental impacts caused by the discharge of treated wastewater into water bodies, contributes to archiving good qualitative and quantitative water status (European Commission, 2020). Combining soilless systems with wastewater improves water resource management. Furthermore, plants uptake nutrients from the wastewater, reducing fertilizer usage and improving the wastewater quality (Rana et al., 2011). Hence, these systems are suggested as ecological wastewater recovery methods, promoting the decentralization of current wastewater treatment systems (Yadav et al., 2020; Yang et al., 2015). Following the principles of circular economy, hydroponically reclaimed water could then be used in irrigation in conventional agriculture, thereby reducing impacts on human health and soil (Cifuentes-Torres et al., 2020).

Wastewater reuse for hydroponic production of various products intended for human consumption or decorative purposes, such as lettuce, tomato, barley, and ornamental flowers has been studied by many authors (Adrover et al., 2013; Carvalho et al., 2018; Maloupa et al., 1997; Rana et al., 2011). Most of these studies used wastewater from domestic treatment plants, considering its chemical constituents, which reduces the risk of heavy metal contamination (Adrover et al., 2013). However, the possibility of fecal contamination through crops is of great concern (Magwaza et al., 2020).

Taking this into consideration, the European Commission and the World Health Organization (WHO) have guidelines for wastewater reuse, with a fit-for-purpose approach, recommending the use of wastewater in agriculture after it has undergone at least a secondary treatment (European Commission, 2020; OMS, 2006). The pre-treatment of the wastewater helps minimize human health and environmental risks through the removal of pathogens and other pollutants such as chemicals (emerging organic contaminants), particles, nutrients, and heavy metals, which could impact crops (Magwaza et al., 2020). Barriers that prevent direct contact of the product with the wastewater (Lopez-Galvez et al., 2014), and the selection of crops that are not consumed raw, are also ways to minimize human health risks (Magwaza et al., 2020), potentially reducing the need for such demanding pre-treatments.

Characterizing the wastewater is essential, not only to evaluate possible environmental risks associated with reuse but also to identify the possible need for supplementation due to the specific requirements of the crops (Santos Júnior et al., 2014). Supplementation usually aims to promote plants' uptake of nutrients, ensuring their nutritional value and increasing their mass (Carvalho et al., 2018). This results in better yields and positive outcomes in wastewater treatment and purification, making the production of hydroponic crops using wastewater economically viable (Magwaza et al., 2020).

Lettuce (*Lactuca sativa L.*) is widely grown and consumed worldwide due to its benefits for the human diet: it is low in calories, fat, and sodium, and a good source of fiber, iron, and vitamin C, among various other health-beneficial bioactive compounds (Kim et al., 2016). This cultivar has been previously grown in hydroponic systems with wastewater reuse (Boyden and Rababah, 1996; Carvalho et al., 2018), promoting the removal of a high percentage of nutrients from wastewater and consequently improving water quality. However, detailed information regarding the health risks was not provided. Since lettuce is generally eaten raw in salads, it is of utmost importance to assess the contamination risk of human exposure to pathogens such as persistent *Escherichia coli*, which can cause acute gastroenteritis (Ungureanu et al., 2020).

This study aimed to assess the growth of green leafy beaded lettuce cultivars (*var. crispa L.*) in a greenhouse hydroponic system using wastewater from an urban treatment plant, following primary (sedimentation) or secondary (activated sludge) treatment, during both summer and winter periods. Secondary treatment is generally preferred for hydroponic systems due to its reduced impact on plant growth and public health (Adrover et al., 2013; WHO, 2006), and primary treated wastewater (PTW) offers higher nutrient concentrations, making it an appealing nutrient source for plants. Despite the associated challenges, the inclusion of PTW in this study aimed to provide deeper insights into potential benefits and contributions to water circularity. If proven feasible, PTW reuse in hydroponics could significantly reduce wastewater treatment requirements and costs. The experimental design intended to investigate the effects of the different levels of wastewater treatment and environmental conditions on crop yield, evaluate sanitary risks, and assess the impact of plant growth on wastewater quality improvements.

2. Material and methods

2.1. Hydroponic system

The study was conducted in a greenhouse, located at the Polytechnic of Leiria (latitude 39°44'37'' N; longitude, 8°48'25'' W; and altitude of 33 m). The greenhouse is covered with transparent polyethylene and has side openings for ventilation on warm days. The workbench was placed on a surface covered by shading screens, to attenuate the intensity of solar radiation on the crop.

The system was a small-scale deep-water culture (DWC), without recirculation, consisting of six 23-liter black opaque plastic containers, and a compressed air aeration pump (HAILEA ACO-328), connected to small wooden diffusers placed at the bottom of each container to provide root aeration. Each hydroponic tank comprised four plants placed in 5.5 cm diameter plastic net pots (Bulsø Plastics, Denmark), containing expanded clay pebbles (SIRO Hydroton) for mechanical support of the plant. The pots were positioned in holes evenly distributed in a floating material, sized to serve as a lid to the hydroponic tanks and prevent the entry of light (Fig. 1).

2.2. Plant seedlings and growth monitoring

The green leafy beaded lettuce cultivar seedlings *(Lactuca sativa var. crispa L.)* were randomly chosen from commercial vegetable gardens and transplanted into the system after gently washing the roots with tap water for soil removal.

Plant growth was evaluated in the summer period, from August 5th to September 9th, 2020 (35 growing days), and in the winter period, from January 1st to March 3rd, 2021 (49 growing days), thus allowing the characterization of growth in the seasonal periods where the environmental conditions reach their extremes. Crops were harvested when plants on the nutrient solution (control) reached a size compatible with that found for market sale, explaining the difference in the duration of the assays.

Fig. 1. : DWC hydroponic system.

Plant growth was evaluated under three different hydroponic water conditions: urban wastewater after primary treatment (PTW); urban wastewater after secondary treatment (STW); and a nutrient solution (NS), used as a positive control. Two hydroponic tanks were used for each experimental condition with a total of 8 plants per hydroponic water.

Once a week, plants' morphological growth was evaluated in terms of number of leaves ($\geq$ 2 cm, $>$ 50 % of dead area), foliage diameter (cm), biggest leaf length (cm), plant height (cm), and root length (cm). After the growth period, plants were harvested and immediately weighed without roots using a 0.0001 g precision scale (KERN 470–36, KERN & Sohn GmbH, Germany). All results were expressed as the average of eight plants per experimental condition.

2.3. Hydroponic Waters

PTW and STW were collected from the Olhalvas Wastewater Treatment Plant (WWTP) in Leiria, Portugal, which predominantly receives wastewater from domestic households. Previous monitoring data indicates that concentrations of heavy metals in the wastewater are below the method quantification limit, and concentrations in the generated sludge are within levels that permit soil application (data not shown). Thus, these wastewaters are potentially suitable for the hydroponic plant growth tested in this study, concerning this issue.

Collection took place in August (dry season) and in January (wet season), thus providing samples with expected differences regarding water quality parameters, due to the type of sanitation network and the time of year (Metcalf and Eddy, 2016).

The NS (Table 1) was based on the solution used by Carvalho et al. (2018), containing the recommended concentrations for lettuce crops, with some modifications: calcium nitrate was replaced by a commercial fertilizer (YaraTera™ CALCINIT), containing 15.5 % total nitrogen, 14.4 % nitrate nitrogen, 1.1 % ammoniacal nitrogen and 26.5 % calcium oxide; and monoammonium phosphate was replaced by monopotassium phosphate (KH_2PO_4). Apart from the calcium nitrate, all the chemicals used were of analytical grade.

As an eco-friendly option, rainwater collected from the greenhouse facilities was used to prepare the NS. The rainwater was previously characterized to ensure no significant differences in water quality parameters were introduced. Additionally, rainwater was used to keep the hydroponic water volume constant in the tanks, added weekly according to the reduction caused by plant consumption and evaporation. The volume added and the consequent water dilution in the thanks was considered in the results shown in Section 3.

2.4. Greenhouse environmental conditions

The daily profile of the environmental conditions inside the greenhouse was characterized during the assays, through continuous measurement of temperature and relative humidity with a HAXO-8 probe (LogTag, New Zealand); and luminous intensity measurement three times a day (morning, 09:30–10:30 h; midday, 13:00–14:00 h; afternoon, 17:00–18:00 h), with a lux meter (KOBAN, KL1330).

2.5. Toxicity and microbiological analyses

Given that the lettuce produced in the hydroponic system can be used for animal/human consumption, it is important to evaluate the toxicity of the plant material to animal/human cells. Hence, Caco-2 cells (human intestinal epithelial cell line) were used as model cells to investigate the toxicity (by the MTT cell viability test) imposed by the plant material (lettuce leaves), according to the procedure reported by Primitivo et al., (2022). High viabilities of Caco-2 cells indicate that the material is free of potentially harmful substances to human health under the tested conditions. Potentially harmful substances can include heavy metals, pesticides, or pathogenic microorganisms.

Considering the significant concern associated with the presence of pathogenic microorganisms in wastewater used in hydroponic systems (Magwaza et al., 2020), total coliforms and *E. coli* were evaluated using the membrane filtration method (ISO 9308–1:2014) in the wastewater samples and both leaves and roots of harvested plants. The extraction process for the leaves and roots was carried out using 4 g samples in 100 ml of OXOID Maximum Recovery Diluent (peptone1.0; sodium chloride 8.5) and rotating it at 150 rpm for 5:30 min, using a Seward laboratory homogenizer (Stomacher® 400 circulator). This procedure was adapted from Mritunjay and Kumar (2017).

2.6. Water quality analysis

Water temperature (T), pH, electrical conductivity (EC), and total dissolved solids (TDS) were measured daily during plant growth using a multiparameter probe (Edge HI-9829, Hanna Instruments, Italy). This procedure allowed to learn about the influence of these physicochemical parameters on growth. According to Furlani et al. (1999), these parameters affect the solubility of water components, compromising nutrient availability and can cause a reduction in the nutrient uptake by the culture.

Biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), total phosphorus (P), ammoniacal nitrogen ($N\text{-}NH_4$), nitrates ($N\text{-}NO_3$), nitrites ($N\text{-}NO_2$), dissolved potassium (K) and dissolved iron (Fe (III)) were monitored weekly, according to standard methods (APHA, 2005) (Table 2). A composite water sample per experimental condition (NS, PTW, and STW) was prepared by mixing an equal volume taken from each hydroponic tank.

Nutrient monitoring (N, P, K, and Fe) aimed to assess their absorption and availability to the crop, given their importance in plant metabolism as a source of energy, in plant respiration, photosynthesis, formation and translocation of sugars, and nitrogen fixation (Furlani et al., 1999). Nitrogen was quantified in three forms ($N\text{-}NH_4$, $N\text{-}NO_3$, and $N\text{-}NO_2$) to monitor the nitrification process *in situ*; and BOD_5 and COD

Table 1
Nutrient solution (Carvalho et al., 2018).

Component	Chemical formula	Concentration (mg/L)
Calcium nitrate	$Ca\,(NO_3)_2.4\,H_2O$	900
Potassium nitrate	KNO_3	134
Potassium sulphate	K_2SO_4	280
Magnesium sulphate	$MgSO_4.7\,H_2O$	495
Potassium chloride	KCl	138
Monoammonium phosphate	$NH_4H_2PO_4$	142
Ferric chloride	$FeCl_3.6\,H_2O$	11.97
Manganese sulphate	$MnSO_4{\cdot}H_2O$	3.39
Boric acid	H_3BO_3	2.92
Zinc sulphate	$ZnSO_4.7\,H_2O$	0.49
Copper sulphate	$CuSO_4.5\,H_2O$	0.08
Sodium molybdate	$Na_2MoO_4.2\,H_2O$	0.12
EDTA-disodium	$C_{10}H_{14}N_2O_8Na_2.2\,H_2O$	16.42

Table 2
Methods and equipment used to monitor the chemical parameters of the hydroponic waters.

Parameter	Method	Equipment
P	Colorimetric method (SMEWW 4500-P-E)	Spectrophotometer (VARIAN-Cary 50 UV–vis)
$N\text{-}NH_4$	Colorimetric method (ISO 7150–1)	
$N\text{-}NO_3$	Colorimetric method (EPA 352.1)	
$N\text{-}NO_2$	Colorimetric method (SMEWW 4500-NO_2^- B)	
BOD_5	5-Day BOD test (SMEWW 5210 B)	Dissolved oxygen meter (Yellow Springs 5000)
COD	Open reflux method (ISO 6060)	COD reactor (Selecta−4000638)
K and Fe	Flame atomic absorption spectrometry (SMEWW 3111B)	Spectrophotometer (VARIAN-SpectrAA 55B)

determination aimed to assess water quality improvement in the hydroponic system concerning organic load.

2.7. Statistical analysis

Descriptive statistics, including average and standard deviation, were used to analyze physical and chemical parameters of the hydroponic water, morphological crop growth parameters, and air temperature and relative humidity inside the greenhouse. Data on morphological crop growth parameters were statistically analyzed using hypothesis tests, including a two-way Analysis of Variance (ANOVA) with repetition (eight plants per hydroponic water), under the influence of the hydroponic water (NS, PTW, and STW) and the period of the year (summer and winter) as two independent factors. For dependent morphological variables, ANOVA analysis assumed data normality (verified by the Kolmogorov–Smirnov test) within factor groups, and the homogeneity of variances was also guaranteed by Levene's test. The most advanced statistical analyses were conducted with IMB SPSS Statistics 2022 software v28.

3. Results and discussion

3.1. Crop growth

Plants' average growth, 35 days after transplanting in summer and, 49 days after transplanting in winter is shown in Fig. 2. As expected, the NS provided the best crop growth performance in both periods. However, the NS-plants developed better in the summer period presenting bigger leaves, thus occupying a larger area (foliage diameter), and increased height (2.0 cm more on average), and root length (11.4 cm more on average).

Results showed that PTW-plants and STW-plants were 6.1 cm and 8.3 cm smaller than NS-plants, in the winter period, respectively, and that the difference was higher in summer (12 cm, PTW-plants, and 9.7 cm, STW-plants). Similarly, the root length varied according to the hydroponic water and seasonal period: they were generally smaller in winter and presented more significant length differences in summer (SN-roots were the longest, and PTW-roots were the smallest). Sapkota et al. (2019) also identified significant differences in the length of leaves and roots of lettuces that grew in different nutrient solutions, referring that lower growth may be related to low concentrations of nutrients (K, N, Ca) and solutions with excessive concentrations of these nutrients lead to short roots. The excess of nutrients in NS seemed not to limit root length in the present work Table 3.

Moreover, considering plant growth in wastewater, a better performance was observed in PTW-plants in winter; and in STW-plants in summer. Wastewater characteristics during winter, such as lower solid load and organic matter (Table 4, Fig. 5, Section 3.4), may have favored the PTW-crop by allowing less accumulation on the root zone and consequently a better uptake of nutrients by the crop. The duration of the assay (49 days) may have contributed to the higher growth in PTW. The STW's higher nutrient concentration (P, N-NO$_3$, and K) in the summer period favored growth.

Weekly monitoring showed that PTW-plants growth was delayed in

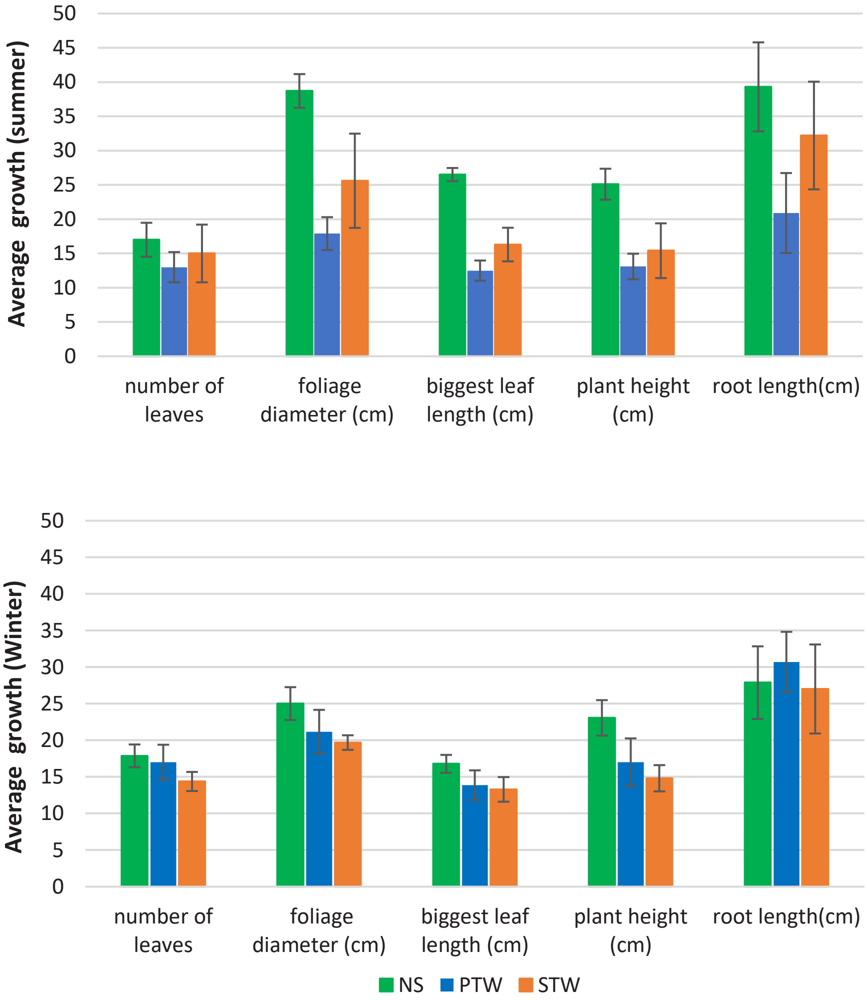

Fig. 2. : Plants' average growth in both winter and summer periods.

Table 3
Coliforms and E. coli enumeration in the wastewater, and plants' leaves and roots.

PTW		*E. coli*	Coliforms	STW		*E. coli*	Coliforms
Leaves	CFU/100 ml	0.0	0.0	Leaves	CFU/100 ml	0.0	36.7
	CFU/g	0.0	0.0		CFU/g	0.0	8.9
Roots	CFU/100 ml	1.1×10^3	1.9×10^4	Roots	CFU/100 ml	267	1.1×10^4
	CFU/g	283	4.7×10^3		CFU/g	65	2.6×10^3
Water	Start	2.0×10^6	6.1×10^6	Water	Start	1.2×10^4	5.1×10^4
(CFU/100 ml)	End	70	1.3×10^3	(CFU/100 ml)	End	77	817

Table 4
Initial concentration of BOD$_5$, COD, P, N-NH$_4$, N-NO$_3$, N-NO$_2$, K, and Fe(III) in the hydroponic waters used for crop growing during the summer and winter periods.

Growing conditions		BOD$_5$	COD	P	N-NH$_4$	N-NO$_3$	N-NO$_2$	K	Fe(III)
Summer	NS	6.0	<30.0*	36.6	10.7	130	0.01	264	2.5
	PTW	110	247	6.0	40.7	<1.0*	0.01	21.3	<0.10*
	STW	4.0	<30.0*	6.9	0.28	13.5	0.03	18.7	<0.10*
Winter	NS	<2.0*	<30.0*	39.1	11.7	145	<0.005*	274	4.0
	PTW	65.3	93.7	4.0	33.9	1.2	0.09	15.0	<0.10*
	STW	7.1	<30.0*	4.6	2.34	10.4	0.06	15.0	<0.10*

* Under the method quantification limit

the first two weeks (data not shown) (Santos, 2021). This was related to the accumulation of solids in the root zone and the high pH and concentration of N-NH$_4$. According to Trejo-Téllez and Gómez-Merino (2012), pH affects the nitrification process. In their work, the authors stated that at pH 8.5 the rate of N-NH$_4$ oxidation increases over the rate of N-NO$_2$ oxidation, resulting in the accumulation of N-NO$_2$ to levels considered dangerous for plants (observed peak of 4.2 mg NO_2^-/L). Based on the maximum values of pH and N-NO$_2$ observed in this study (8.7 pH, and 36.7 mg N-NO$_2$/L = 120.48 mg NO_2^-/L), it can be assumed that this was the case. STW-plants grew properly at the beginning of the assay, but growth slowed in the last two weeks. Since crop growth is largely associated with nutrient availability, this may be related to the insufficient concentration of nutrients in the wastewater that had been intensively consumed up to that time (data not shown) (Santos, 2021).

Average fresh weight results showed that NS-plants and STW-plants were heavier in the summer period with 134.2±20.8 g and 54.2±25.9 g, respectively, and with no significant differences from the winter period (123.1±9.1 g NS and 49.1±5.5 g STW), which corresponds to an average difference of 60 % less fresh weight in STW-plants. PTW-plants grew more in winter thus weighting 65.1±12.7 g (*vs* 27.3±10.1 g in summer), which leads to an average of 47 % less fresh weight than NS-plants (*vs* 80 % in summer).

In line with this study, Boyden and Rababah (1996), observed that lettuce plants could contribute to the removal of approximately 87 % BOD$_5$, 86 % COD, 77 % phosphorus, and 80 % nitrogen in urban wastewater, however, plants' fresh mass would be reduced by 50 % compared to those in a nutrient solution. The authors pointed out the low concentration of nutrients as being the determining factor. To counter this fresh mass reduction Carvalho et al., (2018), suggest the supplementation of the wastewater according to its specific nutrient deficiency, as a way to promote the capture of nutrients by the plant and increase its mass.

Two-way ANOVA results on crop growth showed that the interaction effects between the two factors (hydroponic water and period of the year) significantly impact the average growth of foliage diameter, biggest leaf length, plant height, root length, and fresh weight with *p-values* of 1.63×10^{-7}, 7.70×10^{-11}, 9.27×10^{-3}, 3.30×10^{-5} and 1.99×10^{-4} respectively, for a significance level of 1 %. Concerning the number of leaves, the interaction between the two factors is not significant on the average number for a significance level of 1 % with a *p-value* of 4.28×10^{-2}, however, is significant at a level of 5 %. Nevertheless, when the hydroponic water factor was studied separately, it was found to have a significant effect on the average number of leaves (*p-value* = 0.0102) for a significance level of 5 %. On the other hand, the period of the year showed no statistical evidence of differences in the average number of leaves between summer and winter (*p-value* = 0.131).

3.2. Greenhouse environmental conditions

The environmental conditions inside the greenhouse were dependent on the external environment, as the greenhouse lacked heating or cooling systems aside from side openings that could be used (or not) for ventilation.

Daily average air temperature and relative humidity inside the greenhouse are shown in Fig. 3. In summer, the average temperature and relative humidity of the internal air were 25.6±1.9 °C and 59.6±7.5 %, respectively (*vs* 17.3±1.9 °C and 73.4±7.6 % in winter).

The water temperature was consistently lower than the air temperature, averaging 1.8 °C less in summer and 0.4 °C less in winter (Figs. 3 and 5). According to Tomasi et al. (2014), great differences between water and air temperatures can severely affect the quality of vegetables grown in hydroponic systems, causing incapacity in nutrient uptake. The difference observed in summer led to an impact on nutrient absorption, and episodes of plant wilting, particularly during periods of greater solar intensity. The higher summer temperature led to an acceleration in crop growth (35 days of growth), and mild environmental conditions in winter allowed a greater absorption of nutrients by the crop leading to a significant improvement in water quality (Table 5, Section 3.4).

Higher temperatures and relative humidity in summer lead to the emergence of caterpillars on the crop, as plant transpiration and evaporation in high-temperature environments increase air humidity enhancing the activity of insects and providing suitable conditions for the proliferation of pathogens (Roberts et al., 2020).

Illuminance data revealed an estimated photoperiod of 10 hours in summer and 8 hours in winter. During the period of highest solar intensity (13:00–14:00 h, afternoon), the luminous flux per unit area varied from 7620 to 23100 lux in summer, and from 2160 to 9900 lux, in winter. The differences found did not lead to negative effects on the lettuce, which can be grown all year round, both outdoors and in greenhouses, as it adapts to a wide range of solar radiation intensity achieving maximum productivity when grown under partial shade (Kavga et al., 2018).

3.3. Toxicity analyses and coliforms evaluation

The human cell toxicity potential associated with exposure to lettuce grown in wastewater was investigated using MTT viability tests conducted with Caco-2 model cells (Fig. 4).

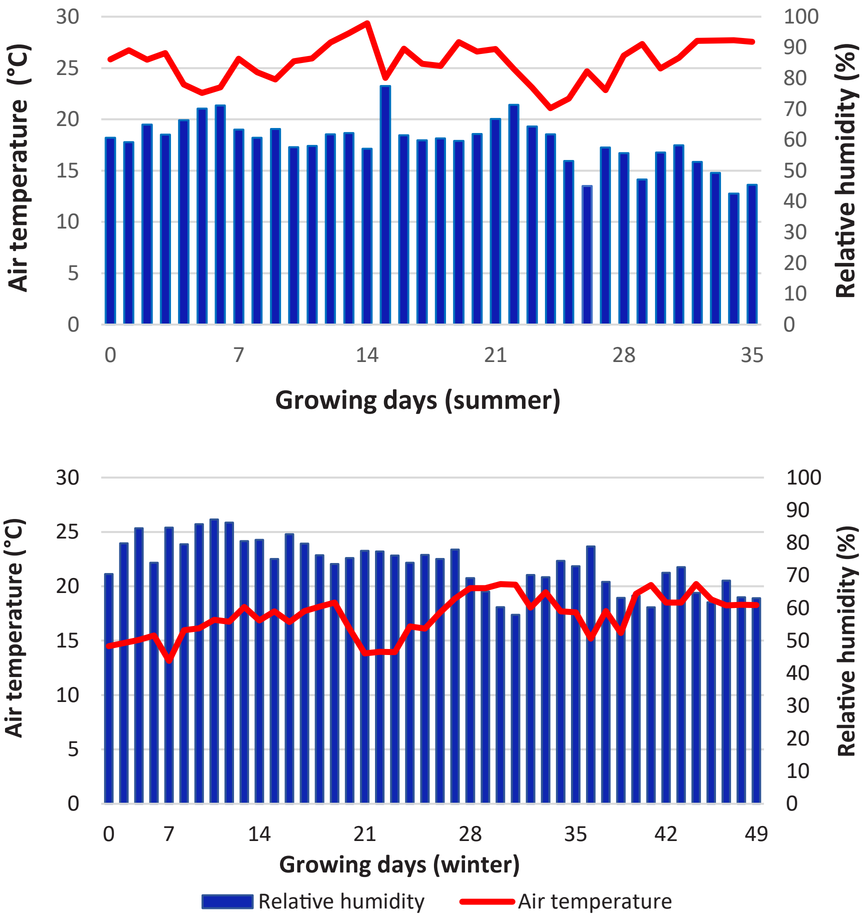

Fig. 3. : Air temperature and relative humidity inside the greenhouse during crop growth.

Table 5
Nutrient removal from the hydroponic waters at the end of the growth periods.

Growing conditions		BOD_5		COD		P		N (NO_2+NH_4+NO_3)		K		Fe(III)	
		%	(mg/L)	%	(mg/L)	%	(mg/L)	%	(mg/L)	%	(mg/L)	%	(mg/L)
Summer	NS	44.5	2.7	—	—	12.0	4.4	1.0	1.4	62.4	164.6	44	1.1
	PTW	98.2	108	83.1	205	43.4	2.6	44.9	18.7	99.8*	21.3*	—	—
	STW	75.2*	2.0	—	—	49.7	3.4	83.9	11.6	99.7*	18.7*	—	—
Winter	NS	—	—	—	—	35.1	13.7	15.0	23.4	31.8	87.1	30	1.2
	PTW	98.5*	63.3*	84.0	78.7	83.9	3.4	60.9	21.4	90.4	13.6	—	—
	STW	85.9*	6.1*	—	—	76.9	3.6	95.9*	11.7*	99.7*	15.0*	—	—

— Not applied, initial concentration under the method quantification limit; *Removal calculated assuming the method quantification limit divided by 2, for final concentration values when below the limit, based on Wiest et al., (2021).

After a 24-hour incubation period, good Caco-2 cell performance, close to 100 % cell viability, was observed in samples of leaf material when extract concentrations were lower than 1 % w/v.

Given that lettuce is typically consumed fresh in salads, with an average daily intake of 3.78 g/person (Ding et al., 2013), and has a high water content (approximately 95 %) (Kim et al., 2016), the daily consumption would correspond to a concentration below 1 % w/v (Primitivo et al., 2022). Thus, results indicate that at low concentrations (less than 1 % w/v), the lettuce extract is free of potentially harmful substances to human health.

Regarding the analysis of total coliforms and *E. coli* in both plant leaves and roots, a notable accumulation of these contaminants was observed in the roots, in both PTW and STW plants. However, contamination in the leaves was exclusively identified in STW-plants (Table 3). The accumulation of microorganisms in the roots corroborates the findings of Ottoson et al.(2005), highlighting plant uptake and adsorption as mechanisms responsible for retaining a portion of the microorganisms. Furthermore, the extended retention time within the hydroponic system is identified as a key factor contributing to the increased removal efficiency.

Considering that the STW presented lower microbial enumeration than PTW (5.1×10^4 CFU/100 ml vs 6.1×10^6 CFU/100 ml), it might be assumed that the STW-leaves contamination occurred through direct contact during sampling. Nevertheless, the risk of pathogen transmission to humans must still be considered given that the contamination can also occur during large-scale hydroponic production, and lettuce is consumed raw. Thus it is recommended to include a disinfection stage before the use of wastewater in the hydroponic system to prevent the presence of pathogens in the water (Ungureanu et al., 2020).

Despite this finding, total coliforms were found within acceptable limits for *Enterobacteriaceae* coliforms ($<10^4$ CFU/g) (Health Protection Agency, 2009), which represents most of the bacterial genera that

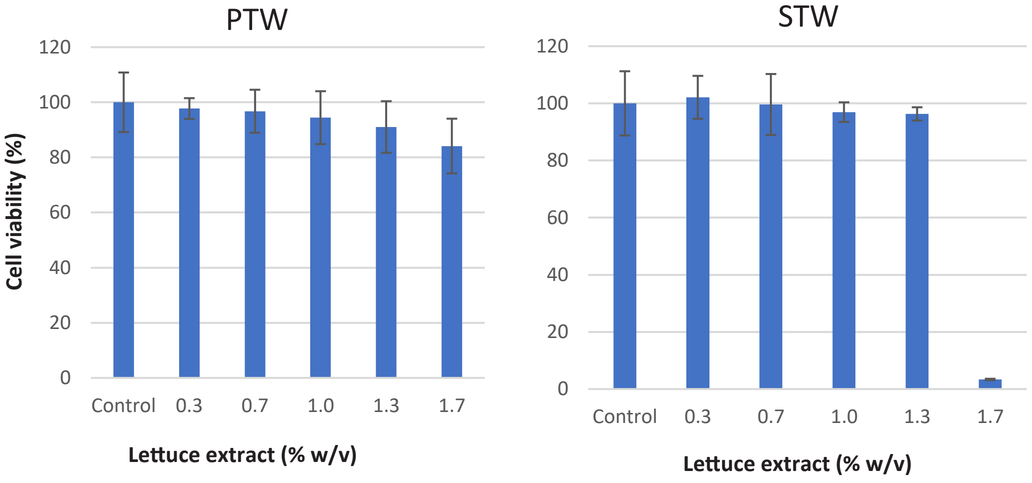

Fig. 4. : Cell viability assays of Caco-2 cells, after exposure to lettuce leaf extract grown in PTW and STW.

comprise the coliform group (e.g., *Escherichia, Klebsiella, and Serratia*) (Martin et al., 2016).

The system allowed a high reduction of these microbiological contaminants in the wastewater (PTW: 99.9 % *E. coli*/coliforms; STW: 99.4 % *E. coli* and 98.4 % coliforms) (Table 3). Ndulini et al. (2018) also reported high removal percentages of fecal coliforms from wastewater in a hydroponic system. They attributed the reduction of microbial population to various mechanisms including antibiosis, which leads to microorganisms harming each other, and microbial predation caused by food scarcity.

3.4. Water quality and nutrient removal

The daily measurement of T, pH, EC, and TDS monitored the physiochemical parameters of the hydroponic water during lettuce growth (Fig. 5).

Water temperature depended on the environmental conditions inside the greenhouse, thus showing similar variations in the hydroponic waters throughout all the assays. In summer, the temperature varied from 20.7 to 31.4 °C, with an average temperature of 24.6±2.2 °C, and in winter, the temperature ranged from 11.5 to 24.2 °C, with an average temperature of 17.7±2.7 °C (Fig. 5). Water temperature directly affects the solubility and bioavailability of nutrients. The recommended temperature for hydroponic solutions should not be higher than 30 ºC, and the ideal for plants is in the range of 18–24 ºC in warm periods (summer), and 10–16 ºC in cold periods (winter) (Furlani et al., 1999). As shown, the average temperature of the water was higher than the maximum recommended for each period in all assays.

During the summer assay, pH was significantly higher in the wastewater (PTW, 6.9–8.7; STW, 8.1–8.4) than in the NS (5.5–7.3) with average values of 7.7±0.4, 8.3±0.1, and 6.5±0.5, respectively. The same pattern was verified during the winter assay, with pH values varying from 7.5 to 8.7 (PTW), 8.3–8.6 (STW), and 4.1–6.2 (NS), corresponding to average values of 8.1±0.3, 8.5±0.1, and 5.3±0.8, respectively (Fig. 5).

In the wastewater (PTW and STW), pH values were always above the maximum recommended value (6.5) in both assays, especially in the STW. Values of pH higher than 6.5 make certain nutrients (Fe^{2+}, Cu^{2+}, Zn^{2+}, B^{2+}, and Mn^{2+}) unavailable for the crop due to precipitation (Trejo-Téllez and Gómez-Merino, 2012), which can be related to the delay observed in crop growth (winter and summer), particularly in the early period of growth in the PTW (first 15 days, data not shown) (Santos, 2021).

The pH of a nutrient solution should be between 5.5 and 6.5, as this range provides greater availability of nutrients for plants (Furlani et al., 1999; Trejo-Téllez and Gómez-Merino, 2012). pH lower than 5.5 leads to competition between H^+ ions and other essential cations (NH_4^+, Ca^{2+}, Mg^{2+}, K^+, Cu^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}), and values below 4 affect the integrity of cell membranes (Furlani et al., 1999). Higher pH values favor the reduction of anions and the precipitation of elements such as Ca^{2+}, Mg^{2+}, PO_4^{3-}, Fe^{2+}, and Mn to insoluble unavailable salts (Trejo-Téllez and Gómez-Merino, 2012). The pH registered in the NS was generally within the recommended range, but by the end of the summer assay values were above 6.5, in the winter values were below the recommended minimum (5.5), during approximately 16 days of growth. Moreover, the NS presented significant changes in pH values during both periods, probably due to the elevated concentration of nutrients (Table 4), making this hydroponic water particularly susceptible to the formation of soluble complexes and nutrient precipitation (Trejo-Téllez and Gómez-Merino, 2012). Unbalanced nutrient uptake by the plants, with preferential absorption of certain cations or anions (Tomasi et al., 2014), and the design used in the hydroponic system without mechanical water agitation can also cause changes in pH values.

The wastewater and NS showed significant differences in EC values during the summer and winter assays, which decreased as lettuce grew. In summer, EC values in the PTW and STW varied from 468.0 to 932.0 μS/cm, with average values of 741.8±85.9 μS/cm and 627.0 ±73.3 μS/cm, respectively; and in the NS, EC varied from 1454.0 to 2256.0 μS/cm, with an average value of 2018.6±220.3 μS/cm. In winter, EC values in the PTW and STW varied from 407.0 to 693.5 μS/cm, with average values of 569.2±79.5 μS/cm and 491.9±50.4 μS/cm, respectively; and in the NS from 1468.0 to 1993.0 μS/cm, with an average value of 1679.3±132.9 μS/cm (Fig. 5).

Generally, EC values of a nutrient solution are recommended to be kept between 1500 and 3500 μS/cm (Furlani et al., 1999). However, lettuce is a salt-sensitive crop, thus specific studies on optimum EC levels indicate that the range varies depending on the selected cultivar (Xu and Mou, 2015) and the growing season, affecting lettuce yield (Sublett et al., 2018). Higher EC values are known to increase nutrient (K, P, $N\text{-}NO_3$, Fe) uptake by the crop (Kappel et al., 2021; Sublett et al., 2018), but can lead to a lower yield (Ding et al., 2018) if a nutrient imbalance occurs in the plants caused by favored uptake of the nutrients present in higher concentration in the solution and inhibited uptake of those in lower concentration (Samarakoon et al., 2006). For this matter, values of 900 and 1200 μS/cm can be found in the literature to promote a better yield of lettuce in terms of the number of leaves, fresh weight, and foliage diameter (Hosseini et al., 2021).

In this study, the EC values in the NS (> 1200 μS/cm) seemed to favor the plants' growth when compared to the EC wastewater values (< 900 μS/cm) (NS-plants *vs* PTW- and STW-plants growth, Fig. 2). As EC

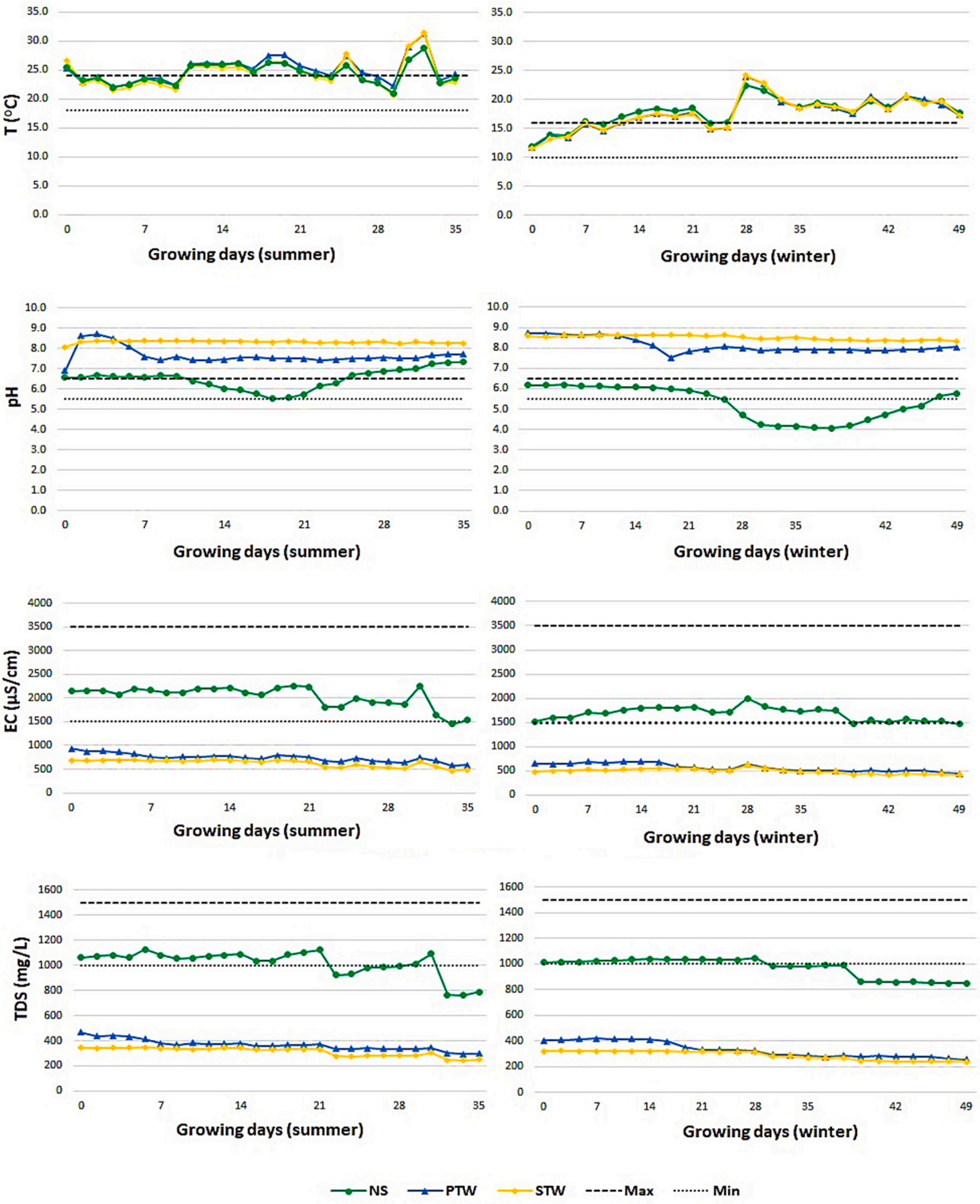

Fig. 5. : Daily values of Temperature(T), pH, EC, and TDS in the nutrient solution (NS), primary treated wastewater (PTW), and secondary treated wastewater (STW); and recommended limits (Max and Min) during crop growth (winter and summer).

measurements are directly related to the dissolved nutrient concentration in the solution this may indicate a deficiency of macronutrients in the wastewater such as Ca, Mg, K, and NO_3, which can severely affect plant health and reduce yield (Trejo-Téllez and Gómez-Merino, 2012).

Summer values of TDS varied from 244.5 to 465.5 mg/L in PTW and STW (average values of 366.5±42.3 mg/L and 313.2±33.7 mg/L, respectively); and from 759.0 to 1126.0 mg/L in the NS, with an average value of 1016.6±102.5 mg/L. Winter values did not vary significantly from the summer values in PTW and STW (238.0–416.5 mg/L; average values of 331.5±58.2 mg/L and 286.9±33.9 mg/L, respectively); but the variation in the NS was higher, considering a range of 851.0–1047.0 mg/L (average value of 971.5±74.5 mg/L).

TDS values recommended by Furlani et al. (1999), corresponding to a total ion concentration of 1000–1500 mg/L (EC in the range of 1500–3500 µS/cm), were considered in this study. However, considering that a lower EC range is expected for lettuce (Hosseini et al., 2021), the optimal values of TDS are likely lower. Results showed that the TDS values varied more significantly in the NS, and were generally higher than those in the wastewaters, during both growth periods. The ion concentration decreased during the growth period (Fig. 5), following the same behavioral pattern as the EC values. This depletion can be related to nutrient uptake by the plants.

The lower values of EC and TDS in the PTW and STW, point to lower nutrient concentrations, which can compromise plant growth (Section 3.1). Moreover, temperature influences chemical precipitation, thus affecting nutrient availability. Partial precipitation can occur due to low temperatures in winter (decreased solubility) and to high temperatures in summer (increased water evaporation and consequent higher nutrient concentration in solution, which can exceed solubility equilibrium) (Tomasi et al., 2014). Therefore pH, EC, and TDS values were higher during the summer assay (and increased on days of higher temperatures) when compared to the winter assay (Fig. 5). This may also explain the variations in EC and TDS in the NS, which is generally more susceptible to precipitation phenomena caused by higher nutrient concentrations and water evaporation.

Although T, pH, EC, and TDS values in the hydroponic waters were not within the recommended ranges, no symptoms of diseases were observed in the plants. However, the results indicate that to promote a better crop yield in the wastewater it is necessary to adjust pH, EC, and TDS to the recommended range, and supplement with nutrients to increase their availability to the crop. NS-plants would also benefit from adjustments to promote better conditions regarding T, pH, EC, and TDS and control the possible excess of nutrients.

The hydroponic waters were tested to evaluate the organic content and nutrient concentration at the beginning and the end of the growing periods (Tables 4 and 5).

As expected, PTW and STW generally presented higher concentrations during the summer period, due to the dry season. Moreover, PTW had a higher organic content (BOD_5 and COD) and nitrogen content than STW, due to the type of treatment applied in the WWTP. The NS exhibited the lowest organic content as it was prepared in the laboratory using inorganic salts; and the highest nutrient concentration (P, N, K, and Fe).

The results at the end of the growing period showed that the hydroponic system contributed to the removal of the organic content present in the wastewater (Table 5), namely: $>$ 98.2 % BOD_5 and $>$ 83.1 % COD (PTW); $>$ 75.2 % BOD_5 (STW). The improvement was more significant in the PTW, where the initial organic content was higher; and seemed to be influenced by the growing period (summer/winter).

The system allowed the removal of nutrients (N, P, K, and Fe), with the lowest removal percentage observed in the NS (Table 5). As the initial nutrient concentration in this medium was much higher than in the wastewater (Table 4), the lower efficiency can be related to an excess of nutrients, considering the number of plants used per hydroponic tank.

According to Grangeiro et al. (2006), lettuce typically requires a low amount of phosphorus to grow. In this study, removal of phosphorus from the wastewater closely resembled values observed in the NS during the summer period (Table 5) suggesting a potential alignment between lettuce's phosphorus needs and its uptake from the wastewater. During the winter period, removal was significantly higher in the NS condition (13.7 mg/L NS vs 3.4 mg/L PTW and 3.6 mg/L STW). Moreover, the increased removal of phosphorus in the wastewater during the winter period (83.9 %, PTW and 76.9 %, STW) compared to the summer period (43.4 %, PTW and 49.7 %, STW) may be attributed to lower initial concentrations in the wastewater resulting from the wet season (Table 4).

As lettuce primarily consists of leaves, it responds favorably to nitrogen supply (Grangeiro et al., 2006), therefore removal of this nutrient should be highly effective. The findings demonstrated the removal of over 11.7 mg/L (95.9 %) of nitrogen in the STW and up to 21.4 mg/L (60.9 %) in the PTW during the winter period. Although the removal was lower in the summer, a similar trend was observed, with the removal of 11.6 mg/L (83.9 %) in STW *vs.* 18.7 mg/L (44.9 %) in PTW (Table 5). In the STW, the nitrogen concentration was lower than in the PTW, consequently limiting consumption, which could justify the similar values in both periods (11.7 and 11.6 mg/L). The biological treatment of the STW in the WWTP implied that this nutrient was partially removed from the STW, and the remaining available nitrogen was mostly in the form of nitrates ($N\text{-}NO_3$, Table 4), which is the preferred form for plant uptake. In contrast, nitrogen in PTW was mostly as $N\text{-}NH_4$, and conversion took place within the hydroponic system, after 21 days (data not shown) (Santos, 2021).

The consumption of potassium in plants exceeds that of other nutrients, such as nitrogen and phosphorus, due to its role in various plant functions, including osmotic properties, photosynthesis, and enzymatic activation, among others (Grangeiro et al., 2006). Therefore, potassium was the most consumed nutrient in the system with removal efficiencies of 90.4 % (PTW, winter) and 99.7 % (PTW, summer, and STW, winter/summer). Although nutrient removal concentrations varied, with higher removal rates observed during summer, the percentages nearing 100 % in both periods suggest that potassium concentration in PTW and STW may be insufficient to meet the crop's requirements. This indicates the potential necessity of supplementation to enhance crop yield. Potassium consumption observed in the NS can serve as a reference for PTW/STW supplementation in future studies (87.1 mg/L winter and 164.6 mg/L summer).

Analysis of iron concentration, Fe (III), in the wastewater (PTW, STW) revealed values below the quantification limit of the analytical method (Table 4). Considering the iron consumption in NS (1.1 mg/L in winter and 1.2 mg/L in summer, resulting in 30.0 % and 44.0 % removal, respectively), it seems necessary to supplement wastewater with this nutrient. Iron is important for plants in processes such as nitrogen fixation and is also one of the nutritional benefits of lettuce for the human diet.

Although crop absorption is expected to be the major responsible for nutrient removal efficiency, nutrient precipitation due to temperature variations and pH, can also affect the process. Therefore, careful analysis of the results is necessary, and it is essential to acknowledge that the decrease in nutrient concentration observed in this study cannot be attributed only to plant uptake. Furthermore, conducting analyses of plant nutrient content would offer a more comprehensive assessment of nutrient consumption.

The nutrient (N and P) removal efficiency was generally higher in the winter period, indicating that the lower water temperature (average 17.7±2.7 °C) favored the uptake of these nutrients by the plants. Detailed analyses of the temperature profile over the growth period showed that after 28 days of growth, the water temperature remained near 20 ºC until the end of the assay (Fig. 5), which could be related to higher nutrient removal efficiencies. This is corroborated by the study of Dalla Costa et al. (2011), which showed that temperature affected not only yield performance but also nutrient uptake of corn salad, referring that plants performed better at 20 °C compared to 15 °C or 25 °C.

The wastewater quality improved significantly after crop growth (winter and summer). PTW met the emission limit values for local discharge of wastewater, namely, $BOD_5 \leq 40$ mgO_2/L, $COD \leq 150$ mgO_2/L, $P \leq 10$ mgP/L, $N \leq 15$ mgN/L, $NH_4 \leq 10$ $mgNH_4/L$, and $NO_3 \leq 50$ $mgNO_3/L$,(Decreto-Lei, 1998). Thus, the hydroponic system can be an effective production and ecological wastewater treatment system.

Weekly water consumption was consistent across all three hydroponic conditions (NS, PTW, and STW) during winter. However, during summer, consumption was significantly higher in the NS condition with a high standard deviation (Table 6). The considerable consumption variability exhibited by the NS in both seasons could be attributed to variable plant needs during growth and environmental factors that

Table 6
Average water consumption (L) per hydroponic tank per week in both winter and summer according to the growth condition.

Growing conditions		Water consumption (L/tank/week)
Summer	NS	4.0±2.7
	PTW	2.3±0.8
	STW	2.8±1.6
Winter	NS	3. 0±1.7
	PTW	2.8±1.4
	STW	2.7±1.2

influence water uptake and evapotranspiration. The PTW and STW conditions exhibit less pronounced seasonal variation in water consumption, suggesting a lower plant uptake of nutrients, and consequently lower yield, which aligns with this study data (Sections 3.1, 3.2, and 3.4).

4. Conclusions

Lettuce plants exhibited satisfactory growth performance in both seasonal periods when using wastewater, although they displayed a lower fresh weight than the reference crop (NS-plants). The level of wastewater treatment (PTW and STW) had varying effects on crop growth during the two seasons, with PTW promoting higher crop growth during winter and STW proving to be more beneficial during summer.

Toxicity analyses revealed that lettuce plants grown in wastewater did not show any signs of toxicity in leaf extracts up to a concentration of 1 % (w/v), *E. coli* was not detected on plants' leaves and total coliforms remained within acceptable limits, suggesting a low contamination risk. However, the nutrient concentration (N and K) in the wastewater appeared to be lower than the level required by the crop (removal rates near 100 %). The winter period allowed the highest removal rate, suggesting that lower temperatures favored nutrient uptake by the crop. Overall, the system efficiently removed nutrients and microorganisms from the wastewater, thereby enhancing its quality.

These findings highlight the potential of urban wastewater as a nutrient source for hydroponic lettuce production, promoting sustainable wastewater management. Nonetheless, enhancements to the hydroponic system, such as wastewater supplementation, temperature acclimatization, and optimization of electrical conductivity and pH in the water, are necessary to achieve better yields. Further investigation of pollutants such as heavy metals, and emergent contaminants such as pharmaceuticals, is crucial to ensure the safety and quality of wastewater hydroponic products. These steps will not only improve crop productivity but also address potential health risks, assuring that urban wastewater is a viable and safe resource for agricultural use.

CRediT authorship contribution statement

Judite Vieira: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Funding acquisition, Conceptualization. **Helena Sousa:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Conceptualization. **Fernando Sebastião:** Writing – review & editing, Formal analysis. **Daniela Vaz:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis. **Ounísia Delgado dos Santos:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization.

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.

Data availability

Data will be made available on request.

Acknowledgment

This work was supported by national funds through FCT/MCTES (PIDDAC): LSRE-LCM, UIDB/50020/2020 (DOI: 10.54499/UIDB/50020/2020) and UIDP/50020/2020 (DOI: 10.54499/UIDP/50020/2020); and ALiCE, LA/P/0045/2020 (DOI: 10.54499/LA/P/0045/2020). The authors also extend their sincere appreciation to Águas do Centro Litoral (AdCL) for supplying the wastewater and for their support throughout this study. They also wish to thank Cristiana L. Pires for her support in conducting the toxicity analyses.

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