Leaf production and quality of sea beet (*Beta vulgaris* subsp. *maritima*) grown with saline drainage water from recirculating hydroponic or aquaculture systems.

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Abstract

The application of greenhouse soilless culture (or hydroponics) and recirculating aquaculture system (RAS) is rapidly growing worldwide as these technologies provide controlled growing conditions for crop plants and aquatic organisms, thus enhancing productivity. The wastewater from RAS and hydroponics is generally rich in many essential plant nutrients and could be reused for crop irrigation, thus reducing the costs for both wastewater treatment and fertilizers. Many wild edible plant species are salt-tolerant glycophytes or halophytes and hence are suitable for cultivation with saline wastewater in cascade cropping systems or decoupled aquaponic systems.

The goal of this work was to investigate the effects of drainage water from semi-closed substrate plant culture or saltwater RAS on leaf production and quality of sea beet plants (*Beta vulgaris* subsp. *maritima*) grown hydroponically in a greenhouse. Two experiments were conducted in autumn with plants cultivated in floating raft system to compare five different nutrient solutions: standard nutrient solution (CNS, control; EC 2.80 dS m^{-1}, Na 0.7 mM); the effluent from a semi-closed substrate culture of tomato used as such (tomato effluent 100%, TE100; EC 6.49 dS m^{-1}, Na 34.9 mM) or diluted (50:50) with CNS (tomato effluent 50%, TE50; EC 4.50 dS m^{-1}, Na 17.8 mM); the effluent from a saltwater RAS with Gilthead sea bream, used as such (aquaculture effluent 100%, AE100; EC 42.00 dS m^{-1}, Na 408.6 mM) or diluted (50:50) with CNS (aquaculture effluent 50%, AE50; EC 25.40 dS m^{-1}, Na 204.6 mM).

In both experiments, leaf production was significantly reduced in plants grown with AE50 (–46.8%, on average) and AE100 (–70.4%, on average) compared to CNS; on the contrary, no or minor differences were found between CNS, TE50 and TE100 plants. The reduction of crop yield was due to the higher salinity and not to abnormal concentration of some mineral nutrients in AE. In the first experiment, the use of TE and AE also resulted in higher leaf antioxidant capacity and concentration (both expressed on a fresh weight basis) of total chlorophylls, carotenoids, flavonoids, and phenols. In both experiments, leaf concentration of Na and oxalate (both total and soluble) significantly increased with the salinity of the nutrient solution. Due to the less favourable light conditions, leaf

nitrate concentration was much higher in the second experiment than in the first one, regardless of the nutrient solution.

In conclusion, sea beet could be grown using hydroponic wastewater with moderate salinity with no or minor effect on leaf production and quality. In contrast, the use of highly saline aquaculture effluents markedly reduced crop yield and negatively affected leaf quality due to increased concentration of sodium, oxalate, and nitrate. In general, sea beet leaves were high in oxalate and their consumption should be limited.

Keywords: cascade cropping system; decoupled aquaponic system; soilless culture; wastewater irrigation; wild edible plants.

1. Introduction

Worldwide aquaculture production in marine or inland water has increased noticeably in the last three decades and in 2020 it accounted for almost half of the total production of aquatic animals (e.g., finfish, crustaceans, mollusks, and other species) (FAO, 2022). Among existing fish farming technologies, recirculating aquaculture system (RAS) is rapidly growing, albeit it still represents a small fraction of the global aquaculture sector (1-2% in EU; (European Market Observatory for Fisheries and Aquaculture Products, 2020). It is a closed system with more controlled growing conditions for aquatic organisms as compared to open systems, such as offshore cages. Moreover, RAS needs much less water than the raceway systems and the effluents daily discharged, which are 5 to 10% of the total volume of recirculating water, must be treated to remove pollutants or could re-used to culture other organisms, such as crop plants (Tom et al., 2021).

In the same way as RAS, soilless culture (or hydroponics) can provide optimal conditions in the plant root zone with positive effects on both crop yield and quality (Raviv et al., 2019). Hydroponic systems currently account for a small fraction of the area devoted to greenhouse crops in the world, but their application is rapidly increasing (Massa et al., 2020). Both open (or free drain) and closed (or recirculating water) hydroponic systems are applied on a commercial scale (Massa et al., 2020). In closed systems, the nutrient solution is normally recirculated until the electrical conductivity (EC) or the concentration of some potential toxic ion (e.g., sodium) reaches a maximum acceptable threshold, afterwards it is discharged, at least partially (semi-closed system; Massa et al., 2010). Both open and semi-closed hydroponic systems, therefore, result in significant amounts of wastewater, which must be treated before release into the environment.

The effluents from both RAS and hydroponic culture are generally rich in many essential plant elements, especially nitrogen (N) and phosphorus (P), and could therefore be reused for hydroponic production of crop species either in cascade cropping systems or in decoupled aquaponic systems,

which combine RAS and hydroponics. In cascade cropping systems, one or more receiving crops are fertigated using the effluent from a more salt-sensitive donor crop (Incrocci et al., 2003), while in decoupled aquaponic systems aquatic organisms and plants are grown in separate water loops and crop water and mineral requirements are satisfied by directing the water from the RAS unit to the hydroponic unit (Incrocci et al., 2003). Both cascade cropping systems and decoupled aquaponic systems allow to reduce the cost for wastewater treatment and fertilizers (Massa et al., 2020; Monsees et al., 2019). Several studies have been recently conducted on greenhouse cascade cropping systems (e.g. Elvanidi et al., 2020; Faliagka et al., 2021; García-Caparrós et al., 2021) and decoupled aquaponic systems with fresh (e.g. Knaus et al., 2022) or saline water (e.g. Beyer et al., 2021).

The main drawbacks of crop irrigation with wastewater from hydroponic and RAS are associated with their chemical characteristics, such as: high salinity, in general due to high NaCl concentration; abnormal concentration of nutritive elements (Samiotis et al., 2022); the presence of phytotoxic roots exudates (Hosseinzadeh et al., 2017), microbial metabolites (Salazar et al., 2021), and residues of plant protection products (Santos et al., 2022) and antimicrobials (Schar et al., 2020). All these factors can negatively affect crop yield and quality.

There is a growing interest in the cultivation of wild edible plants in consideration of their nutritional and nutraceutical attributes (Lombardi et al., 2022). Some wild edible species have recently been shown to adapt well to soilless cultivation, such as *Cichorium spinosum* L. (Voutsinos-Frantzis et al., 2022), *Plantago coronopus* L. and *Picris hieracioides* L. (Puccinelli et al., 2023), and *Scolymus hispanicus* L. (Papadimitriou et al., 2022). Many wild edible plant species are halophytes or salt-tolerant glycophytes (Lombardi et al., 2022) and therefore are good candidates for the cultivation with saline wastewater.

Sea beet (*Beta vulgaris* L. subsp. *maritima*) is a wild edible plant species, which is an ancestor of all beet crops (Rana and Sagwal, 2017). It is a facultative halophyte that grows naturally in the Mediterranean regions and in northern Europe in salt marsh and saline areas (Lombardi et al., 2022). Sea beet leaves are usually eaten cooked (Rana and Sagwal, 2017). Very few studies have been conducted on sea beet grown in hydroponics (e.g. Puccinelli et al., 2022a) or in aquaponics (Pantanella, 2012).

The goal of this work was to investigate the effects of drainage water from a semi-closed substrate culture of tomato or from a saltwater RAS with Gilthead Sea bream (*Spaurus aurata*) on leaf production and quality of sea beet plants (*Beta vulgaris* subsp. *maritima*) grown hydroponically in greenhouse under the typical climate conditions in autumn in a Mediterranean region. Leaf quality was assessed by determining several parameters associated with sensorial, nutritional and nutraceutical quality, and with hazards to human health.

2. Materials and methods

2.1. Plant material and growing conditions

In a glasshouse at the University of Pisa in central Italy (lat. 43°42'42"48N, long. 10°24'52"92 E), two experiments were carried out in the fall of 2021. A weather station within the greenhouse was used to track the climate. Table 1 presents a summary of each experiment's information. Purchased from Magic Garden Seeds (www.magicgardenseeds.it), sea beet seeds were planted in 240-cell trays with stonewool plugs. The trays were kept in a growth chamber at 25 °C for five days, and then seedlings were transplanted into 50-L plastic tanks with standing nutrient solution 28 (for the first experiment) or 43 (for the second experiment) days after sowing. The water depth in the tanks was 25 cm. There were 24 plants per tank, with a crop density of about 96 plants per m^2. The nutritional solution was continually aerated in each tank, and throughout the experiment, the concentration of dissolved oxygen kept above 6 mg L^{-1}.. Leaves were harvested 29 (first experiment) or 41 (second experiment) days after transplanting (DAT) by cutting the leaves approximately 1 cm above the collar level.

2.2. Experimental design and nutrient solutions

In each experiment, five different nutrient solutions were compared in a randomized design with three replicates, each consisting of one hydroponic tank: standard nutrient solution (CNS, control); the effluent from a tomato substrate culture (tomato effluent, TE) of a parallel and independent experiment conducted in a glasshouse nearby used as such (TE100) or diluted (50:50) with CNS (TE50); the effluent from an experimental saltwater RAS (aquaculture effluent, AE) with Gilthead sea bream used as such (AE100) or diluted (50:50) with CNS (AE50). The experiments on tomato and fish were not reported herein.

The CNS was prepared dissolving an appropriate amount of technical-grade inorganic salts in tap water, which contained 0.65 mM Na.

The TE was collected from an independent experiment on the effects of salinity on tomato growth and fruit quality and consisted of the nutrient solutions discharged after 31 days of recirculation, on occasion of the first discharge, when tomato plants were 75-day old (from the sowing date). Tomato plants were cultivated in stonewool slabs in a recirculating drainage water system with a crop density of 3.2 plant m^{-2}. Each growing unit had a mixing tank with a volume of 130 L (17.3 L m^{-2}) and the total volume of the recirculating solution was 250 L (33.3 L m^{-2}). **Table S1** shows the mineral

composition of the starter and refill nutrient solutions used in the first stage of the experiment with tomato.

The AE was collected from a RAS that consisted of: six cylindrical tanks with conic bottom (tank volume: 0.425 m^3; total volume: 2.55 m^3); a nitrifying biofilter (1 m^3 gross volume) filled with 0.5 m^3 of carriers (Bioballs® with a specific surface area of 600 m^2 m^{-3}); a blower for water aeration (dissolved oxygen ranged between 3.0 and 7.9 mg L^{-1}); a heat pump for water temperature control (set point temperature: 23 °C); UV lamps for water disinfection. The fish density in the rearing tanks varied from 15.1 kg m^{-3} (3 August 2021) up to 30.5 kg m^{-3} (2 December 2021). When the aquaculture effluent was collected, gilthead sea bream fish were at on-growing stage, with a fish density in the rearing tanks of 25.2 kg m^{-3} and an average individual weight of 249.3 g. In the RAS, the water was prepared dissolving 25 g L^{-1} of the synthetic sea salt Instant Ocean in tap water. The mineral composition of this salt has been reported by Puccinelli et al. (2022a).

Commentato [AP1]: Userei l'abbreviazione, perché è stata già usata e si usa anche qualche riga sotto.

Commentato [MP2R1]: Si, però in questa fase non è ancora il trattamento AE, ma l'effluente dell'acquacoltura che poi viene usato per i trattamenti AE50 e AE100

Both TE and AE were collected two days before the beginning of the first experiment; they were filtered to remove solid debris and then stored at 7-8 °C in the dark after pH adjustment to 5.5 with sulphuric acid. The electrical conductivity (EC) and the concentration of nutritive elements and Na in the five nutrient solutions are shown in **Table 2.** The aquaculture effluent also contained 0.2, 19.8 and 18.9 mg L^{-1} of organic N, and total and dissolved organic C, respectively.

In both experiments, the pH and EC of each solution were regularly checked, and the pH was adjusted to 5.5-6.0 with sulphuric acid when needed. The EC did not change substantially during the first and second experiment.

2.3. Determinations

2.3.1. Plant growth

Crop yield was determined by recording the fresh weight (FW) of the leaves of 20 plants collected in each tank. Leaf area, dry weight (DW) and succulence, and root DW were determined on four individual plants sampled in each tank. Dry weight was measured after drying fresh samples in a ventilated oven at 70 °C till constant weight. Leaf area was measured using a digital planimeter (DT Area Meter MK2, Delta T-Devices) and leaf succulence was calculated as the ratio between leaf FW and area. Leaf area index (LAI) was calculated as the leaf area of individual plants multiplied by the number of plants per square metre.

2.3.2. Leaf quality attributes

The concentration of mineral elements, nitrate, and oxalate was determined in dry leaf samples while the antioxidant capacity and the concentration of total chlorophylls, carotenoids, flavonoids, and phenols were analysed in fresh samples, each consisting of the leaves of four individual plants collected in each tank.

For the determination of leaf mineral concentration, dried and ground samples were mineralized with a mixture (5:2 v/v) of 65% HNO_3 and 35% $HClO_4$ at 240 °C for 1 h or extracted with distilled water at room temperature for 2 h. The mineralized samples were used for the determination of the concentration of K, Ca, Mg, Na, Cu, Fe, Mn, and Zn by atomic absorption spectroscopy, and P by UV/VIS spectrometry (Olsen's method). Leaf water extracts were also analysed spectrophotometrically for nitrate concentration using the salicylic sulphuric acid method as reported by Puccinelli et al. (2022a).

Dried leaf samples were also extracted with 0.25 M HCl (50 mg DW in 6 mL) at 100 °C for 15 minutes for the determination of the total oxalate concentration. The mixture was allowed to cool, filled to a volume of 10 mL with 0.25 M HCl, and then filtered through filter paper. The oxalate concentration was determined by adding 0.20 mL of extract to 1 mL of 1 M H_2SO_4 and 0.40 mL of 3 mM $KMnO_4$; after 10 minutes at room temperature, the absorbance of the solution was read at 528 nm and the oxalate concentration was calculated using a calibration curve of oxalic acid (Naik et al., 2014). The concentration of soluble oxalate in each leaf sample was determined as above, using distilled water instead of 0.25 M HCl.

Fresh samples were extracted with methanol 99% v/v, sonicated for 60 min (frequency 28-34 kHz, power peak 350 W), and then stored at −18 °C for 24 h.; afterwards, the concentration of total chlorophylls, carotenoids, and flavonoids, and the antioxidant capacity (FRAP index) were determined spectrophotometrically as reported by Puccinelli et al. (2022b).

2.4. Statistical analysis

Data were tested for the normality of distribution using Shapiro Wilk's test and for the homogeneity of variances using Levene's test, and then subjected to 2-way ANOVA followed by Tukey's post-hoc test (P <0.05) for mean separation. The percent ratios between soluble and total oxalate were arcsine transformed for statistical analysis but shown in tables as indicated. Regression analysis was performed for the relationship between the leaf concentration of soluble oxalate and Na, and between the Na concentration in leaf tissues and in the nutrient solution. Statistical analysis was performed using JMP Statistical Software (JMP Pro 17.0.0; SAS Institute, Cary, NC Software).

3. Results

3.1. Plant growth and leaf production

In the first experiment, sea beet plants were grown under more favourable light conditions (**Table 1**) and, on average, the production of fresh leaves was greater (+60.9%) than in the second experiment (**Fig. 1A**). The plants harvested in the first experiment also showed greater leaf area, moisture content, succulence (**Fig. 1B,E,F**) and antioxidant activity (FRAP index); they also contained more carotenoids, but less chlorophylls, flavonoids, nitrate, oxalate (**Table 3**), and mineral elements (except Cu and Zn; **Table 3 and 4**). Root DW was greater in the first experiment than in the second (**Fig. 1D**).

Compared to the controls, crop yield was markedly reduced using AE in both experiments (on average, –46.8% and –70.4 % in AE50 and AE100 plants, respectively), although the difference between CNS and AE50 plants was not significant in the second experiment (**Fig. 1A**). In contrast, a slight but significant reduction (–21.2%) of crop yield was observed in TE100 plants only in the first experiment (**Figure 1A**). Similar results were found for LAI (**Fig. 1D**). Leaf DW was not affected by TE and diluted AE, but it significantly decreased in AE100 plants compared to CNS plants (**Fig. 1D**). Root DW was significantly reduced in AE50 and AE100 plants in the first experiment only (**Fig. 1D**).

3.2. Leaf quality

In both experiments, leaf moisture content significantly decreased in AE plants with the lowest value found in AE100 treatment (**Fig. 1E**). In the first experiment, leaf succulence was significantly reduced in TE and AE plants compared to the controls (**Fig. 1F**) while less prominent effects of the nutrient solution on this parameter were found in the second trial.

In the first experiment, using TE and AE resulted in higher leaf antioxidant capacity and concentration (on a FW basis) of total chlorophylls, carotenoids, flavonoids, and phenols compared to the control, while no significant differences across the treatments were found in the second experiment (**Table 3**).

The use of AE100 significantly increased the leaf concentration of N, P, K, Fe, and Zn compared to the other treatments in the first experiment, and the concentration of Ca, Mg, and Cu in both experiments (**Table 4**). A lower leaf Mn concentration were detected in TE plants than in the other plant groups (**Table 4**).

The use of TE and AE markedly increased leaf Na concentration in both experiments, although the difference between CNS and TE50 plants was not significant in the first run (**Table 5**).

In the first experiment, a higher nitrate concentration was detected in the leaves of AE100 plants, with no differences across the other treatments. In contrast, in the second experiment leaf nitrate concentration was significantly higher in AE50 plants and lower in AE100 plants compared to the controls, while no significant differences were detected between CNS and TE plants (**Table 5**).

In both experiments, the leaf concentration of both total and soluble oxalate was significantly higher in AE plants than in those grown with CNS and TE50 **(Table 5)**. There were no important differences across the treatments in the percent ratio between soluble and total oxalate, which ranged between 69.6% and 90.1% **(Table 5)**. The oxalate/Ca molar ratio was increased using TE100, AE50 and AE100 only in the first experiment **(Table 5)**. A highly significant (R^2 = 0.902) positive linear relationship were found between the leaf concentration of soluble oxalate and Na, both expressed as equivalent concentration per unit of leaf water (**Fig. 2**).

4. Discussion

4.1. Crop growth and yield

The salinity and the mineral composition of the nutrient solutions used in the present work were quite different (**Table 2**). Both TE and AE were more saline than CNS due to the higher concentration of Ca, Mg, and Na, and they contained less P, Mn, and Zn. The AE also contained much more B and less Fe than CNS and TE. The ion compositions of TE and AE were similar to those previously reported for effluents from hydroponic culture of tomato (e.g. Massa et al., 2010; Puccinelli et al., 2023) and saltwater inland aquaculture systems (Campanati et al., 2022).

In TE100 plants a significant reduction (–11%) of leaf production was detected only in the first experiment, while in AE plants crop yield was markedly reduced in both runs (**Fig. 1A**). A similar yield reduction of c. 10% was found in cascade cropping systems with basil (Elvanidi et al., 2020)as receiving crop and cucumber as donor crop (Elvanidi et al., 2020). In contrast, crop yield was much lower (–50%, approximately) in rosemary and peppermint grown with effluents from a cucumber culture (Elvanidi et al., 2020).

The reduction of crop yield in TE100, AT50 and AT100 plants was due to the higher salinity of these effluents and not to insufficient or excessive concentrations of some mineral nutrients with respect to the control solution. This interpretation is corroborated by the following observations. Firstly, in both experiments no plant displayed recognisable signs of nutrient deficiency or salt toxicity (e.g., leaf chlorosis and necrosis) and leaf concentration (as expressed on a DW basis) of macronutrients and trace elements (**Table S3**) were within or above the adequate levels reported for beet leaves in all the treatments (Hochmuth and Hanlon, 2022). Secondly, crop yield did not differ significantly between

TE50 and TE100 plants and between AE50 and AE100 plants despite large differences in the concentration of some nutrients in the culture solutions (P and Mn in TE treatments; B and Mn in AE treatments; **Fig. 1A**).

The reduction of leaf FW in AE50 and AE100 plants was due to the leaf dehydration induced by the high salinity of aquaculture effluent, and not to a reduction of dry matter production. Indeed, in both experiments leaf moisture was lower in AE plants than in the other plants (**Fig. 1E**), while no significant differences were observed across the treatments as regards leaf DW, apart from a moderate but significant decrease of this parameter in AE100 plants compared to CNS and TE50 plants (**Fig. 1C**). It is known that a major effect of high salinity is the osmotic stress causing a reduced root water uptake and leaf dehydration (Arif et al., 2020). These findings are in agreement with those of previous work conducted with sea beet and Swiss chard (*B. vulgaris* var. *cicla*) grown in floating raft system with nutrient solutions prepared with freshwater or diluted seawater (salinity of 10 g L^{-1}) (Puccinelli et al., 2022a). In both species, the greater effect of salinity on leaf FW than DW, could be due to the hydroponic system used in these studies. Indeed, the stress induced by salinity can be alleviated in water culture, where the root uptake of water and nutrients is facilitated, due to the high availability of nutrients and water, and it is easier to prevent salt accumulation in the root zone compared to soil or substrate cultivation. For example, basil was more tolerant to salinity stress when grown with nutrient film technique rather than in stonewool cubes (Faliagka et al., 2021).

4.2. Leaf quality

In **Tables 3-5** the concentration of nutraceuticals, minerals, and oxalate has been expressed on a FW basis because the possible effects of vegetables on human health depend on the daily intake of fresh material. However, in this work large differences were found across the treatments and the experiments in terms of leaf moisture content (**Fig. 1E**) and therefore the concentration of these substances was also expressed on a DW basis (**Table S2-S4**) to distinguish the effects of different nutrient solutions that can be ascribed to the genuine root uptake of mineral elements or biosynthesis of nutraceuticals and oxalate rather than to the reduction of leaf water content.

4.2.1. Leaf moisture and succulence

In leafy vegetables, the tolerance to post-harvest handling and storage often decreases concomitantly with increasing tissue moisture content, due to the easier water loss and tenderness of leaves (Clarkson et al., 2003). Therefore, the leaves of AE plants could be longer lasting than those of the other treatments.

Succulence can affect leaf texture, which is an important sensory attribute (Damerum et al., 2020). contrasting results on the effect of salinity on leaf succulence have been reported in the literature. In Swiss chard and sea beet grown in floating raft system with a nutrient solution prepared with freshwater or diluted seawater (10 g L^{-1}), the latter solution resulted in a greater leaf succulence in sea beet while no effects were observed in Swiss chard (Puccinelli et al., 2022a). Moreover, the use of brackish water with salinities up to EC 7.5 dS m^{-1} increased leaf succulence in spinach plants grown in soil, compared with the control (EC 0.8 dS m^{-1}), but the opposite result was observed in plants grown hydroponically (Leal et al., 2020). Therefore, the effect of salinity on leaf succulence depends on plant species and growing conditions. In the present work, leaf succulence significantly decreased in plants grown with saline effluents compared to the controls in the first experiments, with no clear trend in the second run (**Fig. 1F**). The water culture could have alleviated the stress induced by salinity, and consequently have prevented the increase of leaf succulence. The different light intensity in the two experiments could explain the higher leaf succulence in the first experiment compared to the second one (**Fig. 1F**). In spinach grown at two different light levels, leaf thickness, which is related with succulence, was greater in plants grown at higher light intensity (Proietti et al., 2004).

4.2.2. Nutraceuticals

The nutraceutical value and positive effects on human health of leafy vegetables are mostly related to their content of antioxidant compounds, such as carotenoids, flavonoids, and phenols, which play a crucial role in protecting plants from the oxidative stress caused by many kinds of stress (Yang et al., 2022).

The leaf concentration (expressed on a FW basis) of total chlorophyll, carotenoids, and phenols detected in this study (**Table 3**) was similar to the values reported in Swiss chard leaves by other authors, for instance by Gamba et al. (2021) for phenols, and by Libutti et al. (2020) and Hajnal-Jafari (2020) for pigments.

In general, salt stress increased leaf antioxidant activity and concentration of antioxidant compounds in plant leaves, as found in sea beet and sugar beet (Gholipor et al., 2022). However, a reduction of leaf phenol concentration was observed in *Hibiscus sabdariffa* L. exposed to salinities ranging from 60 to 160 mM NaCl (Hashemi and Shahani, 2019). The leaf concentration of chlorophylls and carotenoids generally decreases in salt stress conditions (Mostafa Heidari, 2011). In Swiss chard and sea beet plants, for instance, salt stress induced a reduction of leaf chlorophyll concentration (Yolcu et al., 2021).

In the first experiment, the higher leaf antioxidant capacity and concentration of pigments, flavonoids, and phenols in TE and AE plants (**Table 3**) were due to a reduction of leaf water content (**Fig. 1E**). In fact, if expressed on a DW basis (**Table S2**), these parameters were reduced or not affected by TE and AE as compared to the control.

4.2.3. Mineral nutrients

Leafy vegetables are important components of the human diet as they are among the major sources of minerals. The EU Regulation No. 1169/2011 (European Parliament and Council of the European Union, 2011a) states that the contribution to the diet of a food serving is significant if it provides at least 15% of the recommended daily intake (**Table S5**).

The daily intake (EDI_{50}) of P, K, Ca, Mg, Cu, Fe, Mn, and Zn resulting from the consumption of sea beet leaves was estimated considering a serving size of 50 g and expressed as percent of percentage of the reference intake (RI) for an average adult, (Table S5) (European Parliament and Council of the European Union, 2011a). Sea beet leaves have proved to be a significant source of Ca and Mn; however, the availability of Ca strongly depends on the oxalate concentration, as discussed later. The leaves of AE100 plants were also a significant source of Mg (**Table S5**).

In this study, the leaf concentration (expressed on a FW basis) of P, Ca, K, Mg, Cu, Fe, and Zn increased in AE100 plants (**Table 4**); Cu concentration also increased in AE50 plants. This result was a consequence of a reduced leaf moisture as the leaf concentration of these minerals expressed on a DW basis did not significantly change (Ca) or was reduced (all the other mineral nutrients) in TE or AE plants **(Table S3).** The reduction of K, Mn, and Zn concentration on a DW basis in TE and AE plants was most likely the result of the lower concentration of these minerals in the nutrient solution (**Table 2**). The antagonism between Na and K could also explain the reduction of K uptake in plants grown with a higher Na in the nutrient solution (TE and AE treatments). Indeed, the uptake of K is inhibited by Na (Marschner, 2012).

4.2.4. Sodium

A high Na consumption increases the cardiovascular risk (European Food Safety Authority, 2019). The health risk index (HRI) due to excessive intake of Na was calculated as the percent ratio between EDI_{50} and the allowed daily intake for adults of 2 g day^{-1} of Na (European Food Safety Authority, 2019). In all the treatments, however, leaf Na level was safe as its maximum daily intake with a serving of 50 g of fresh leaves with the highest Na concentration (10.8 g kg^{-1} FW in AE100, second experiment) would be slightly more than 0.5 g per day (**Table S6**).

Leaf Na concentration increased when the plants were irrigated with TE and AE (**Table 5**), not unexpectedly, since these effluents contained much more Na than the control solution (**Table 2**). A

significant (R^2 = 0.887) linear regression was found between the Na concentration in leaf tissues (expressed in meq L^{-1} of leaf water) and in the culture solution (**Fig. S1**). These results agree with those of previous studies with Swiss chard (Puccinelli et al., 2022a), sea beet (Puccinelli et al., 2022a; Yolcu et al., 2021), and spinach (Leal et al., 2020) grown hydroponically with different NaCl concentrations in the nutrient solution.

4.2.5. Nitrate

Nitrate may have several negative effects on human health and because leafy vegetables are among the main sources of nitrate for human nutrition, in the European Union limits have been imposed to the nitrate concentration of some leafy species such as lettuce, spinach, and rocket salad (European Parliament and Council of the European Union, 2011b).

In this work, leaf nitrate levels in plants grown in the second experiment were invariably higher (**Table 5**) than the maximum level established for spinach by the European Union (3.5 mg kg^{-1} FW). Moreover, leaf nitrate concentration was significantly higher in AE plants than in the other treatments (**Table 5**). Similar results were recently found in Swiss chard and sea beet plants grown in floating raft system with standard or saline nutrient solutions (Puccinelli et al., 2022a): leaf nitrate concentration was higher in salinized plants than in non-salinized plants. On the other hand, sea beet is generally consumed cooked, and this significantly reduces the risk of an excessive intake of nitrate, as cooking has been found to reduce the nitrate level in vegetables (Salehzadeh et al., 2020).

In the first experiment, the higher leaf nitrate concentration of AE plants was due to the lower leaf moisture content, as in both experiments the level of nitrate expressed on a DW basis was significantly lower in AE plants than in the controls and TE plants (**Table S4**). A large accumulation of nitrate in plant leaves is due to an imbalance between the uptake and assimilation of this ion and depends on plant species and growing conditions (Colla et al., 2018). Sodium chloride salinity decreases the root uptake and leaf accumulation of nitrate due to the antagonistic interaction between this ion and chloride (Colla et al., 2018). Moreover, it is well known that excessive accumulation of nitrate can occur in plants grown under poor light conditions (Colla et al., 2018) and this explains why leaf nitrate concentration was much higher in the second experiment (**Table 5**), when mean daily solar radiation was lower than in the first run (**Table 1**).

4.2.6. Oxalate

Oxalic acid naturally occurs in many plants and its content ranges between 3% and 80% of plant DW weight depending on plant genotype and organ as well as on growing conditions (Li et al., 2022). Due its strong acidity, in plant tissues oxalic acid generally exists in the form of insoluble oxalate of Ca or Mg, and soluble oxalate of Na or K (Li et al., 2022).

Oxalate contained in food is considered an ‘antinutrient' since it affects the absorption of Ca and increases the risk of developing kidney stones (Petroski and Minich, 2020). Although there are neither official guidelines on daily intake of oxalate nor specific regulations on the oxalate concentration of fresh vegetables, there is a consensus that the maximum daily intake should be 0.2 g day^{-1} in normal individuals (Coe and Harris, 2019) and much lower, 0.04-0.05 g day^{-1} in people predisposed to kidney stones (Marcason, 2006). While insoluble oxalate largely passes through the digestive tract and is not absorbed, soluble oxalate is absorbed and can bind Ca, thus reducing its bioavailability (Simpson et al., 2009). Foods with an oxalate/Ca molar ratio higher than one are not good sources of Ca and can make Ca unavailable in other foods eaten at the same time (Combo et al., 2020). Moreover, oxalate may affect organoleptic quality of foods, because it combines with Ca contained in saliva to generate calcium oxalate crystals, which cause an odd sensation known as "spinach teeth” (Iskandar et al., 2018).

The leaf concentration of total oxalate found in CNS, TE, and AE50 plants (3.65-13.82 g kg^{-1} FW; **Table 5**) were close to the values previously reported in Swiss chard leaves by Freidig and Goldman (2011) (10.19 g kg^{-1} FW) and Simpson et al. (2009) (10.93 g kg^{-1} FW), and in spinach by Joshi et al. (2021) (2.87-7.86 g kg^{-1} FW). Much higher concentration of total oxalate was found in AE100 plants (19.75-22.18 g kg^{-1} FW). In all the plants, most of the oxalate was present in the soluble form (**Table 5**).

The HRI due to excessive intake of oxalate is calculated as the percent ratio between daily ingestion of soluble oxalate, for a serving of 50 g of fresh leaves, and the recommended maximum daily intake is 0.2 g day^{-1} (Coe and Harris, 2019). In all the treatments, apart from the control and TE50 in the first experiment, the daily ingestion of soluble oxalate would always be higher than the recommended maximum daily intake (**Table S6**). The amount of fresh leaves with the highest oxalate concentration (i.e. those of AE plants in the second experiment) that could be consumed daily in order to not exceed this dose was 13.0 g. Moreover, the oxalate/Ca ratio was higher than one in all the treatments in both experiments (**Table 5**) and therefore sea beet leaves cannot be considered a good source of Ca and might make the Ca in other foods unavailable.

As for nitrate, soluble oxalate is leached into cooking water and this reduces its content in the eaten leaves (Savage and Klunklin, 2018). For instance, boiling reduced by 85% the content of soluble oxalate in Swiss chard leaves (Chai and Liebman, 2005). Also, appropriate modification of the hydroponic growing technique could reduce leaf oxalate concentration at harvest. For instance, the addition of ammonium to the nutrient solution reduced the oxalate concentration in purslane (Fontana et al., 2006) and in spinach (Song and Liu, 2015).

Commentato [AP3]: NON C'E' UN UNICO LAVORO CHE DICE TUTTE QUESTE COSE DEGLI OSSALATI?

Commentato [MP4R3]: Purtroppo no

Commentato [AP5]: CONTENT OR CONCENTRATION? CONTROLLA IL LAVORO

In our work, leaf concentration of total and soluble oxalate was greater in plants grown with TE and AE than in the controls (**Table 5**). In TE100 and AE100, the greater oxalate concentration was also observed when expressed on a DW basis, thus suggesting that oxalic acid was accumulated in response to high salinity. A significant (R^2=0.902) positive relationship was found between the equivalent concentration of soluble oxalate and Na in leaf water (**Fig. 2**). This suggests that oxalate may play a role in ion homeostasis regulation of cells (Li et al., 2022). Indeed, oxalic acid is involved in many metabolic processes such as the regulation of intercellular $pH_,$ ion homeostasis, and tolerance to biotic or abiotic stress (Li et al., 2022). A role for oxalate in plant tolerance to salt or alkali stress is suggested by previous findings in several halophytic species, such as *Kochia sieversiana* (Ma et al., 2011), *Suaeda glauca* (Yang et al., 2008), *Portulaca oleracea* (Camalle et al., 2020), and *Chloris virgata* (Yang et al., 2010) grown with Na levels in the growing medium up to 400 mM. In these works, the concentration of total (*S. glauca*) or soluble (*K. sieversiana, P. oleracea* and *C. virgata)* oxalate in fresh leaves significantly increased with Na level in the root zone, thus contributing to osmotic adjustment and balancing excess intake of cations (e.g., Na^+, K^+) over anions (e.g.. $Cl^–$, $SO_4^{2–}$). However, leaf oxalate concentration significantly decreased in purslane plants grown hydroponically with nutrient solutions containing more than 20 mM NaCl as compared to plants grown in NaCl-free solutions (Carvalho et al., 2009). *Salicornia europaea* also showed the largest accumulation of oxalate when grown without NaCl (Austenfeld, 1974). Thus, salinity stress could have different effect of oxalate accumulation depending on plant species and salinity level. In this work, the large accumulation of oxalate in sea beet leaves could also be ascribed to the high nitrate concentration, since this ion has been shown to inhibit the breakdown of oxalate by oxalate oxidase (Libert and Franceschi, 1987).

In spinach grown in a growth chamber with two photosynthetically photon flux densities (200 and 800 µmol $m^{–2}$ $s^{–2}$), Proietti et al. (2004) found that the plants grown under high light conditions contained less oxalate. The authors ascribed this result to the degradation of oxalate by oxalate oxidase, whose activity is stimulated by light (Loewus, 1999). Our results agree with these findings, as in all the treatments leaf oxalate concentration was much lower in the first experiment (**Table 4**), when light conditions were more favourable than in the second run (**Table 1**).

5. Conclusions

The use of wastewater from in-land salt water aquaculture or greenhouse production systems for hydroponic cultivation of fresh vegetables has the main advantages of saving water and fertilisers and reducing the discharge of nutrients (in particular, nitrate and phosphate) to the environment.

According to the results of this work, sea beet plants can be grown in floating raft system, in greenhouse using as nutrient solution the drainage water from a semi-closed tomato substrate culture, with limited reduction of crop yield and no or minor effects on leaf quality, even when the effluent was used without dilution with fresh nutrient solution. In contrast, the use of the effluent from saltwater aquaponics , as such or after dilution, markedly reduced crop yield and quality due to the large accumulation of sodium, nitrate, and oxalate. In general, sea beet leaves were high in oxalate and should be consumed moderately and/or after cooking. Future research could be carried out with the aim of developing cultivation protocols that allow the reduction of leaf oxalate concentration in this and other leafy species

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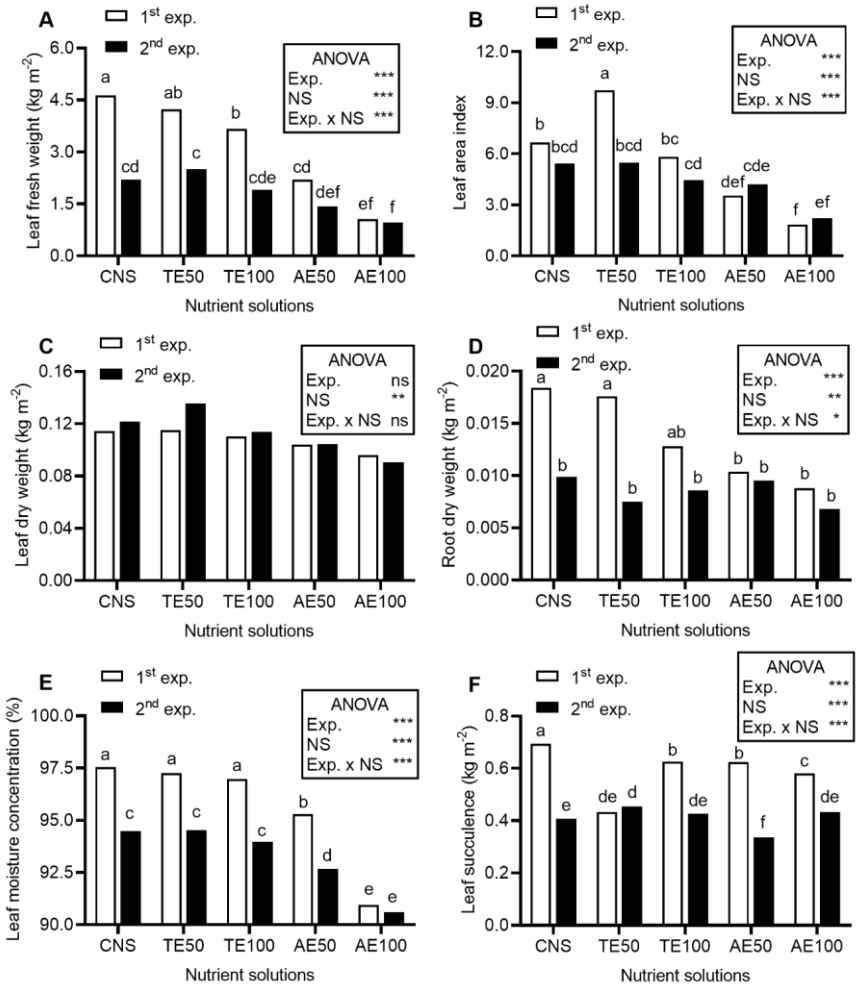

673

674 **Fig. 1.** Leaf fresh weight (A), leaf area index (B), leaf (C) and root (D) dry weight, leaf moisture
675 content (E) and succulence (F) in *Beta vulgaris* subsp. *maritima* plants grown in floating raft system
676 with different nutrient solutions. CNS: standard nutrient solution; TE50: tomato effluent diluted
677 (50:50) with CNS; TE100: tomato effluent used as such; AE50: aquaculture effluent diluted (50:50)
678 with CNS; AE100: aquaculture effluent used as such. Means (n = 3) flanked by the same letter are
679 not statistically different at 5% level after Tukey's test.

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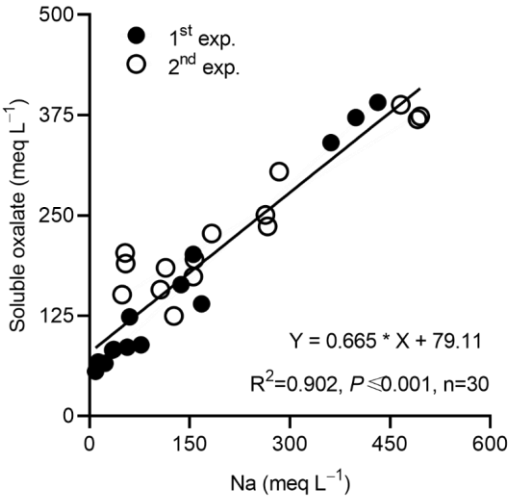

Fig. 2. Linear regression between the equivalent concentration of soluble oxalate and sodium (Na) per unit of leaf water in *Beta vulgaris* subsp. *maritima* plants grown in floating raft system with different nutrient solutions.

Table 1. Basic information on the experiments with *Beta vulgaris* subsp. *maritima* plants grown in floating raft system in greenhouse in 2021.

	First experiment	Second experiment
Sowing date	6 September	29 September
Transplant date	4 October	17 November
Start of treatment	18 October	1 December
Harvest date	2 November	22 December
Days of treatment	15	21
Mean air temperature (°C)	20.80*	19.62*
Mean daily solar radiation (MJ m^{-2} day^{-1})	4.10*	1.52*
Cumulative solar radiation (MJ m^{-2})	118.90*	53.20*

* The values were computed for the period from transplanting to harvest: 29 and 35 days in the first and second experiment, respectively

693 **Table 2.** Mineral composition, electrical conductivity (EC), and pH of the nutrient solutions used in
694 the experiments with *Beta vulgaris* subsp. *maritima* plants grown in floating raft system in
695 greenhouse. The nutrient solutions were the following: standard nutrient solution (CNS, control)); the
696 effluent from a tomato substrate culture used as such (TE100) or diluted (50:50) with the CNS (TE50);
697 the effluent from a saltwater aquaculture system with Gilthead Sea bream used as such (AT100) or
698 diluted (50:50) with the CNS (AT50).

	Nutrient solutions				
	CNS	TE50	TE100	AE50	AE100
$N\text{-}NO_3$ (mM)	10.00	10.25	10.50	7.80	5.60
P (mM)	1.50	0.88	0.25	1.10	0.70
K (mM)	9.00	6.95	4.90	8.60	8.20
Ca (mM)	4.50	5.70	6.90	5.95	7.40
Mg (mM)	2.00	3.55	5.10	21.05	40.10
Na (mM)	0.65	17.78	34.90	204.63	408.60
Fe (µM)	40.00	35.25	30.50	22.70	5.40
B (µM)	40.00	30.00	20.00	170.50	301.00
Cu (µM)	3.00	4.00	5.00	1.90	0.80
Zn (µM)	10.00	6.65	3.30	8.55	7.10
Mn (µM)	10.00	5.35	0.70	5.45	0.90
Electrical conductivity (dS m^{-1})	2.80	4.50	6.49	25.40	42.00
pH	5.5	5.5	5.5	5.5	5.5

699

700 **Table 3.** Leaf antioxidant capacity (FRAP index) and concentration of nutraceuticals (both expressed
701 on a fresh weight basis), in *Beta vulgaris* subsp. *maritima* plants grown in floating raft system with
702 different nutrient solutions.

Experiment	Nutrient solutions	FRAP (mmol Fe(II) kg^{-1})	Chlorophylls (g kg^{-1})	Carotenoids (g kg^{-1})	Flavonoids (g kg^{-1})	Phenols (g kg^{-1})
First	CNS	8.408 bc	0.766 de	0.076 d	0.207 d	0.857 b
	TE50	9.356 b	0.718 e	0.113 cd	0.637 cd	1.133 b
	TE100	10.518 ab	0.893 bcde	0.189 a	0.763 bc	1.196 ab
	AE50	10.604 ab	0.816 cde	0.145 abc	0.780 bc	1.328 ab
	AE100	12.877 a	0.976 abcde	0.180 ab	1.479 a	1.725 a
Second	CNS	6.694 c	1.216 a	0.126 bcd	1.325 a	1.266 ab
	TE50	6.802 c	1.023 abcd	0.115 cd	1.231 ab	1.324 ab
	TE100	6.448 c	1.102 ab	0.118 cd	1.266 a	1.240 ab
	AE50	6.388 c	1.045 abcd	0.110 cd	1.417 a	1.320 ab
	AE100	6.617 c	1.056 abc	0.135 abcd	1.026 abc	1.139 b
First		10.353 a	0.834 b	0.140 a	0.774 b	1.248
Second		6.590 b	1.089 a	0.121 b	1.253 a	1.258
	CNS	7.551 b	0.991	0.101 b	0.766 c	1.062
	TE50	8.079 b	0.871	0.114 b	0.934 bc	1.229
	TE100	8.483 ab	0.998	0.153 a	1.015 abc	1.218
	AE50	8.496 ab	0.931	0.127 ab	1.099 ab	1.324
	AE100	9.747 a	1.016	0.158 a	1.253 a	1.432
ANOVA						
Experiment		***	***	*	***	ns
Nutrient solutions		**	ns	***	***	ns
Experiment x Nutrient solutions		**	*	***	***	**

703 CNS: standard nutrient solution; TE50: tomato effluent diluted (50:50) with CNS; TE100: tomato effluent used as such;
704 AE50: aquaculture effluent diluted (50:50) with CNS; AE100: aquaculture effluent used as such. Means (n = 3) flanked
705 by the same letter are not statistically different at 5% level after Tukey's post-hoc test.. Significance level: *** $P \leq 0.001$;
706 ** $P \leq 0.01$; * $P \leq 0.05$; ns = not significant.

707

Table 4. Leaf concentration (on a fresh weight basis) of mineral nutrients in *Beta vulgaris* subsp. *maritima* plants grown in floating raft system with different nutrient solutions.

Experiment	Nutrient solutions	N-tot (g kg^{-1})	P (g kg^{-1})	K (g kg^{-1})	Ca (g kg^{-1})	Mg (g kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})
First	CNS	1.521 de	0.067 d	2.476 f	1.233	0.307	1.231	6.449 d	10.983	12.196 b
	TE50	1.628 de	0.067 d	3.046 ef	1.096	0.324	0.832	8.314 cd	8.153	3.770 de
	TE100	1.478 e	0.074 cd	3.088 ef	1.036	0.333	0.605	7.794 cd	4.852	4.349 de
	AE50	2.334 d	0.107 cd	4.329 de	1.650	0.517	1.731	10.861 bcd	11.943	6.748 cde
	AE100	4.669 a	0.164 c	5.830 bc	2.954	1.120	1.810	18.372 a	12.687	18.721 a
Second	CNS	4.107 abc	0.371 b	7.284 a	3.892	0.553	0.547	15.665 ab	14.911	2.891 e
	TE50	3.465 c	0.351 b	6.041 abc	3.214	0.483	0.545	9.249 cd	7.990	3.642 e
	TE100	3.790 bc	0.296 b	5.056 cd	4.509	0.573	0.602	10.852 bcd	4.624	8.456 bcd
	AE50	4.513 ab	0.331 b	6.544 ab	4.826	0.622	0.733	12.964 abc	16.348	5.873 de
	AE100	4.364 ab	0.482 a	5.845 bc	6.129	1.271	0.933	15.533 ab	16.124	11.232 bc
First		2.326 b	0.096 b	3.754 b	1.594 b	0.520 b	1.242 a	10.358 b	9.724 b	9.157 a
Second		4.048 a	0.366 a	6.154 a	4.514 a	0.700 a	0.672 b	12.852 a	11.999 a	6.419 b
	CNS	2.814 c	0.219 b	4.880 bc	2.563 bc	0.430 bc	0.889 b	11.057 b	12.947 a	7.544 b
	TE50	2.546 c	0.209 b	4.543 cd	2.155 c	0.403 c	0.688 bc	8.782 b	8.071 b	3.706 c
	TE100	2.634 c	0.185 b	4.072 d	2.772 bc	0.453 bc	0.604 c	9.323 b	4.738 c	6.402 bc
	AE50	3.424 b	0.219 b	5.437 ab	3.238 b	0.570 b	1.232 a	11.912 b	14.145 a	6.311 bc
	AE100	4.517 a	0.323 a	5.838 a	4.541 a	1.195 a	1.372 a	16.953 a	14.406 a	14.977 a
ANOVA										
Experiment		***	***	***	***	***	***	**	**	***
Nutrient solutions		***	***	***	***	***	***	***	***	***
Experiment x Nutrient solutions		***	*	***	ns	ns	ns	***	ns	***

CNS: standard nutrient solution; TE50: tomato effluent diluted (50:50) with CNS; TE100: tomato effluent used as such; AE50: aquaculture effluent diluted (50:50) with CNS; AE100: aquaculture effluent used as such. Means (n = 3) flanked by the same letter are not statistically different at 5% level after Tukey's post-hoc test. Significance level: *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; ns = not significant.

713 **Table 5.** Leaf concentration (on a fresh weight basis) of oxalate, nitrate, sodium and soluble/total
714 oxalate ratio and molar ratio between oxalate and Ca, in *Beta vulgaris* subsp. *maritima* plants grown
715 in floating raft system with different nutrient solutions.

Experiment	Nutrient solutions	Sodium (g kg^{-1})	Nitrate (g kg^{-1})	Total oxalate (g kg^{-1})	Soluble oxalate (g kg^{-1})	Soluble/total oxalate (%)	Oxalate/Ca molar ratio
First	CNS	0.257 g	1.323 e	3.651 f	2.725 e	75.2	1.32 de
	TE50	0.700 fg	1.503 e	4.735 ef	3.388 e	71.4	1.96 bcd
	TE100	1.440 f	1.414 e	5.772 e	4.358 de	75.2	2.53 ab
	AE50	3.358 de	1.983 e	8.022 d	7.253 cd	90.1	2.18 abc
	AE100	8.306 b	2.714 d	19.750 b	15.120 a	76.6	2.99 a
Second	CNS	1.129 fg	4.417 b	9.266 d	7.753 c	83.5	1.06 e
	TE50	2.506 e	4.171 bc	9.526 d	6.650 cd	69.9	1.32 de
	TE100	3.568 d	4.106 bc	12.100 c	8.449 bc	69.6	1.24 de
	AE50	5.783 c	5.165 a	13.824 c	11.047 b	80.1	1.29 de
	AE100	10.087 a	3.572 c	22.177 a	15.442 a	69.7	1.63 cde
First		2812 b	1.787 b	8.386 b	6.569 b	77.7	2.20 a
Second		4615 a	4.286 a	13.379 a	9.868 a	74.6	1.31 b
	CNS	693 e	2.870 b	6.459 d	5.239 c	79.4 ab	1.19 c
	TE50	1603 d	2.837 b	7.130 d	5.019 c	70.7 b	1.64 bc
	TE100	2504 c	2.760 b	8.936 c	6.403 c	72.4 ab	1.88 ab
	AE50	4571 b	3.574 a	10.923 b	9.150 b	85.1 a	1.74 b
	AE100	9197 a	3.143 b	20.964 a	15.281 a	73.2 ab	2.31 a
ANOVA							
Experiment		***	***	***	***	ns	***
Nutrient solutions		***	***	***	***	*	***
Experiment x Nutrient solutions		***	***	***	***	ns	*

716 CNS: standard nutrient solution; TE50: tomato effluent diluted (50:50) with CNS; TE100: tomato effluent used as such;
717 AE50: aquaculture effluent diluted (50:50) with CNS; AE100: aquaculture effluent used as such. Means (n = 3) flanked
718 by the same letter are not statistically different at 5% level after Tukey's post-hoc test. Significance level: *** $P \leq 0.001$;
719 ** $P \leq 0.01$; * $P \leq 0.05$; ns = not significant.