ECOTOXICOLOGICAL RISK ASSESSMENT AS A DECISION TOOL FOR THE VALORISATION OF WINERY WASTEWATER TREATMENT BYPRODUCTS TO AGRICULTURAL PURPOSES

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ABSTRACT

A simple and economical process consisting in the precipitation with slaked lime ($Ca(OH)_2$) was applied to winery wastewater. The process not only removed organic matter and other contaminants but also simultaneously captured atmospheric CO_2. In order to help the wine industry to advance to the objective of circular economy, treatment byproducts (supernatant and sludge) have been reused for agricultural purposes due to their physicochemical properties compatible with these applications. In addition to an exhaustive study of the physicochemical characteristics, the ecotoxicological impact of these by-products were also evaluated through the bioindicators: *Thmanocephalus platyurus* (mortality bioassay after 24h), *Vibrio fisheri*

(luminescence inhibition after 30 min) and *Pseudokirchneriella subcapitata* (growth inhibition after 72h).

Raw winery wastewater showed high toxicity to all bioindicators, being classified as class 4 (Toxic Unit (TU); classification proposed by Personne, 1999). According values of TU of 20% for *T. platyurus* (24h), 9.17% for *V. fisheri* (30 min) and 100% for *P. subcapitata* (72h) were found. After treatment (treated WW), the toxicity significantly dropped to class 2, with values of TU of 1.34% for *T. platyurus* (24h), 2.92% for *V. fisheri* (30 min) and 3.97% for *P. subcapitata* (72h).

After the immediate one-step lime precipitation process (treated WW), the supernatant was diluted 1:4 (v/v) with groundwater (GW) to generate the so-called effluent nutrient solution (WWNS) which did not present any type of toxicity to the bioindicators used.

Accordingly, an assay was carried out to the production of red pak choi, using three different conditions: irrigation with groundwater (control); pH soil correction with organomineral fertilizer and groundwater irrigation; and irrigation with wastewater nutrient solution (WWNS). After the assay none of the soils showed any type of indirect ecotoxicity.

The pigment of the red pak choi cultivar produced in this study was evaluated through the analysis of chlorophyll a, chlorophyll b and carotenoids, showing the benefits to the healthy development of the plant.

Keywords: Wine industry; ecotoxicological assays; wastewater industry; slaked lime.

1. INTRODUCTION

The Mediterranean area is one of the most important regions in wine production worldwide (Amor et al. 2019; Oliveira et al. 2019), with the European countries representing 61% of total world production (Bolzonella et al. 2019). Portugal is the 11th wine producer in the world, with 7.4 million hL in 2021 (IVV IP, 2022), being Alentejo (one of the 14 wine regions, located in the South of Portugal) the 3rd wine producing region of the country, with almost 1.2 x 10^{6} hL of wine produced in 2021/2022 (IVV IP, 2022). In the Alentejo region, the vineyards crops are irrigated, due to the meteorological conditions of the area with low precipitation and high temperatures (Costa et al. 2020).

Because of climate change and water scarcity, growth of the wine industry can be questioned (Bolzonella et al. 2019). Reuse of resources, namely water, is of paramount importance to the sustainability of this activity. During the winemaking process (from the reception of the grape to bottling), many resources are used, mainly energy and water (cleaning tanks and barrels, washing

equipment and floors) (Amor et al. 2019). In the case of water, consumption can raise up to 450 L of water per liter of wine produced (Costa et al. 2020). In addition to the water used in the process, effluents generated are of major concern for the sector, with high variation in composition and flow generated throughout the natural year (Ngwenya et al. 2022). Additionally, the effluent generated contains a wide diversity of substances such as grape films and seeds, yeasts, alcohols (methanol, ethanol and glycerol), soluble acids (acetic, tartaric), tannins, lignin's, polyphenols, residues and cleaning and disinfection products (Ngwenya et al. 2022; Beer et al. 2017; Solís et al. 2017). In general, winery effluents present high values of organic matter measured as chemical oxygen demand (COD) 31.37 - 38.39 g L^{-1}, total suspended solids (TSS) 34.90 - 76.60 g L^{-1}, as well as high turbidity 319-782 NTU (Ioannou et al. 2015). Due to the seasonal character of this crop, the pH of the effluent varies throughout the year. Additionally, this effluent is characterized by low levels of nutrients such as nitrogen and phosphorus (Bolzonella et al. 2019) and high levels of sodium and potassium (Arienzo et al. 2009). Winery effluents are frequently discharged into the environment or into the sewer system without an adequate pre-treatment stage (Calheiros et al. 2018), presenting a strong environmental impact (Amor et al. 2019). If properly treated, winery wastewater can be reused for agricultural irrigation (Mosse et al. 2012). Winery wastewater treatment studies have already been presented in the specialized literature, such as, coagulation-flocculation (Braz et al. 2010; Rizzo et al. 2010); coagulation-flocculation-decantation and ozonization process (Jorge et al. 2021a) membrane processes (nanofiltration and reverse osmosis) (Ferrarini et al. 2001; Ioannou et al. 2013a); ozonation (Beltrán et al. 1999; Lucas et al. 2009a, 2009b); combination of ozone and radiation and/or hydrogen peroxide (Lucas et al. 2009 a); photo-Fenton (Mosteo et al. 2006; Ormad et al. 2006); photo-Fenton with solar radiation (Monteagudo et al. 2012); wet oxidation (Domínguez et al. 2014); anodic oxidation (Lauzurique et al. 2022; Candia-Onfray et al. 2018), anaerobic digestion (Kaira et al. 2022), combination of adsorption and thermocatalytic processes (Jorge et al. 2021b) advanced oxidation processes based on sulfate radicals (Amor et al. 2019), photocatalytic/ photolytic processes (Agustina et al. 2008) and microalgae and immobilized TiO_2/UV-A LEDs (Marchão et al. 2021). However, the application of alkaline precipitants for the treatment of wastewater in wineries still needs to be further studied. Luz et al. 2021, developed a simple and cheap treatment process for winery wastewater which consists of a single precipitation step with slaked lime ($Ca(OH)_2$) to remove organic matter and other contaminants with simultaneous atmospheric CO_2 capture. That application results in an abundant and insoluble precipitate, capable of sweeping and removing contaminants and the instantaneous combination of calcium and magnesium salts, through the addition of an excess of calcium ions (Luz et al. 2021). In addition to the process studied and to the analysis of chemical parameters, an integral evaluation of the treatment efficiency in terms of reuse or disposal is recommended. This process

could help the winery industry to achieve circular economy through the reuse of generated residues (supernatant and sludge).

Application of byproducts for agricultural purposes requires a previous ecotoxicological analysis. The assessment of ecotoxicological endpoints allow a preliminary analysis of the environmental impact of these wastewaters (Mendonça et al. 2009). In this line, Sousa et al. (2019) studied the impact of vinasses wastewater obtained from the distillation of winemaking by-products on several species, such as *Aliivibrio fisheri*, *Daphnia magna* and *Zebrafish sp*. The acute results revealed a high toxicity for all species tested, indicating a strong impact in aquatic ecosystem (see Table 1).

Arienzo et al. (2009) used Garden cress (*Lepidium sativum*) and Onion (*Allium coepa*) in phytotoxicity bioassays to determine the quality of the winery wastewater (see Table 1). The authors claimed again a high toxicity, inhibiting the development and growth of the species under study correlated to high levels of COD, phenols and salinity, and low pH.

Ioannou et al. (2013b) carried out the treatment of winery wastewater by a membrane bioreactor (MBR) and Fenton's solar oxidation processes. The complete process generated an effluent without toxicity to the aquatic crustacean *D. magna*, as well as without phytotoxicity (Table 1).

In summary, the main objectives of this study were: (i) to assess the environmental impact of wastewater from wineries; (ii) analyze the potential reuse of the supernatant and precipitate in food production.

Table 1. Ecotoxicological effects of raw and treated winery wastewater

TYPE OF WASTEWATER	BIOINDICATORS	BIOASSAY	ECOTOXICOLOGICAL ENDPOINTS (EC_{50})	STUDY
Raw winery	*D. magna*	Mortality/ immobility	93.3±2% (24h)	Ioannou et al. 2013b
Winery MBR + Fenton solar oxidation process	*D. magna*	Mortality/ immobility	26.7±1.5% (24h)	
	D. magna	Mortality/ immobility	4.8% (48 h)	
Raw vinasse	*Zebrafish* embryos	Mortality	0.34% (96 h)	Sousa et al. 2019
	A. fisheri	Luminescence inhibition	7.0% (30 min)	
Raw winery	*Lepidium sativum*	Germination	2.5-15%	Arienzo et al. 2009
	Allium coepa	Germination	0.25-2%	

EC_{50} - concentration with effect in 50% of the exposed population.

2. MATERIALS AND METHODS

This article is a complement of the article of Luz et al. (2021), where a study of the best efficient processes of the immediate lime precipitation and carbonatation with atmospheric CO_2 process was reported. This work adds the description of the preparation of the nutritive solution based on the wastewater from the treated wine cellar and all the ecotoxicological characterization of the different waters, to be applied in agricultural, with correction soil properties and growth of the Red Pak Choi (*Brassica rapa L. var. chinensis (L.) Kitam*), through irrigation with diluted supernatant and incorporation of sludge into the soil.

2.1. Winery Wastewater

Winery wastewater was collected from a winery located at Vidigueira (Beja; Portugal), that has an integrated production regime, consisting of more than 100 ha of vineyards in sustainable planning, with about 10 grape varieties (Antão Vaz, Síria, Verdelho, Alvarinho, Arinto, Chenin Blanc, Aragonez, Touriga Nacional, Alicante Bouschet and Tinta Miuda).

The sampling was carried out in a single day in September 2017, at the winery effluent treatment station in the wastewater (WW) storage tank. Wastewater samples were collected in 5 L polyethylene (PET) packages, subjected to refrigeration at 4ºC or freezing at -20ºC (the specific procedure was detailed previously in Luz et al. 2021), until analysis.

2.2. Soil Characterization

Soil samples were collected from a vineyard, whose main characteristic is its acidic nature. After collection, soil samples were air-dried and sieved through a 2 mm sieve. Physicochemical characterization was carried out in triplicate. Extractable P and K were determined using the Egnere-Riehm Method (Riehm, 1958).

After air-drying, soil samples were subjected to a water leaching test at room temperature for 24 h, under constant stirring, using deionized water to form a solid-liquid solution 1:5 (w/v) ratio. The leachate was separated by centrifugation and filtered through a membrane filter of 0.45 µm pore size. Leachate was analyzed for pH, Electrical Condutivity (EC; dS m^{-1}) and ecotoxicologycal bioassays.

2.3. Metals analysis

Metal ions Pb, Cd, Zn, Fe, Ni, Mn, Cu, Ca and Mg were determined in the wastewater samples and leached from the soil, by atomic absorption spectrometry (AAS) after digestion of the samples with aqua regia, according to ISO 11466 (1995). Samples were digested with a mixture of HCl (37%) and HNO_3 (70%), in a ratio 3:1 (v/v), at room temperature for 16 h, and then at 130 ° C for 2 h, under reflux conditions.

Each suspension was then filtered and diluted to 100 mL with 0.5 mol L^{-1} HNO_3. Three independent replicates were analyzed for each sample and blanks were measured in parallel.

2.4. Ecotoxicological characterization

A battery of bioassays was performed in the following samples: Raw Wastewater (WW); Treated WW (TWW); Groundwater (GW); WW nutrient solution (WWNS) (see Table 2) assessing lethal and sub-lethal endpoints, with representative species of key taxonomic and functional groups: i) lethal effects assessed in crustaceans; and ii) two sublethal effects, light and growth inhibition assessed in bacterial and algae.

Raw WW is water directly collected from the winery's treatment plant; treated WW is the water treated through the immediate one-step lime precipitation process ((optimal dosage of 5 g L^{-1} of slaked lime (Luz et al. 2021)), and GW is the water that will be further used to dilute the TWW; WWNS is the combination TWW and GW (dilution 1:4), in order to obtain the optimum pH and EC for plant growth.

All bioassays considered have been largely used in ecotoxicological studies and, most of them, have already been standardized. The bioassays were: i) 30 min of luminescence inhibition of *V. fischeri* (ISO 11348-2, 1998); ii) 24-h mortality test with *T. platyurus* (Persoone, 1999); iii) 72-h population growth of the green microalgae *P. subcapitata* (OECD 201, 2006). All reported ecotoxicological tests fulfilled the validity requirements established by the respective guidelines.

2.4.1. *V. fischeri* bioassay

The Luminotox® bioassay was completed by using a marine bacterium *Vibrio fischeri* (NRRL B-11177). Luminescence inhibition was monitored with bacteria supplied as a dry liquid solution (Dr. Lange GmbH & Co. KG, Düsseldorf, Germany, ISO 11348-2 standard (1998) and protocol "DR LANGE luminescent bacteria test"). The solution was stored at -20 °C and rehydrated before tests. These tests were carried out with two replicates of 100% (v/v) of the samples and successive dilutions of 2% NaCl at 50, 25, 12.5, 6.25 and 3.125% (v/v) and non-toxic control of 2% NaCl solution at a fixed temperature of 15±0.5°C. In each sample, bioluminescence was measured

before and after the desired incubation period (30 min.) and EC_{50} (%; the concentration of each sample that reduced 50% of bacterial luminescence) was determined. The decrease in luminescence is due to the inhibition of the bacterial luciferase enzyme by exposure to the samples.

2.4.2. *T. platyurus bioassay*

The Thamnocephalus platyurus bioassay was carried out following the protocol used in the THAMNOTOXKIT F™ kit (Persoone, 1999). This test evaluates the mortality of shrimp larvae (<24h) incubated in 24-well plates, with 1.0 mL of the test solution and ten crustaceans per well. Four repetitions per treatment were competed at 25 °C for 24 hours in the absence of light, without feeding the animals during the time of the test. Concentrations of 12.5, 25, 50, 100% (v/v) were tested using the samples of supernatant. And concentrations of 25, 50, 100% (v/v) were tested using the samples (included in the test kit, also used as a non-toxic control) for the soils. The 24 hour contact with each test solution was used as the selected end point, and the EC_{50} (%) was calculated.

2.4.3. Growth inhibition of green microalgae *P. subcapitata*

The determination of the fluorospectrometric growth rate of the unicellular microalgae *P. subcapitata* was carried out for 72 hours, (protocol of OECD 201 (2006)), with the MBL Medium Woods-Holecontrol medium. At the beginning of the test, *P. subcapitata* (100 µL of an inoculum with $3\text{-}5\times10^4$ cells mL^{-1}) was exposed to water samples (900 µL) in a 24-well microplate. The test flasks were randomly incubated in an orbital shaker for 72 hours at a constant temperature of 21±2°C and with a constant light intensity (60–120 µE m^{-2} s^{-1}, equivalent to 6.000–10.000 lx). Concentrations were calculated using the microscope and Neubauer chamber.

The average rate of specific growth for a specific period was calculated as the logarithmic increase in biomass (after 72 hours) using the equation:

$$\mu_{i-j} = (\ln B_j - \ln B_i)/t_j\text{-}t_i \quad (1)$$

Where: μ_{i-j} is the average specific growth rate from time i to j; t_i is the time for the start of the exposure period; t_j is the time for the end of the exposure period, B_i is the biomass concentration at time i, and B_j is the biomass concentration at time j.

The inhibition of algae growth was estimated as the percentage of reduction in the growth rate compared to the control:

$$\% I = [(\mu_c - \mu_t)/ \mu_c] \times 100 \quad (2)$$

Where: % I is the mean percentage of inhibition for specific growth rate; μ_c is the mean value for the growth rate in the control, and μ_t is the mean value for the growth rate in water samples.

2.5. Red Pak Choi cultivation. Experimental design

Red Pak Choi was grown in pots with 1.5 kg of acidic soil under three different conditions: i) irrigated with groundwater (soil control); ii) correction of soil pH with organomineral fertilizer + underground irrigation; iii) irrigation with wastewater nutrient solution (WWNS). The experimental design was randomized with nine replications, and the experiment was conducted outdoors under uncontrolled temperature conditions, from May to July. The plants were placed in the tests when they had only cotyledonary leaves.

The studied acid soil had a pH of 5.3±0.1, K 152.7±4.2 mg L^{-1} K_2O fine soil, P 32.9±3.6 mg L^{-1} P_2O_5 fine soil classified as sandy loam, 0.8±0.1% organic matter, with 50.6% coarse sand, 30.1% fine sand, 8.8% silt and 10.2% clay (Table 4).

Monitoring of plant growth was through marking and measurements. Leaf biomass was sampled for determination of moisture, chlorophylls (a, b) and carotenoids.

2.6. Preparation of WW nutrient solution

WWNS was prepared from the TWW, with the addition of GW, until the ideal pH and conductivity characteristics for plants growth (*Brassica rapa L. var. chinensis (L.) Kitam*) were obtained. These vegetables are considered moderately salinity sensitive with a threshold EC of 1.3 dS m^{-1}, and the salinity of the irrigation water can compromise the vegetative growth and root development of the plant. (Ünlükara et al. 2008).

Dilution with GW was optimized to achieve the reduction of water salinity (see Table 2) as well as its toxic effect. According to Figure 1, toxicity is eliminated after dilution above 25%. Thus, the ideal condition for WWNS is the addition of 1:4 groundwater, obtaining a pH of 7.2±0.13 and EC of 1.1 ± 0.63 dS m^{-1} (Table 2).

Table 2. Characterization of WW Samples

Parameter	Units	Raw WW*	TWW*	GW	WWNS
pH	Sorensen scale	4.26±0.03	12.41±0.24	7.79±0.05	7.17±0.13
Electric Conductivity	dS m^{-1}	1.243±0.034	6.545±0.62	0.525±0.16	1.140±0.63
Total hardness	mg $CaCO_3$ L^{-1}	424.78±53.10	1804.97±350.75	319.17±5.52	345.69±23.77
Magnesium	mg Mg L^{-1}	98.71±13.33	179.89±58.63	32.01±2.07	76,75±1.86
Calcium	Mg Ca L^{-1}	7.09±6.14	426.76±84.46	75.12±5.35	134.65±8.59
COD	mg O_2 L^{-1}	3225±25	2509±166	0	448±48
BOD_5	mg O_2 L^{-1}	1266 ±208	280±0	11±1	17 ±2
BOD_5/COD	-	0.39	0.11	---	0.04
Turbidity	NTU	159.77±7.68	2.00±0.84	---	---
N- NH_4^+	mg NH_4 L^{-1}	0.72±0.00	5.38±0.00	0	0
N Kjeldhal	mg N L^{-1}	22.3±1.6	7.58±0.00	0.19±0.32	1.86±0.32
Ptotal	mg P L^{-1}	155.69±6.53	20.3±8.65	4.41±1.78	9.54±0
Phosphates	mg PO_4 L^{-1}	---	---	---	---
Chlorides	mg L^{-1}	124.7±1.5	121.9±23.8	23.3±0.15	78.9±5.8
Nitrates	mg NO_3^- L^{-1}	5.9±0.4	12.6±2.7	86.7±0.3	21.2±0
Fluorides	mg L^{-1}	166.7±2.3	176.0±11.1	0.03±0.0	36.3±0.1
Sulphates	mg SO_4^{2-} L^{-1}	95.4±0.5	56.5±14.3	34.4±0.2	52.5±0.3
Iron	mg Fe L^{-1}	5.725±1.591	0.030±0.009	---	0.030±0.001
Manganese	mg Mn L^{-1}	0.173±0.018	0.005±0.007	---	0.005±0.001
E. Coli	ufc/100mL	0	0	0	---
Fecal coliforms	ufc/100mL	45±29.8	0	0	---
Enterococus	NMP/mL	3.7×10^{-3}	0	0	---

* Luz et al. 2021 (mean±SD; n = 3; $p<0.05$)

2.7. Sludge

After applying the processes of immediate precipitation of lime, used in the study, the sedimentation and decantation process is carried out, where the TWW is separated from the precipitate (sludge), which, due to its characteristics, pH 12.40±0.02, EC 7.25±0.15 dS m -1, Magnesium 0.0381±0.0067 mg L^{-1} (Luz et al. 2021), was used as an agricultural corrective in the studied acid soil.

2.8. Statistics

In the *V. fischeri* bioluminescence inhibition test, the EC_{50} (%) values were determined using LUMISsoft 4 Software™. The EC_{50} (%) for the *T. platyurus* and *D. magna* mortality were determined using the probit analysis (Finney 1971). Data for the inhibition growth bioassay were checked for homogeneity of variance by the Kolmogorov–Smirnov test and, when possible, subjected to one-way analysis of variance (ANOVA). Data which did not satisfy the assumption for ANOVA, were analyzed non-parametrically using Kruskal–Wallis ANOVA by ranks test. Whenever significant differences were found ($p<0.05$), a post hoc Dunnett's test was used to compare treatments with the control, for an alpha value of 0.05 (Zar 1996).

For a better interpretation of toxicity data, all measurements (EC_{50}) were converted into Toxicity Units (TU), i.e. the inverse of EC_{50} values, expressed in percent:

$$TU\ (\%) = 1/EC_{50} \times 100 \qquad \text{(Equation 5)}$$

With the toxicity classification system (TCS) reported by Personne et al. (2003), it is possible to classify the samples as: class 1 (TU < 1) exhibiting no significant toxicity; class 2 (1 < TU < 10) exhibiting significant toxicity; class 3 (10 < TU < 100) exhibiting high toxicity; and class 4 (TU > 100) exhibiting very high toxicity.

The results of the parameters analyzed in the remaining tests were submitted to analysis of variance (ANOVA), and their means were compared by Tukey's test, at the 0.05% level of significance, using the GraphPad.

In the ANOVA analysis of variance, it was determined that whenever the F value was greater than the critical F, there was a significant effect between treatments. Whenever F was less than the critical F, there was no statistically significant difference.

3. RESULTS

3.1. Ecotoxicological Assessment

This study presents results of the ecotoxicological analysis of several water matrices obtained from the chemical treatment applied to winery wastewaters, and the assessment of their potential use (diluted with groundwater) as irrigation water. The ecotoxicological assessment allowed to

analyze the risk to the aquatic ecosystem by using the irrigation solution prepared (produced from treated winery wastewaters). The ecotoxicological results using the bioindicators *T. platyurus*, *V. fischeri* and *P. subcapitata* are summarized in Table 3. Further, the results for growth inhibition of *P. subcapitata* (a sub-lethal parameter) are shown in Figure 2.

The lethal ecotoxicological results (Toxic Unit (TU); Table 3) indicated that the winery effluent was very toxic to the three bioindicators, being classified as class 4 (TU> 100; with very high toxicity), constituting a serious danger to the receiving environment. The green microalgae was the organism most sensitive to this effluent with a 72h-EC_{50} = 0.02% for growth inhibition. The toxicity detected may be correlated to: (i) the concentrations of chlorides (125 mg L^{-1}), sulphates (195 mg L^{-1}), ammonium (0.72 mg L^{-1}), and fluorides (7 mg L^{-1}) (see Table 2); and (ii) unknown organic contaminants. Authors such as, Alzahrani et al. (2013), Palma et al. (2009) and Alvarenga et al. (2009), verified the influence of concentrations of ammonia, chlorides and sulfates in the growth inhibition or mortality of aquatic organisms. The ecotoxicological effect of the one-step lime precipitation process, i.e., the capacity to reduce the wastewater ecotoxicity, was evident, resulting in a treated wastewater classified as class 2 (using the TU classification) with a lower acute toxicity for all the bioindicators used. The lime precipitation treatment led to the decrease of the concentrations of chlorides, sulphates, iron and manganese (see table 3). Despite that, ammonia concentrations are still high 5.4 mg L^{-1}, as well as sulfates concentrations (52.5 mg SO_4^{2-} L^{-1}) and fluorides (36.3 mg L^{-1}), contributing, therefore, to the remaining observed toxicity. Dilution of the treated wastewater with groundwater (1:4) resulted in a water matrix classified as class 1 (TU<1) in terms of toxicity. Although most studies conducted with wastewaters reported *V. fisheri* as the most sensitive bioindicator to ecotoxicological assays (Alzahrani et al., 2013; Latorre et al. 2007; Zgórska 2011; Palma et al. 2016), these results showed that *P. subcapitata* was in this case the most sensitive organism. This fact highlighted the need to include these two bioindicators in a battery of ecotoxicity tests to assess the environmental risk of industrial effluents, as well as in the discussion of the effectiveness of different effluent treatments.

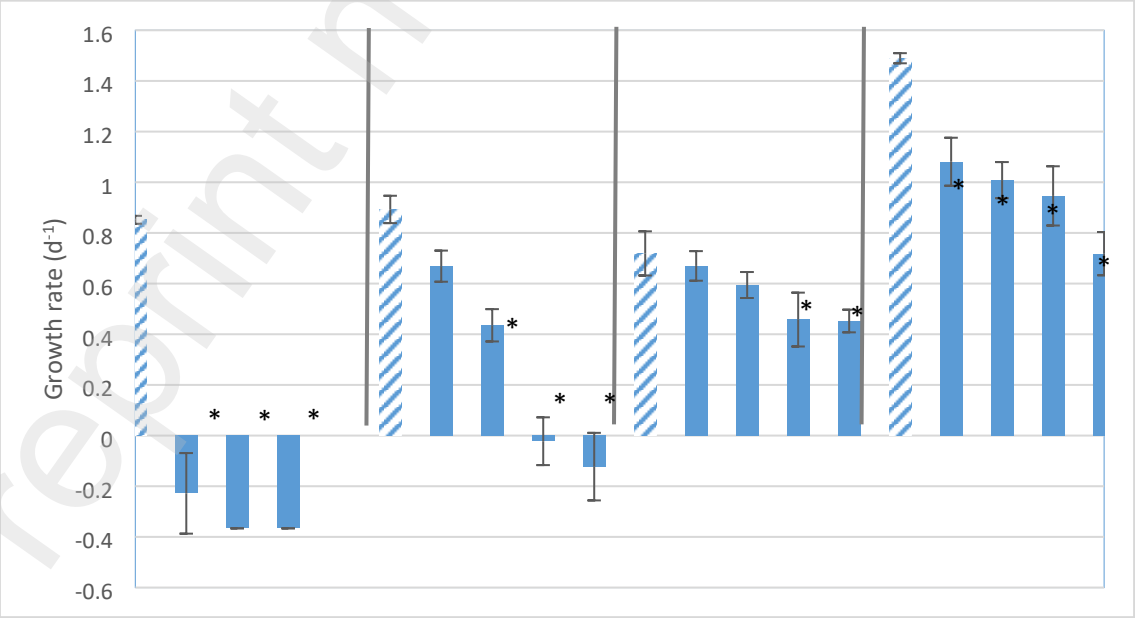
Table 3. Values of TU (%) obtained with *V. fischeri, P. subcapitata* and the crustacean *T. platyurus* when exposed to the winery wastewater (WW), treated wastewater (TWW); groundwater (GW) and wastewater nutritive solution (WWNS). The matrices were classified into toxicity classes according to their value of toxicity units (TU).

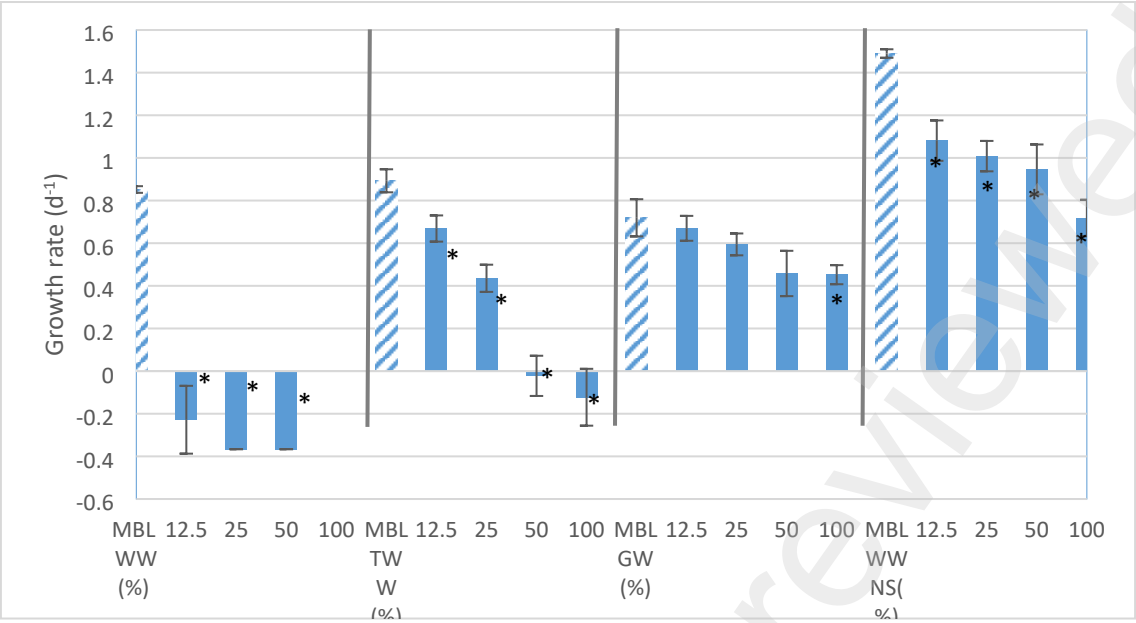
	TU (%)			
	***T. platyurus* (24h)**	***V. fisheri* (30 min)**	***P. subcapitata* (72h)**	
WW	20	9.17	100	Class 4

Treated WW	1.34	2.92	3.97	Class 2
GW	non toxic	non toxic	non toxic	Class 1
WWNS	non toxic	non toxic	non toxic	Class 1

The ecotoxicological assessment through sublethal parameters showed that the winery wastewater significantly induced growth inhibition to the microalgae *P. subcapitata* (one-way ANOVA; $p<0.05$), with 100% of inhibition for all the concentrations exposed (Figure 1). The results indicated that all matrices induced some growth inhibition of microalgae, less accentuated in groundwater, with growth inhibition rates always below 30% (significantly different to the control; one-way ANOVA; $F_{4,18}=10.353$, p=0.00016).

WWNS (obtained from treated wastewater diluted with groundwater) induced a growth inhibition rate of 45%, indicating toxicity for the evaluated sublethal endpoints (significantly different to the control; one-way ANOVA; $F_{4,20}=48.423$, p=0.00000). Therefore, despite the decrease in toxicity, the irrigation solution still exhibited some toxicity to microalgae. The results can be explained considering the sensitivity of the bioindicator and the toxicological endpoints used (sublethal bioassays assess different stages of the bioindicator's life cycle, being more sensitive to lower concentrations of contaminants). In fact, several authors reported toxic effects in the microalgae *P. subcapitata*, when exposed to low concentrations of organic compounds such as polycyclic aromatic hydrocarbon (PAHs; Eom et al. 2007), pharmaceuticals (Yang et al., 2008) and herbicides (Pérez et al. 2011).





366

367 **Figure 1.** Growth rate (d^{-1}) of the microalgae *P. subcapitata* after 3 days exposed to the winery
368 wastewater (WW), treated wastewater (TWW); ground water (GW) and Wastewater nutrient
369 solution (WWNS) (mean±SD; n = 6; * p<0.05, Dunnett's test with a control (MBL).

370

371 **3.2. Use of sludge amended soils in cultivars**

372 Soil pH correction was carried out by addition of different amounts of sludge to raw soil. Figure
373 2 depicts the final pH achieved as a function of the percentage of added sludge. As observed with
374 just an approximated 1.2% (w/w) of sludge the pH of the soil can be corrected to circumneutral
375 conditions, more adequate to red pak choi growth.

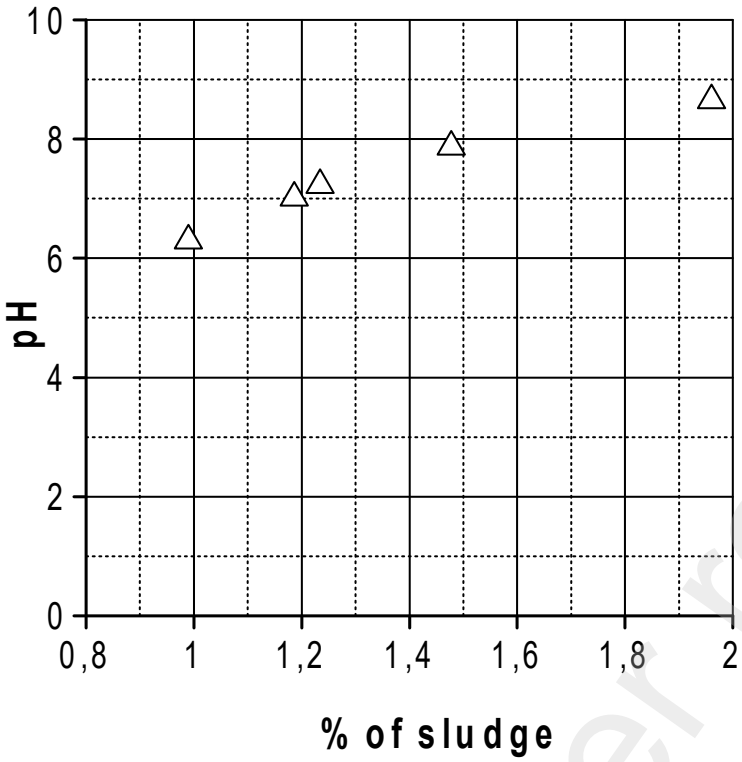


Figure 2. pH correction of acidic soil as a function of sludge percentage addition.

Table 4 compares the characterization of raw soil and corrected soil. Apart of pH, main differences are found in P content increasing from roughly 33 mg g^{-1} to 64 mg g^{-1} as P_2O_5 and K raising from 153 to 168 mg g^{-1} as K_2O. These results are sustained by the content in phosphorus and potassium of the obtained sludge after treatment, specifically 895 and 990 mg g^{-1} in P_2O_5 and K_2O, respectively (Luz et al. 2021). The critical level of nutrients differs depending on environmental conditions, soil type, plant type, method of extraction, etc. In any case, broadly speaking, P in corrected soil (14 mg g^{-1} as P) seems to be in the range of medium level content assuming that all P was available for plants while K content (139 mg g^{-1} as K) is considered slightly low if all K were extractable. The other remarkable change in concentration corresponds to Ca, which was expected given the sludge origin. Calcium deficiencies usually are found only on very acidic soils.

393 **Table 4.** Characterization of raw (acidic) and pH corrected (1.2 %) soils

	Acidic Soil		Corrected soil	
	Mean	SD	Mean	SD
pH	5.31	0.12	7.23	0.08
EC, mS cm^{-1}	0.27	-	0.40	-
% TOC	0.45	0.03	0.49	0.02
% Organic matter	0.8	0.1	0.8	-
K, mg g^{-1} K_2O	152.7	4.2	168.1	10.5
P, mg g^{-1} P_2O_5	32.9	3.6	63.7	1.7
Pb, µg g^{-1} DW	32.40	1.15	34.10	1.10
Cd, µg g^{-1} DW	2.20	0.20	2.20	0.50
Zn, mg g^{-1} DW	0.2366	0.1744	1.0197	0.5317
Fe, mg g^{-1} DW	96.647	9.1393	100.368	4.374
Ni, µg g^{-1} DW	77.30	5.00	73.90	2.00
Mn, mg g^{-1} DW	1.5116	0.0329	1.4345	0.0659
Cu, mg g^{-1} DW	0.2283	0.0118	0.2207	0.0088
Ca, mg g^{-1} DW	10.013	0.6908	102.00	20.274
Mg, mg g^{-1} DW	13.677	0.3470	15.011	0.6881

394 DW = Dry weight

395

396 The ecotoxicological evaluation of the different soil leachates (acid soil, soil irrigated with
397 WWNS and corrected soil), allowed the analysis of the risk to the ecosystem after the application
398 of the prepared irrigation solution (WWNS) and the incorporation of the precipitate as a way of
399 correcting the pH of the soil. ground. Ecotoxicological results using the bioindicators *T. platyurus,*
400 *V. fischeri* are summarized in Table 5 and the bioindicator *P. subcapitata* in Figure 3. Lethal
401 ecotoxicological results (Toxic Unit (TU); Table 5) indicated that both irrigation and correction
402 did not induce the increment of soil toxicity.

403

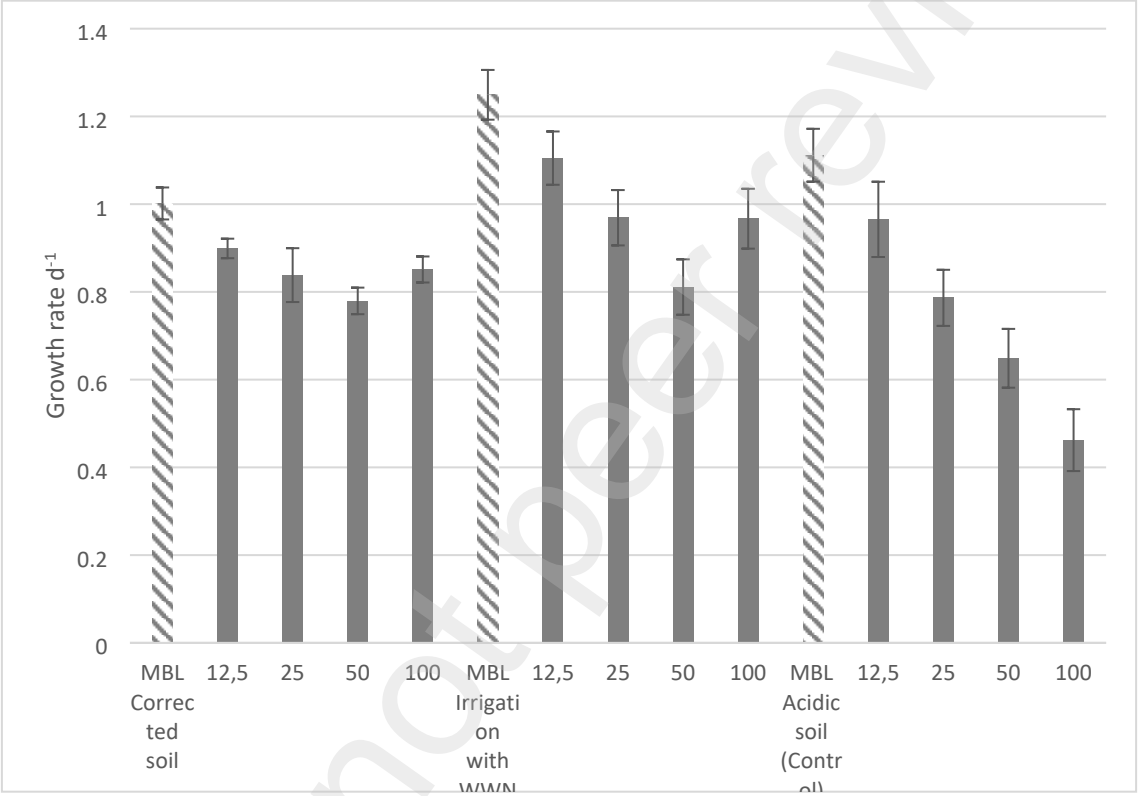
404 **Table 5.** Values of TU (%) obtained with *V. fischeri,* and the crustacean *T. platyurus* when
405 exposed to the soils, acidic soil (control); irrigation with WWNS and corrected soil. The matrices
406 were classified into toxicity classes according to their value of toxicity units (TU).

	TU (%)	
	T. Platyurus	***V. fisheri***

	(24h)	(30 min)	
Acidic soil (Control)	non toxic	non toxic	Class 1
Irrigation with WWNS	non toxic	non toxic	Class 1
Corrected soil	non toxic	non toxic	Class 1

407

408



409

410 **Figure 3.** Growth rate (d^{-1}) of the microalgae *P. subcapitata* after 3 days exposed to the corrected
411 soil, irrigation with WWNS and acidic soil (mean±SD; n = 6; * p<0.05, Dunnett's test with a
412 control (MBL).

413

414 Once soils were characterized, the potential benefits of soil sludge amendment were assessed by
415 analyzing the evolution of a control cultivar (red pak choi) under three different scenarios. Hence,
416 red pak choi was grown in the raw acidic soil by using either groundwater or treated wastewater
417 in the irrigation. The wastewater used was obtained after dilution (1:4) and neutralization of
418 treated wastewater with groundwater (Luz, 2021).

Several parameters were measured to compare the effect of soil amendment and water irrigation nature on red pak choi (see Figure 4). Hence, regarding plant size a positive effect of soil correction and use of wastewater was experienced in stem diameter, plant heigh and leaf length/area while a decrease in stem heigh was obtained when the plants were cultivated under the studied systems if compared to values in acidic soil.

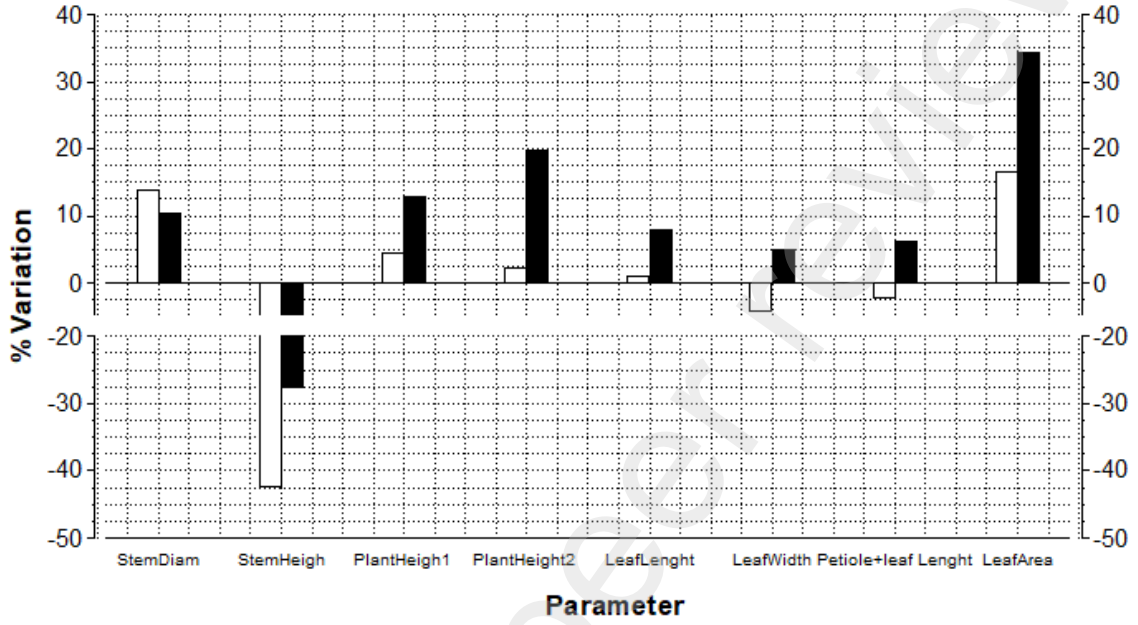


Figure 4. Use of irrigation with WWNS on acid soil (white bars) and irrigation with GW on amended soil (black bar) on red pak choi growth. Parameters compared to results obtained in uncorrected acidic soil irrigated with GW (PlantHeigh1 measured from ground to the longest leaf stretched, PlantHeigh2 measured from ground to the largest leaf without stretching).

Broadly speaking, cultivars grown in amended soil (irrigated with GW) presented better characteristics than those produced by irrigating with WWNS the uncorrected soil. Moreover, wastewater watering led to a reduction in leaf width and petiole+leaf length if compared to control experiments. The most remarkable differences in amended soil were found in plant height and leaf area with an increase of roughly 20% (PlantHeigh2) and 34%, respectively, contrasted to results obtained in the acidic soil. The only parameter negatively affected by soil amendment was stem heigh with a reduction of 28% (see Figure 5).

The higher size of plants was translated to a notable increase in the dry weight of the aerial part of the vegetables, experiencing a 23% and 42 % raise in plants grown with WWNS and corrected soil, respectively.

Contrarily to aerial data, use of corrected soil led to a decrease of root weight although an increase in length was experienced. Watering with WWNS increased the size and weight of roots, especially fresh weight with a raise of 25%.

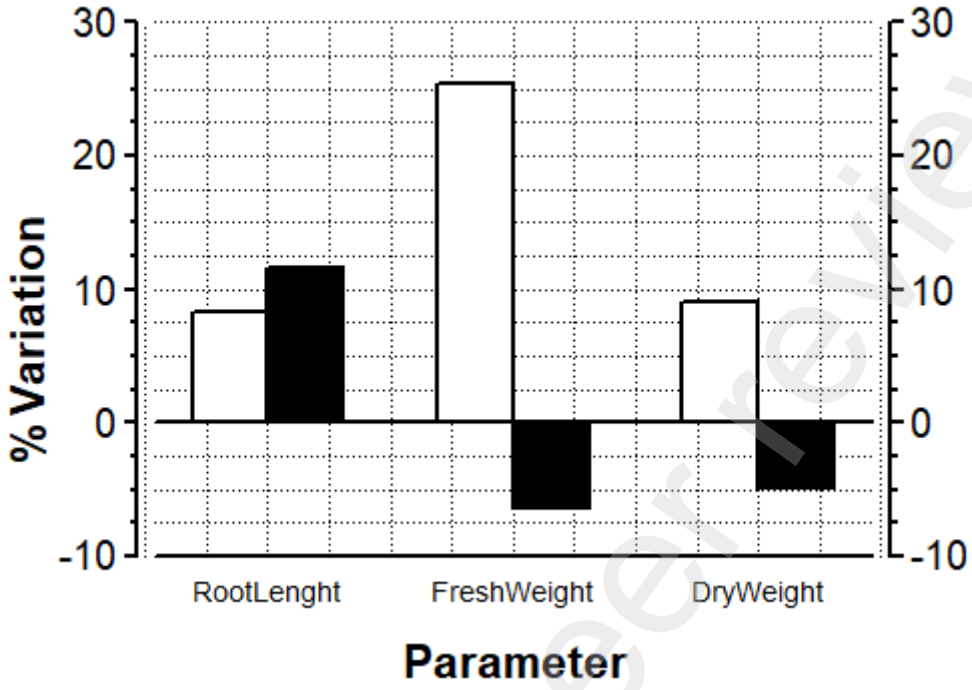


Figure 5. Use of irrigation with WWNS (white bars) or amended soil (black bar) on red pak choi growth. Root parameters compared to results obtained in uncorrected acidic soil.

3.3. Pigment content in red pak choi grown

Chlorophyll is the designation of a group of photosynthetic pigments found in the chloroplasts of plants. In green plants, chlorophyll appears in two forms: chlorophyll a and b.

This pigment, together with carotenoids, has the ability to capture solar radiation and allow plants to carry out photosynthesis, a vital function of plants, which allows the production of carbohydrates, which they use to produce essential energy for their development. and productivity.

During photosynthesis, CO_2 is consumed, and oxygen is released, hence the importance of plants for maintaining oxygen and sequestering carbon on the planet (Moreira, 2013)

As can be seen in table 6, the plants watered with WWNS, as well as those grown in the soil, where the pH correction was carried out, had higher levels of chlorophyll, which is a positive aspect, since they are plants with higher development capacity and greater productive capacity.

Nitrogen is part of chlorophyll and is directly involved in photosynthesis (Rodrigues and Coutinho, 2000).

The content in pigments (chlorophyll and carotenoids) can be an indirect tool to assess nitrogen availability in soils. Photosynthetic pigments can, therefore, detect deficiencies in nitrogen (Balasu-Bramanian et al., 2000; Torres Netto et al., 2005). As a rule of thumb, deficiencies in N, P and K affect photosynthesis and growth (Muñoz Huerta et al., 2013), and, chlorophyll and carotenoids content is a valuable tool to reveal the shortage of these essential elements (Sanchez et al., 2018). As inferred from table xx, cultivars grown with irrigation with WWNS or in amended soils present higher levels of chlorophyll than those obtained in acidic soil. Carotenoids content does not significantly vary with the procedure of cultivation. These results suggest a positive influence of TWW and sludge amendment on NPK availability to plants.

Table 6. Pigment content in red pak choi grown under three scenarios

	Chlorophyll a mg g^{-1}		Chlorophyll b mg g^{-1}		Carotenoids mg g^{-1}	
	Mean	SD	Mean	SD	Mean	SD
Acidic soil (control)	1.55	0.265	1.06	0.262	0.24	0.040
Irrigation with WWNS	1.925	0.095	1.53	0.235	0.264	0.081
Corrected soil	1.845	0.178	1.38	0.123	0.25	0.106

4. CONCLUSION

Winery wastewater represents a serious environmental problem when not treated. Since the annual production of these tributaries is very significant, their use can be a solution for the future, offering profitability and promoting a circular economy for this industry. The raw effluent (WW) showed high toxicity, which decreased significantly after the application of the slaked lime treatment. To use it for irrigation, it was essential to meet the pH and conductivity characteristics that the plant needs, being necessary to dilute the TWW with GW, thus making it an ideal nutrient

solution for the cultivar. Ecotoxicologically, WWNS did not show any toxicity. An agronomic study was then carried out using three different conditions: irrigation with groundwater (soil control); correction of soil pH with organomineral fertilizer and underground water irrigation; irrigation with WWNS, for the production of red pak choi.

All soils were analysed ecotoxicologically using indirect bioassays, and none showed toxicity. After the cultivar reached the optimal harvesting conditions, it was analysed in terms of pigmentation, through the study of chlorophylls a, b and carotenoids. All parameters presented favourable conditions for healthy plant growth.

DECLARATIONS

Consent for publication

All authors agreed with the content and that all gave explicit consent to submit.

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