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Nitrogen-Rich Sewage Sludge Mineralized Quickly, Improving Lettuce Nutrition and Yield, with Reduced Risk of Heavy Metal Contamination of Soil and Plant Tissues

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Abstract: Sewage sludge should primarily find use in agriculture, reducing the quantity directed towards alternative disposal methods like incineration or deposition in municipal landfills. This study evaluated the agronomic value and the risk of soil and plant tissue contamination with heavy metals in sewage sludge obtained from two wastewater treatment plants (WWTP). The experiment was arranged as a 2 × 5 factorial (two sewage sludges, five sanitation treatments), involving lettuce cultivation in pots over two growing cycles. The two sewage sludges were sourced from the WWTPs of Gelfa and Viana do Castelo and underwent five sanitation and stabilization treatments (40% and 20% calcium oxide, 40% and 20% calcium hydroxide, and untreated sewage sludge). The Gelfa sewage sludge, characterized by a higher initial nitrogen (N) concentration, resulted in greater dry-matter yield (DMY) (12.4 and 8.6 g plant^{-1} for the first and second growing cycles, respectively) compared to that from Viana do Castelo (11.0 and 8.1 g plant^{-1}), with N release likely being a major factor influencing crop productivity. The high N concentration and the low carbon (C)/N ratio of sewage sludge led to rapid mineralization of the organic substrate, which additionally led to a higher release of other important nutrients, such as phosphorus (P) and boron (B), making them available for plant uptake. Alkalizing treatments further stimulated sewage sludge mineralization, increasing soil pH and exchangeable calcium (Ca), thereby enhancing Ca availability for plants, and indicating a preference for use in acidic soils. Cationic micronutrients were minimally affected by the sewage sludge and their treatments. The concentrations of heavy metals in the sewage sludge, soils, and lettuce tissues were all below internationally established threshold limits. This study highlighted the high fertilizing value of these sewage sludges, supplying N, P, and B to plants, while demonstrating a low risk of environmental contamination with heavy metals. Nevertheless, the safe use of sewage sludge by farmers depends on monitoring other risks, such as toxic organic compounds, which were not evaluated in this study.

Keywords: *Lactuca sativa*; organic fertilization; nitrogen mineralization; circular economy; wastewater treatment plants

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1. Introduction

The utilization of improved varieties, expansion of irrigated areas, and the widespread application of pesticides and fertilizers have facilitated the production of food and the provision of other goods and services at acceptable quantities and prices to a world population that continues to increase. It is estimated to reach 9.7 billion in 2050 and could peak at nearly 10.4 billion in the mid-2080s [1]. Associated with this increase in the world population, food demand is expected to increase by 50% and global demand for the three main cereals (maize, rice, and wheat) by 70% [2]. However, although soil productivity on a global scale must continue to increase, it must be based on sustainable agricultural practices that cause less impact on soil quality and the environment.

The intensification of agriculture has caused damage to the soil, favoring erosion, acidification and salinization and other problems that reduce the sustainability of agricultural systems [3,4]. Regarding crop fertilization, while fertilizers can contribute to soil acidification and salinization, the main concerns are the contamination of water bodies with nitrates and other nutrients leached from agricultural fields [5,6]. Additionally, there is concern about the pollution of the atmosphere with N oxides and other greenhouse gases [7,8].

Soil organic matter, an important indicator of soil quality and a vital component for the sustainability of agro-systems, has also declined. This decline is associated with the widespread adoption of monocultures and the intensification of agricultural practices [9,10]. Conversely, the specialization of agriculture has resulted in the reduction of mixed farming systems. In vast areas of intensive agriculture, no animals are raised, leading to the absence of manure—a crucial resource for maintaining the level of organic matter in the soil [11,12]. An adequate level of organic matter improves soil resilience and provides favorable conditions for plant growth. Overall, it improves the water-holding capacity, aeration, and buffering power of the soil, while supporting the life of heterotrophic soil microorganisms with a relevant role in nutrient cycling [13,14].

The need to reduce the use of industrial synthetic fertilizers, both due to their environmental impacts and increasing market prices, along with the reduction in the availability of farmyard manure, tremendously increases the importance of other available organic fertilizing resources. It should not be ignored that agriculture is an extractive activity, with part of the nutrients not being replaced in the soil by natural processes at the rate at which they are removed from cultivated crops, creating the need to balance with external inputs [9,15]. From this perspective, sewage sludge, residues from agro-industrial activities, and many other organic materials can play an increasingly important role in crop fertilization. This aligns with a circular economy strategy, where nothing goes to waste and everything must be recycled and reused [16–18].

Sewage sludge is a mud-like residue from wastewater treatment [19]. The increase in the world's population and the massive migration of rural populations to urban areas, associated with the rapid increase in industrialization, have resulted in the generation of large quantities of wastewater and sewage sludge [18,20]. Annually, the European Union produces an estimated 2 to 3 million tons of sewage sludge [19]. This sludge is typically rich in organic matter and contains considerable amounts of mineral elements, especially N and P [21–23], which are nutrients commonly applied in larger quantities to crops [3,4].

Sewage sludge can be directed to various destinations, including incineration, deposition in municipal landfills, or composting [20,22,24]. Additionally, researchers have explored its applications in various engineering fields [18]. However, use as fertilizer or soil amendment has been the most common destination in several parts of the world [18,20,21]. For example, in the European Union, approximately 40% of the generated material, equivalent to 17 kg per hectare, is annually applied in agricultural fields [19].

The widespread application of sewage sludge in agriculture is hindered by its potential to contain high concentrations of heavy metals [20,25–27], pathogenic microorganisms [22,28], and/or toxic organic compounds [22,29]. These contaminants have the potential to cause harm to soil, the environment, and human health. However, the level of contamination depends on the origin of the wastewater, with the risk of heavy metal contamination being greater when municipal sewage treatment plants accept industrial wastewater [30]. Nevertheless, European legislation will impose stringent restrictions on the agricultural use of sewage sludge, including limits on heavy metal concentrations in the sludge and the receiving soils, specifications for crop species, application timings, and quantities. Additionally, it will be mandatory to treat sewage sludge before its application onto the land [19].

Alongside legislative progress on the conditions for applying sewage sludge in agriculture, various studies have assessed its agronomic value and associated risks. Some studies have reported favorable effects on crop growth and yield [18,23,31], as well as other beneficial impacts on the physical, chemical, and/or biological properties of soils [18,24]. While the most reported risks are associated with soil and/or plant contamination by heavy metals, most studies have indicated that contamination levels have remained below the threshold values set by respective national legislation [20,23,32].

This study aimed to investigate the impact of sewage sludge from two WWTPs, treated with two rates of calcium oxide and calcium hydroxide, on the growth of lettuce (*Lactuca sativa* L.). Additionally, the research assessed the levels of heavy metals in both soil and plant tissues. The study also evaluated how treatments with alkalizing materials influenced the agronomic value of sewage sludge by examining their effects on soil properties, plant elemental composition, and overall plant growth. The test plant chosen for this study was lettuce, as it is widely recognized as a good indicator of nitrogen availability and heavy metal presence in the soil.

2. Materials and Methods

2.1. Experimental Conditions

The agronomic evaluation of sewage sludge was based on a pot experiment over two consecutive cycles of lettuce (cv. Summer Wonder) and took place in Bragança in the northeast of Portugal in 2022. The climate of the region is Mediterranean, although with some Atlantic influence. According to the Köppen and Geiger classification, it is of type Csb [33]. The average annual temperature is 12.6 °C, and the precipitation is 772.7 mm. The monthly records of air temperature and precipitation for the year 2022 are presented in Figure 1. The soil used in this trial is a Eutric Regosol [34]. The texture is sandy clay loam (543.7, 238.3, and 218.0 g kg^{-1} sand, silt, and clay, respectively), the pH is 6.1, the organic C content is 22.5 g kg^{-1}, the value of extractable P is 109.4 mg (P_2O_5) kg^{-1} and that of K is 180.0 mg (K_2O) kg^{-1}.

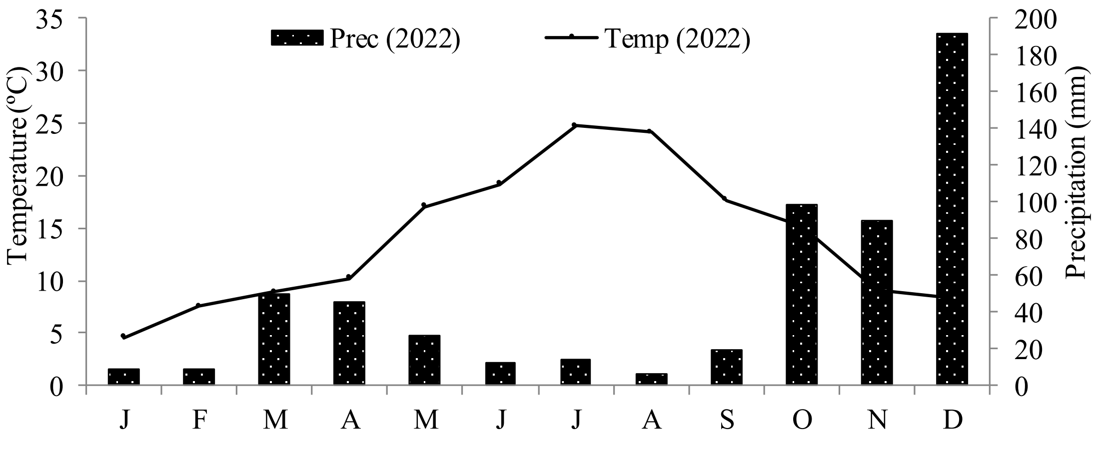

Figure 1. Monthly average values of air temperature and precipitation for the year 2022.

2.2. Experimental Design and Characterization of Sewage Sludge

The experiment was arranged in a factorial design, with two factors: the origin of sewage sludge (two WWTPs, Gelfa and Viana do Castelo) and five sewage sludge stabilization and disinfection treatments [40% and 20% calcium oxide, 40% and 20% calcium hydroxide (mass/mass), and untreated sewage sludge]. Three replicates (three pots) were included for each combination of factors. In this way, 30 pots were used, comprising 2 (WWTPs) × 5 (treatments) × 3 replicates. Sewage sludge from the Gelfa and Viana do Castelo WWTPs were used because they are representative of the type of sewage sludge that is produced by the WWTPs in northern Portugal and the treatments to which they are subjected.

The dose of sewage sludge to be applied in each pot was determined to achieve an application rate of 50 kg N ha^{-1}, considering a commercial lettuce planting density of 140,000 plants ha^{-1}, and considering that only one lettuce was cultivated in each pot. The calculations considered the N concentration in the sewage sludge and its moisture content.

The Gelfa WWTP provides activated sludge with prolonged aeration in the water line, with thickening and mechanical dewatering of the sludge. This results in a solid material with about 80% moisture and some odor. The WWTP of Viana do Castelo provides sewage sludge from a liquid line of primary settling, with the sludge being activated at average load. The sewage sludge undergoes treatment through cold digestion and mechanical dewatering. The process results in solid material with approximately 80% moisture. The sewage sludge from the two WWTPs underwent elemental chemical analysis, and the results are presented in Table 1.

Table 1. Composition of sewage sludge (average ± standard deviation, n = 3, dry-weight basis) used in the pot experiment.

	Gelfa	Viana do Castelo
Dry matter (%)	19.2 ± 1.41	20.3 ± 1.61
pH	6.2 ± 0.12	7.3 ± 0.22
Carbon (g kg^{-1})	362.5 ± 2.83	318.0 ± 16.60
Nitrogen (g kg^{-1})	69.6 ± 8.10	45.5 ± 2.72
Carbon/nitrogen ratio	5.3 ± 0.66	7.0 ± 0.05
Phosphorus (g kg^{-1})	12.5 ± 2.00	15.0 ± 0.81
Nitrogen/phosphorus ratio	5.7 ± 1.58	3.1 ± 0.35
Potassium (g kg^{-1})	3.1 ± 0.20	1.6 ± 0.25
Calcium (g kg^{-1})	8.4 ± 0.55	21.8 ± 8.95
Magnesium (g kg^{-1})	2.3 ± 0.33	2.3 ± 0.76
Boron (mg kg^{-1})	13.6 ± 0.94	21.0 ± 1.34
Iron (mg kg^{-1})	3425.8 ± 403.30	27,080.5 ± 2829.45
Copper (mg kg^{-1})	117.0 ± 3.47	137.3 ± 18.59
Manganese (mg kg^{-1})	56.1 ± 1.99	341.6 ± 17.24
Zinc (mg kg^{-1})	419.4 ± 23.08	565.9 ± 17.27
Cadmium (mg kg^{-1})	0.6 ± 0.08	1.2 ± 0.21
Chromium (mg kg^{-1})	18.1 ± 2.50	128.8 ± 9.87
Lead (mg kg^{-1})	16.5 ± 0.81	38.0 ± 4.40
Nickel (mg kg^{-1})	9.6 ± 1.59	154.5 ± 9.91

2.3. Pot Management

The pots were filled with 3 kg of dry soil, sieved through a 2 mm mesh, and mixed with the corresponding dose of sewage sludge. In each pot, a lettuce seedling was planted at the "3rd true leaf unfolded" phenological stage [35]. In the first growing cycle, the planting took place on 19 May. In the days following planting, the pots were kept free of weeds as they germinated. The pots were kept outdoors throughout the growing cycle. Whenever necessary, the pots were watered; 100 mL of water was applied with highly variable frequency, depending on the size of the lettuce and prevailing environmental conditions. Lettuces from the first growing cycle were harvested on 23 June 2022, at the phenological stage 49, "typical size, form, and firmness of heads" [35]. On 31 August 2022, the second growing cycle began, repeating the entire process described earlier. The same soil was used, to which a new dose of sewage sludge equivalent to that of the first growing cycle was added. This second growing cycle concluded on 13 October with the harvesting of the lettuces.

2.4. Leaf Gas Exchange Measurements

CO_2 and water exchange measurements were carried out during the second growing cycle on a cloudless day and on two sun-exposed and fully expanded leaves per plant. Atmospheric conditions consisted of a photosynthetic photon flux density of 1470 ± 30 μmol m^{-2} s^{-1}, an air temperature of 26.6 ± 0.7 °C, and a CO_2 concentration of 410 ± 2.3 μmol CO_2 mol^{-1}. Measurements were made around midday with a portable photosynthesis system (LCpro+, Analytical Development Co., Hoddesdon, UK), operating in the open mode. Net photosynthetic rate (A), stomatal conductance to water vapor (g_s), and the ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a) were calculated using the equations of von Caemmerer and Farquhar [36].

2.5. Sampling Plant Tissues and Soils

At the end of each growing cycle, two circles with a diameter of 3.7 cm were cut from each lettuce, from selected mature leaves with fully developed blades from the middle part of the rosette. The tissues were placed in screw-capped jars, sealed tightly, packed in a thermal bag, and transported to the laboratory, where fresh weight and dry weight were determined after drying in a forced-air oven set at 70 °C. Subsequently, the lettuces were cut at ground level and taken to the laboratory, where they were also dried at 70 °C until constant weight. With the dry mass and area of the circles, as well as the dry mass of the whole lettuce, the total leaf area of the lettuce was estimated. Following the lettuce harvest, the soil from the pots was properly homogenized, and a subsample of ~250 g was taken to the laboratory, where it was again sieved through a 2 mm mesh and oven-dried at 40 °C.

2.6. Laboratory Analyses

The lettuce was ground in a mill with a 1 mm mesh. For the determination of nitrate concentrations in the tissues, 1 g of the sample and 50 mL of distilled water were used, followed by agitation for 1 h and subsequent filtration with Whatman No. 42 filter paper. Nitrate concentrations in the extracts were analyzed by UV–Vis spectrophotometry [37].

Tissue N concentration was determined using the Kjeldahl method, involving sample digestion with sulfuric acid and selenium as a catalyst. B was determined by colorimetry using the azomethine-H method after sample incineration with calcium oxide, and the ash was diluted with sulfuric acid. P was determined by colorimetry, employing the blue ammonium molybdate method with ascorbic acid as a reducing agent. The analysis of other elements [potassium (K), Ca, magnesium (Mg), iron (Fe), copper (Cu), zinc (Zn), manganese (Mn), cadmium (Cd), chromium (Cr), lead (Pb), and nickel (Ni)] was conducted using atomic absorption spectrophotometry. For a comprehensive description of these analytical procedures, refer to Temminghoff and Houba [38].

Soil samples underwent analysis for various variables, including pH (H_2O and KCl) using a soil-to-solution ratio of 1:2.5, cation-exchange capacity (ammonium acetate, pH 7.0), organic C (wet digestion, Walkley–Black method), and extractable P and K using the Egner–Riehm method. Soil B was extracted using hot water and determined by the azomethine-H method. For detailed information on these analytical procedures, refer to Van Reeuwijk [39]. Other micronutrients and heavy metals (Cu, Fe, Zn, Mn, Cd, Cr, Pb, and Ni) in the soil were determined by atomic absorption spectrometry. This involved extraction with ammonium acetate and diethylenetriaminepentaacetic acid (DTPA) buffered at pH 7.3, following the standard procedure outlined by FAO [40].

2.7. Data Analysis

The data underwent analysis to assess normality and homogeneity of variance using the Shapiro–Wilk and Bartlett's tests, respectively. The effect of treatments was compared using two-way ANOVA. In cases where significant differences between treatments were observed ($p < 0.05$), means were separated using the multiple range Tukey HSD test ($\alpha = 0.05$).

3. Results

3.1. Dry Matter Yield and Total Leaf Area

The DMY clearly differed between the two growing cycles, with the second cycle producing only 71% of the first one (Figure 2). When comparing the effects of sewage sludge from the two WWTPs on lettuce DMY, significant differences were observed in both growing cycles. The higher values were found for the sewage sludge from Gelfa (12.4 and 8.6 g plant^{-1} for the first and second growing cycles, respectively) compared to V. Castelo (11.0 and 8.1 g plant^{-1}) WWTP. In the first growing cycle, no significant differences were found between treatments, with average values ranging from 11.3 (control) to 12.1 (CH 40%) g plant^{-1}. However, in the second growing cycle, significant differences emerged between treatments. The highest average values were observed for sewage sludge treated with calcium oxide (8.9 and 8.7 g plant^{-1} for CO 20% and CO 40%, respectively), while the lowest values were recorded for the control (8.1 g plant^{-1}) and CH 20% (8.0 g plant^{-1}).

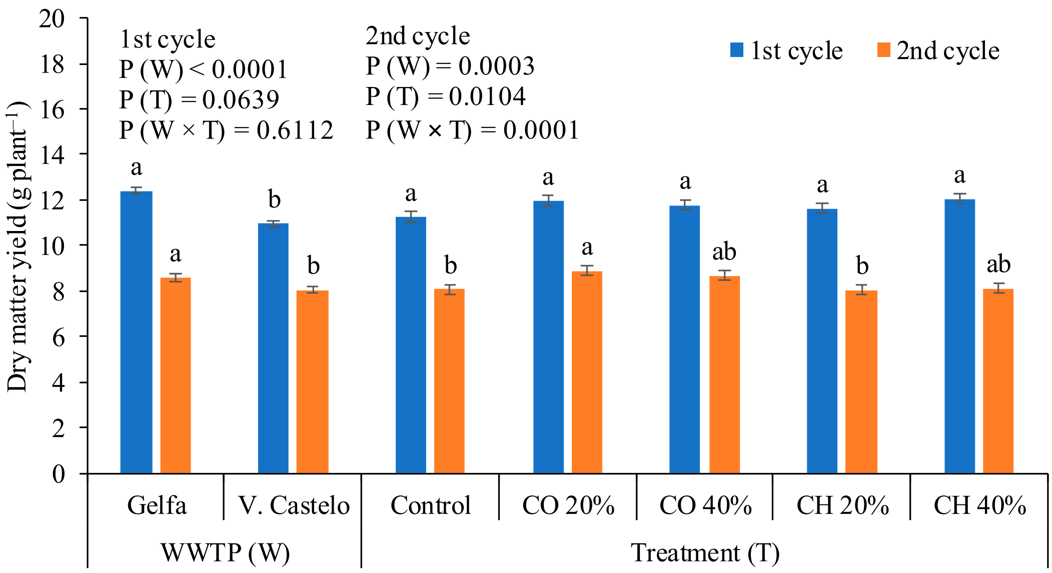

Figure 2. Lettuce dry-matter yield in the first (1st) and second (2nd) growing cycles by wastewater treatment plant (WWTP) and treatment [control, calcium oxide (CO) 20% and 40%, and calcium hydroxide (CH) 20% and 40%]. By growing cycle, WWTP or treatment, means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05). Error bars are the standard errors.

Total lettuce leaf area showed a pattern somewhat similar to DMY, albeit with some differences (Figure 3). Lettuces grown in the second cycle exhibited only 65% of the leaf area recorded in the first growth cycle. The average total leaf area of lettuces was also higher when treated with sewage sludge from Gelfa, but significant differences were only observed in the second growth cycle (29.6 and 25.5 dm^2 plant^{-1} in the first and second growth cycles, respectively). No significant differences were observed among treatments, indicating that the total leaf area estimate is a variable with less discriminative power than DMY.

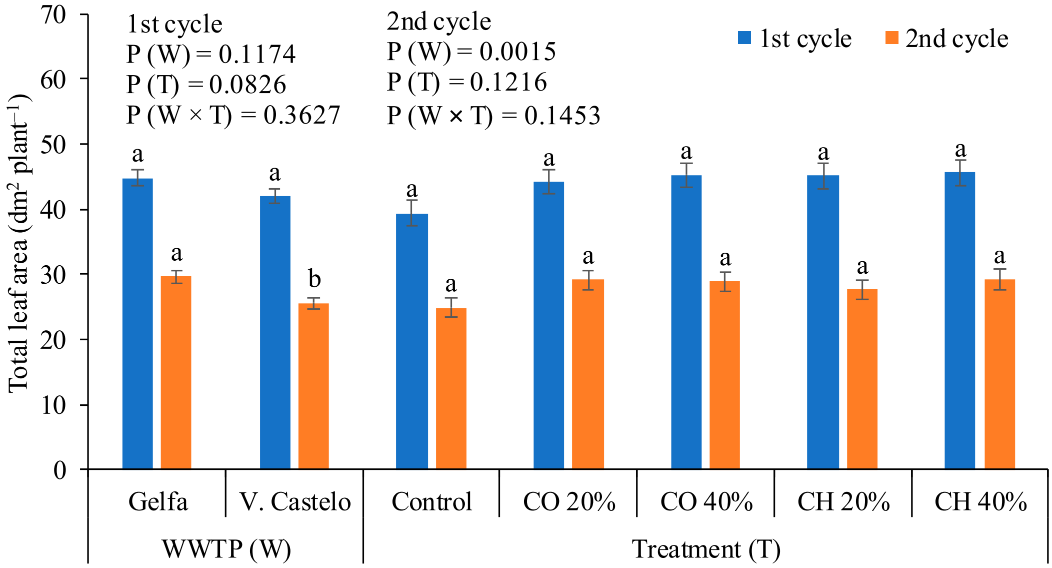

Figure 3. Total leaf area of lettuces of the first (1st) and second (2nd) growing cycles by wastewater treatment plant (WWTP) and treatment [control, calcium oxide (CO) 20% and 40%, and calcium hydroxide (CH) 20% and 40%]. By growing cycle, WWTP or treatment, means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05). Error bars are the standard errors.

3.2. Leaf Gas Exchange

Leaf gas exchange variables differed significantly between WWTPs (Table 2). The values of A, g_s and C_i/C_a for Gelfa were 14.8%, 30.4% and 7.4% higher, respectively, than those for V. Castelo. On the other hand, no significant differences were observed between treatments.

Table 2. Net photosynthetic rate (A, μmol m^{-2}-s^{-1}), stomatal conductance (g_s, mmol m^{-2}-s^{-1}) and the ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a) in lettuce leaves from the second growing cycle by wastewater treatment plant (WWTP) or treatment [control, calcium oxide (CO) 20% and 40%, and calcium hydroxide (CH) 20% and 40%].

	A	g_s	C_i/C_a
WWTP (W)			
Gelfa	12.1 a	153.9 a	0.584 a
V. Castelo	10.5 b	118.0 b	0.544 b
Treatment (T)			
Control	10.9 a	131.3 a	0.570 a
CO 20%	11.8 a	144.9 a	0.559 a
CO 40%	11.4 a	135.1 a	0.560 a
CH 20%	11.1 a	133.1 a	0.569 a
CH 40%	11.2 a	135.7 a	0.562 a
Prob. (W)	0.0002	<0.0001	0.0031
Prob. (T)	0.1674	0.1295	0.2564
Prob. (W × T)	0.6426	0.7013	0.5642

In columns, by WWTP or Treatment, means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05).

3.3. Plant Nitrogen Nutritional Status and Nitrogen Recovery

The N concentration in plant tissues differed significantly between WWTPs in both growing cycles (Table 3). Average values for Gelfa (28.0 and 28.3 g kg^{-1} in the first and second growing cycles, respectively) were higher than those for V. Castelo (21.3 and 22.4 g kg^{-1}). The N concentration in tissues also varied significantly between treatments in the first growth cycle, with values for the control treatment (21.7 g kg^{-1}) being lower than those for other treatments (between 25.7 and 26.0 g kg^{-1}). In the second cycle, no significant differences were observed between treatments, with average values ranging from 23.9 to 26.4 g kg^{-1}. The amount of N recovered followed the pattern of N concentration in tissues, with the magnitude of differences accentuated as the variable incorporated the effect of DMY (Figure 1), which varied in a direction very similar to N concentration in tissues. Significant interaction was observed for N concentration in tissues in the first growing cycle and N recovery in the second cycle, indicating that treatment responses depend on the origin of the sewage sludge.

Table 3. Nitrogen concentration in plant tissues and plant nitrogen recovery in the first (1st) and second (2nd) growing cycles by wastewater treatment plant (WWTP) or treatment [control, calcium oxide (CO) 20% and 40%, and calcium hydroxide (CH) 20% and 40%].

	Nitrogen Concentration (g kg^{-1})		**Nitrogen Recovery (mg plant^{-1})**	
	1st Cycle	**2nd Cycle**	**1st Cycle**	**2nd Cycle**
WWTP (W)				
Gelfa	28.0 a	28.3 a	347.2 a	243.6 a
V. Castelo	21.3 b	22.4 b	233.7 b	180.9 b
Treatment (T)				
Control	21.7 b	26.3 a	246.0 b	211.7 a
CO 20%	25.7 a	23.9 a	310.0 a	215.6 a
CO 40%	25.8 a	26.4 a	306.7 a	229.4 a
CH 20%	26.0 a	24.8 a	305.4 a	202.6 a
CH 40%	25.8 a	24.8 a	313.1 a	202.3 a
Prob. (W)	<0.0001	<0.0001	<0.0001	<0.0001
Prob. (T)	<0.0001	0.1791	<0.0001	0.0961
Prob. (W × T)	0.0095	0.0684	0.1822	<0.0001

In columns, by WWTP or Treatment, means followed by the same letter are not significantly different by Tukey HSD test ($\alpha = 0.05$).

3.4. Macro, Micronutrient, and Heavy Metals Concentration in Plant Tissues

The concentration of P in lettuce tissues was significantly higher in Gelfa than in V. Castelo in both growing cycles (Table 4). Average values in Gelfa were 4.4 and 4.3 g kg^{-1}, and in V. Castelo, they were 3.5 and 3.7 g kg^{-1} in the first and second growing cycles, respectively. The effect of the treatments was not statistically significant, with average values varying between 3.6 and 4.1 g kg^{-1} in the first growing cycle and 3.7 and 4.2 g kg^{-1} in the second growing cycle. The K concentration in plant tissues did not vary significantly between WWTPs or treatments in any of the growing seasons. The average values for each WWTP or treatment ranged between 37.7 and 39.7 g kg^{-1} in the first growing cycle and between 36.0 and 39.1 g kg^{-1} in the second growing cycle. Ca concentration in tissues showed higher average values in the V. Castelo WWTP, but no significant differences were observed for the Gelfa WWTP. Significant differences among treatments occurred in the Ca concentration in tissues in the first growing cycle. The lowest values were observed in the control treatment (5.7 g kg^{-1}), and the highest values were recorded in the CO 40% treatment (6.5 g kg^{-1}). The concentration of Mg in tissues did not differ significantly between WWTPs or treatments for either of the growing cycles.

The concentration of B in lettuce tissues varied significantly between WWTPs in the second growing cycle but not in the first, and it did not vary significantly between treatments in either of the growing cycles (Table 5). Average values by WWTP or treatments ranged from 31.3 to 33.4 mg kg^{-1} in the first growing cycle and from 34.8 to 40.9 mg kg^{-1} in the second. The concentrations of iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) did not vary significantly between WWTPs or between treatments for any of the growing cycles, despite differences in the concentration of these elements in the sewage sludge (Table 1). Across the two growing cycles, average Fe values ranged from 479.3 to 707.7 mg kg^{-1}, and average Mn, Zn, and Cu values ranged from 47.4 to 56.9 mg kg^{-1}, 90.1 to 204.4 mg kg^{-1}, and 6.9 to 8.5 mg kg^{-1}, respectively.

Table 4. Phosphorus, potassium, calcium, and magnesium concentration in plant tissues in the first (1st) and second (2nd) growing cycles by wastewater treatment plant (WWTP) or treatment [control, calcium oxide (CO) 20% and 40%, and calcium hydroxide (CH) 20% and 40%].

	Nutrient Concentration (g kg^{-1})							
	Phosphorus		Potassium		Calcium		Magnesium	
	1st Cycle	2nd Cycle	1st Cycle	2nd Cycle	1st Cycle	2nd Cycle	1st Cycle	2nd Cycle
WWTP (W)								
Gelfa	4.4 a	4.3 a	37.7 a	38.7 a	6.0 a	7.3 a	2.4 a	3.4 a
V. Castelo	3.5 b	3.7 b	38.4 a	36.7 a	6.3 a	7.5 a	2.3 a	3.3 a
Treatment (T)								
Control	4.0 a	4.2 a	38.5 a	38.8 a	5.7 b	7.1 a	2.2 a	3.3 a
CO 20%	3.9 a	3.8 a	39.7 a	36.4 a	6.1 ab	7.4 a	2.2 a	3.1 a
CO 40%	4.1 a	3.7 a	39.3 a	36.0 a	6.5 a	7.7 a	2.4 a	3.3 a
CH 20%	4.1 a	4.1 a	39.3 a	37.6 a	6.3 ab	7.5 a	2.4 a	3.5 a
CH 40%	3.6 a	4.2 a	38.7 a	39.1 a	6.5 ab	7.5 a	2.6 a	3.5 a
Prob. (W)	<0.0001	<0.0001	0.1352	0.0694	0.1766	0.3636	0.4383	0.5340
Prob. (T)	0.3506	0.0538	0.9418	0.3045	0.0255	0.5455	0.1092	0.0640
Prob. (W × T)	0.8895	0.0582	0.0894	0.1161	0.5519	0.3346	0.5281	0.3791

In columns, by WWTP or Treatment, means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05).

Table 5. Boron and metallic micronutrient concentrations in plant tissues in the first and second growing cycles by wastewater treatment plant (WWTP) or treatment [control, calcium oxide (CO) 20% and 40%, and calcium hydroxide (CH) 20% and 40%].

	Nutrient Concentration (mg kg^{-1})									
	Boron		Iron		Manganese		Zinc		Copper	
	1st Cycle	2nd Cycle	1st Cycle	2nd Cycle	1st Cycle	2nd Cycle	1st Cycle	2nd Cycle	1st Cycle	2nd Cycle
WWTP (W)										
Gelfa	32.3 a	36.0 b	588.7 a	657.1 a	52.1 a	52.7 a	124.6 a	175.0 a	7.8 a	8.3 a
V. Castelo	33.0 a	40.9 a	514.6 a	638.0 a	49.5 a	49.1 a	111.5 a	200.7 a	7.5 a	7.9 a
Treatment (T)										
Control	33.3 a	39.9 a	606.3 a	602.3 a	50.1 a	47.7 a	123.8 a	204.4 a	6.9 a	8.0 a
CO 20%	31.3 a	34.8 a	479.3 a	614.8 a	47.4 a	49.5 a	121.0 a	167.2 a	8.4 a	7.9 a
CO 40%	33.4 a	37.8 a	584.7 a	707.7 a	56.9 a	50.9 a	90.1 a	156.7 a	8.5 a	8.3 a
CH 20%	33.1 a	39.2 a	509.8 a	688.3 a	48.7 a	51.7 a	126.6 a	197.9 a	7.4 a	8.3 a
CH 40%	31.9 a	39.5 a	541.6 a	654.9 a	51.4 a	56.7 a	125.0 a	201.9 a	7.7 a	8.2 a
Prob. (W)	0.5074	<0.0001	0.0785	0.8857	0.1577	0.0806	0.0716	0.1185	0.3449	0.2027
Prob. (T)	0.2829	0.096	0.2613	0.2131	0.0515	0.0699	0.1543	0.1418	0.1284	0.8320
Prob. (W × T)	0.5109	0.2811	0.2264	0.1080	0.5490	0.2680	<0.0001	0.0762	0.3875	0.5190

In columns, by WWTP or Treatment, means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05).

The concentration of heavy metals in lettuce tissues varied very little, both when comparing between WWTPs and when comparing between treatments of sewage sludge (Table 6), even though the initial sewage sludge showed somewhat dissimilar levels of heavy metals (Table 1). Only in the second lettuce cycle were significant differences observed between treatments in the levels of Cd in the tissues. The average values for Cd were found below 0.11 mg kg^{-1}, those for Cr below 13.1 mg kg^{-1}, those for Pb below 0.35 mg kg^{-1}, and those for Ni below 15.6 mg kg^{-1}.

Table 6. Cadmium, chromium, lead and nickel concentration in plant tissues in the first (1st) and second (2nd) growing cycles by wastewater treatment plant (WWTP) and treatment [control, calcium oxide (CO) 20% and 40%, and calcium hydroxide (CH) 20% and 40%].

	Metal Concentration (mg kg^{-1})							
	Cadmium		Chromium		Lead		Nickel	
	1st Cycle	2nd Cycle	1st Cycle	2nd Cycle	1st Cycle	2nd Cycle	1st Cycle	2nd Cycle
WWTP (W)								
Gelfa	0.09 a	0.09 a	9.9 a	6.1 a	0.26 a	0.19 a	14.5 a	7.7 a
V. Castelo	0.09 a	0.09 a	9.0 a	6.3 a	0.27 a	0.17 a	12.2 a	9.1 a
Treatment (T)								
Control	0.08 a	0.08 b	8.1 a	6.1 a	0.34 a	0.15 a	11.6 a	8.5 a
CO 20%	0.09 a	0.11 a	9.8 a	5.4 a	0.17 a	0.13 a	15.0 a	8.1 a
CO 40%	0.09 a	0.09 ab	13.1 a	5.5 a	0.16 a	0.30 a	15.6 a	8.5 a
CH 20%	0.09 a	0.10 ab	9.1 a	6.3 a	0.26 a	0.22 a	11.3 a	8.3 a
CH 40%	0.10 a	0.10 ab	8.0 a	5.1 a	0.35 a	0.13 a	14.6 a	8.4 a
Prob. (W)	0.3583	0.7097	0.4331	0.6090	0.9813	0.9108	0.0621	0.0767
Prob. (T)	0.0591	0.0213	0.0758	0.4519	0.1426	0.616	0.1321	0.9924
Prob. (W × T)	0.9505	0.0429	0.0927	0.7588	0.218	0.0123	0.5357	0.2685

In columns, by WWTP or treatment, means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05).

3.5. Soil Properties

Organic C did not vary significantly between WWTPs or among treatments, with average values ranging between 17.69 and 19.42 g kg^{-1} (Table 7). The pH(H_2O) significantly varied among treatments but not among WWTPs. The control, untreated with calcium oxide or calcium hydroxide, exhibited the lowest average pH value, while treatments receiving the highest dose of calcium oxide or calcium hydroxide showed higher average pH values. Soil P levels differed significantly between WWTPs but not among treatments. Exchangeable Ca levels did not vary significantly between WWTPs but did vary among treatments. The control treatment, without calcium oxide or calcium hydroxide, had the lowest exchangeable Ca levels, and treatments receiving the higher dose of the oxidant (CO 40%, CH 40%) tended to have higher average exchangeable Ca values. Mg values did not differ significantly between WWTPs or among treatments. Soil K levels varied among WWTPs and treatments, with higher average values for Gelfa and for treatments CO 20% and CH 20%. Exchangeable sodium and exchange acidity did not vary between WWTPs or treatments. Cation exchange capacity (CEC) did not vary significantly between WWTPs or among treatments. However, a clear trend was observed for higher average values in V. Castelo, especially in treatments receiving calcium oxide or calcium hydroxide.

The concentration of micronutrients B, Fe, Cu, and Mn in the soil did not vary significantly between WWTPs or treatments (Table 8). Zinc levels in the soil varied significantly between WWTPs but not between treatments. Cadmium levels varied significantly between WWTPs but not between treatments, whereas chromium levels varied significantly between WWTPs and treatments. On average, Cd values were below 0.3 mg kg^{-1}, and Cr levels were below 0.08 mg kg^{-1}. The levels of Pb and Ni in the soil did not vary between WWTPs or between treatments. The average levels of Pb were found below 1.06 mg kg^{-1}, and the levels of Ni were below 3.63 mg kg^{-1}.

Table 7. Organic carbon (C), pH, extractable phosphorus, exchangeable bases, exchangeable acidity (EA) and cation exchange capacity (CEC) in soil after the second growing cycle of lettuce by wastewater treatment plant (WWTP) and treatment [control, calcium oxide (CO) 20% and 40%, and calcium hydroxide (CH) 20% and 40%].

	Organic C		Phosphorus	Calcium	Magnesium	Potassium	Sodium	EA	CEC
	g kg^{-1}	pH (H_2O)	mg kg^{-1}, P_2O_5	cmol$_+$ kg^{-1}					
WWTP (W)									
Gelfa	18.46 a	7.41 a	121.44 b	15.58 a	5.17 a	0.24 a	0.37 a	0.12 a	21.47 a
V. Castelo	18.80 a	7.42 a	138.44 a	17.92 a	5.46 a	0.22 b	0.33 a	0.13 a	24.07 a
Treatment (T)									
Control	17.69 a	7.09 c	109.86 a	13.65 b	5.59 a	0.22 ab	0.34 a	0.15 a	19.95 a
CO 20%	19.42 a	7.52 a	140.02 a	16.00 ab	4.97 a	0.25 a	0.41 a	0.12 a	21.74 a
CO 40%	19.34 a	7.61 a	136.29 a	19.96 a	5.43 a	0.23 ab	0.30 a	0.12 a	26.03 a
CH 20%	18.83 a	7.27 b	137.19 a	16.08 ab	5.63 a	0.25 a	0.40 a	0.12 a	22.47 a
CH 40%	17.86 a	7.59 a	126.35 a	18.05 ab	4.94 a	0.21 b	0.31 a	0.13 a	23.64 a
Prob. (W)	0.4955	0.7898	0.0206	0.0511	0.3536	0.0113	0.2163	0.3817	0.0842
Prob. (T)	0.0903	<0.0001	0.1367	0.0244	0.4551	0.0030	0.0684	0.5394	0.1328
Prob. (W × T)	0.4741	0.0121	0.1249	0.5582	0.7287	0.3159	0.1908	0.0538	0.7867

In columns, by WWTP or treatment, means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05).

Table 8. Boron, micronutrient cations and heavy metals in soil after the second growing cycle of lettuce by wastewater treatment plant (WWTP) and treatment [control, calcium oxide (CO) 20% and 40%, and calcium hydroxide (CH) 20% and 40%].

	Boron	Iron	Zinc	Copper	Manganese (mg kg^{-1})	Cadmium	Chromium	Lead	Nickel
WWTP (W)									
Gelfa	0.9 a	131.5 a	8.2 b	17.4 a	165.0 a	0.02 b	0.06 b	1.04 a	3.06 a
V. Castelo	0.7 a	124.3 a	10.0 a	16.9 a	161.3 a	0.03 a	0.07 a	0.98 a	2.48 a
Treatment (T)									
Control	0.8 a	122.6 a	9.0 a	17.0 a	164.3 a	0.03 a	0.04 c	1.06 a	2.50 a
CO 20%	1.0 a	128.3 a	9.3 a	17.6 a	167.0 a	0.03 a	0.06 b	1.01 a	3.62 a
CO 40%	0.8 a	133.6 a	9.3 a	17.5 a	160.2 a	0.03 a	0.08 a	1.02 a	2.70 a
CH 20%	0.8 a	131.5 a	9.3 a	16.8 a	165.6 a	0.03 a	0.06 b	1.02 a	2.54 a
CH 40%	0.6 a	123.5 a	8.6 a	16.7 a	158.5 a	0.02 a	0.07 b	0.95 a	2.49 a
Prob. (W)	0.1345	0.1643	<0.0001	0.1836	0.4007	<0.0001	0.0001	0.1922	0.0763
Prob. (T)	0.3734	0.5804	0.6745	0.5199	0.6938	0.1687	<0.0001	0.6159	0.1370
Prob. (W × T)	0.4968	0.8823	0.7656	0.4316	0.0026	0.0903	<0.0001	0.1365	0.1531

In columns, by WWTP or treatment, means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05).

4. Discussion

4.1. Lettuce Dry Matter Yield and Nitrogen Nutrition

The DMY differed between lettuce growth cycles, being higher in the spring compared to the late summer cycle. Long-sunlight days, increased radiation, and mild temperatures are very favorable environmental conditions for lettuce growth [41]. In previous studies conducted with lettuce in the environmental conditions of this region, higher lettuce productivity had already been recorded in spring compared to other seasons of the year [16,42].

The DMY of lettuce was significantly higher in the sewage sludge from Gelfa compared to Viana do Castelo. Gelfa's sewage sludge exhibited a higher N concentration than that from Viana do Castelo, resulting in significant differences in N concentration, N recovery, and nitrate concentration in the lettuce tissues, as well as in leaf gas exchange traits. N availability is generally low in agricultural soils, necessitating regular applications to maintain crop productivity [3,4]. In this study, N concentrations in lettuce tissues tended to be low compared to the sufficiency range established for mature lettuce heads by Bryson et al. [43], which emphasizes the crucial role of N in DMY. Tissue N concentration was below optimal levels, and N is required in large quantities in plant tissues, participating in the chemical structures of proteins, nucleic acids, chlorophylls, and secondary metabolites [44]. Furthermore, previous studies demonstrated that nitrogen-limited growth of lettuce is associated with lower stomatal conductance [45], while the photosynthetic capacity is related to the nitrogen content primarily because the proteins of the Calvin cycle and thylakoids represent most of the leaf nitrogen [46].

It is noteworthy that sewage sludges have a very low C/N ratio compared to conventional organic amendments. The C/N ratio is one of the best indicators predicting the rate and extent of mineralization of organic substrates in the soil, being higher as the C/N ratio decreases [3,4,47]. Furthermore, sewage sludge probably does not contain high levels of lignin, cellulose, and hemicellulose, which confer resistance to the attack of soil microorganisms that is common in conventional organic amendments containing plant residues [3,4,47]. This dual characteristic may have contributed to the ease of sewage sludge decomposition, resulting in high mineralization rates even during the relatively short lettuce growth cycles.

4.2. Macronutrients Other Than Nitrogen in Plant Tissues and Soil

The P concentration in lettuce tissues was higher in pots with sewage sludge from Gelfa than with those from Viana do Castelo. However, the original P concentration in the sludge was not higher in Gelfa than in Viana do Castelo (Table 1). Furthermore, the N/P ratio was greater for Gelfa compared to Viana do Castelo sewage sludge. Thus, the result suggests that the main cause of the greater P availability for plants was a more extensive mineralization of the sewage sludge of Gelfa due to its high N content and low C/N ratio. The treatments with calcium oxide or calcium hydroxide had little influence on the bioavailability of P for plants, although they did influence soil pH. P tends to become more bioavailable as pH increases, since the reaction with Fe and Al oxides in acid soils leads to its precipitation as $AlPO_4$ and $FePO_4$ [3]. In this study, the increase in pH caused by sewage sludge treated with alkalizing materials occurred within a pH range close to neutrality, which has little effect on the bioavailability of P, as the reactions of P precipitation occur especially in soils with very low initial pH [48]. Therefore, it appears that the prevailing factors influencing P availability were the origin of the sewage sludge and the varying rates of mineralization.

Taking into account that the phosphate rocks from which phosphate fertilizers are manufactured are running out, and that difficulties are expected in supplying phosphate fertilizers to agriculture in the relatively short term [44,49], the use of sewage sludge as a soil amendment will have increased importance, as it tends to be very rich in P [21–23] that can quickly become available to plants, as was proven in this study.

The Ca concentration in lettuce tissues varied significantly among treatments in the first growing cycle and showed the same trend without significant differences in the second. The control treatment exhibited lower average values, while the treatments receiving 40% of calcium oxide or calcium hydroxide showed higher values. These results are consistent with soil pH and exchangeable Ca values, which were higher in sewage sludge treatments receiving more Ca (CO 40%, CH 40%). Thus, the results provide evidence of the direct supply of Ca in sewage sludge due to the stabilization and disinfection treatment. Considering the vast areas of the globe where soils are acidic [50,51], and that acid soils pose several problems to plant growth, including low Ca supply [4,48,52], sewage sludge, when stabilized with alkalizing materials, can be a very suitable soil amendment, since it has a liming effect in addition to the release of N, P, and other nutrients.

4.3. Micronutrients in Plant Tissues and Soil

The treatments had little influence on the concentration of micronutrients in the lettuce tissues, as well as in the final soil samples. However, B tended to show a higher concentration in lettuce tissues in sewage sludges with higher initial B concentrations. The availability of B in the soil is highly dependent on the dynamics of organic matter [53,54], especially in alkaline pH soils where B is adsorbed by organic colloids with a binding strength even greater than that of inorganic colloids [4]. Thus, it was the intense mineralization of the organic substrate that probably facilitated access to more B by the plants.

The concentrations of metallic micronutrients (Fe, Mn, Zn, and Cu) in plant tissues did not exhibit any relationship with their initial concentrations in the sewage sludge. While these elements are abundant in the soil in absolute terms, their presence in the soil solution and, consequently, their bioavailability to plants depend on environmental variables, especially soil pH and aeration [4,55].

The pH of the soil is a crucial factor in the solubility of those nutrients. When pH increases, the ionic forms available for plants, such as elements like Fe and Mn, first change to hydroxide ions and, ultimately, to the insoluble oxides [4,56]. In this study, soil pH did not vary between WWTPs, nor did the concentration of elements in lettuce tissues, despite significant differences in the initial concentration of elements in the sewage sludge. However, differences were found between treatments in soil pH due to the application of alkalized sewage sludge, although this pH variation also did not significantly influence the concentration of nutrients in the tissues. The effect of pH is usually significant for values close to 5, where large amounts of Fe and Mn are present in the soil solution [56]. The pH of the soil in this study was above 7 in treatments that received sewage sludge stabilized with calcium oxide or calcium hydroxide. At these pH values, the availability of Fe and Mn is generally low [4,56]. However, in this study, their concentrations in the tissues were high, considering the sufficiency ranges established for the crop by [43].

Another variable that strongly influences the solubility of cationic micronutrients, particularly Fe and Mn, is the soil redox potential. Reduction conditions may increase the soluble forms of Fe and Mn in the soil due to the dissolution of Fe and Mn oxides, leading to a strong uptake by plants [4,55]. In this study, the pots were regularly watered throughout the growing cycle. Despite irrigation being scheduled to prevent situations of water excess or drought that could affect plant growth, the watering process creates cycles of wetting and drying, leading to fluctuations in the soil redox potential. These conditions contribute to high soil nutrient availability, possibly resulting in the relatively high levels of cationic metal concentrations recorded in lettuce tissues. On the other hand, the intense biological activity associated with the rapid decomposition of organic matter, leading to oxygen consumption, is also a known cause of increased bioavailability of metallic cations in the soil [4,17]. Therefore, even though pH conditions might suggest low levels of cationic micronutrients in tissues, they appear at relatively high levels, likely because of wetting and drying cycles associated with pot irrigation and the high rate of decomposition of the organic substrate.

4.4. Heavy Metals in Plant Tissues and Soil

The concentrations of heavy metals in lettuce tissues showed little variation, both with WWTPs and with the stabilization and disinfection treatments. However, the concentrations of heavy metals in the original sewage sludge varied between WWTPs, although they are generally low when compared to the threshold values established in the European Union [19]. In fact, the amounts of heavy metals in sewage sludge depend on the origin of the wastewater, with other studies reporting concerningly high [27] or acceptably low [21,25] values. The values of Cd in the soil varied significantly with the origin of the sewage sludge, and Cr values varied significantly with both the origin and the treatment of the sludge. However, both metals presented low concentrations in the soil compared to international legislation [19]. Researching the results of previous studies, the most common trend is the record of increases in the extractable fraction of heavy metals in the soil due to sewage sludge application, but often below the threshold values for contaminated soil [20,23,24].

Of great relevance to this study was the observation that the concentrations of heavy metals in lettuce tissues did not vary significantly with the treatments. This is particularly noteworthy, as the levels of Cd and Pb are quite low when compared to the established limits for leafy vegetables [57]. High levels of heavy metals in food have been a concern in the use of sewage sludge. Swain et al. [26] reported high Cd values in spinach (*Spinacea oleracea* L.) leaves when applying 20 t ha^{-1} of sewage sludge, although other studies have not revealed significant contamination problems in plants after sewage sludge application [31,32]. On the other hand, these results did not provide a complete view of the problem. Even though the sludges used in this study did not present microbiological contamination issues [58], this type of material may contain toxic organic compounds [22,29] that must be considered for its safe use by farmers. Nevertheless, international legislation should continue to progress to specify the best conditions for applying these materials safely, given that they are rich in valuable nutrients that agriculture should not overlook, especially N and P.

5. Conclusions

The sewage sludge influenced the growth of lettuce mainly due to its N concentration, resulting in a higher DMY observed in lettuce treated with Gelfa sewage sludge, which had the highest N concentration. The sewage sludges used in this study had a high N concentration and a low C/N ratio, leading to rapid mineralization and N supply for the plants. Although sewage sludge was originally rich in P, it seems that the supply of P to plants was more influenced by the initial concentration of N, promoting its mineralization rate, than by the initial P concentration.

Sewage sludge also contains interesting levels of B, which, like N and P, becomes readily available due to the rapid mineralization of the organic substrate. Regarding cationic micronutrients, the effect of applying sewage sludge on their concentration in tissues was limited, appearing to be more dependent on the soil conditions in which the lettuce was grown. Stabilization and disinfection treatments of sewage sludge had a positive impact on its fertilizing value, notably increasing soil pH and available Ca and providing an additional stimulus to the rapid mineralization of sewage sludge. Thus, sewage sludge subjected to this type of treatment should preferably be used in acidic soils, where the benefits of its application are enhanced.

The sewage sludges used in this study had very low initial concentrations of heavy metals, and the levels in the soil where they were applied and in the lettuce tissues were also low when compared to international legislative limits. Based on the information gathered in this study, sewage sludges with such characteristics can be used in agriculture, as they pose a reduced environmental and human health risk and have high agronomic value. Although lettuce was selected as the test plant for this study, it does not necessarily mean it is the most suitable species for using these fertilizers, which may introduce various other risks, such as toxic organic compound content. Taller plants, like cereals, which are not typically consumed fresh by humans, might be more appropriate. Given the wide range of origins and treatment methods of sewage sludge and the associated risks of their use, further studies will be needed to establish the optimal conditions for utilizing these materials, despite their clear fertilizing value.

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