



Article

Sewage Sludge Increased Lettuce Yields by Releasing Valuable Nutrients While Keeping Heavy Metals in Soil and Plants at Levels Well below International Legislative Limits

Manuel Ângelo Rodrigues^{1,2,*} , Almeida Sawimbo^{1,3} , Julieta Moreira da Silva⁴, Carlos Manuel Correia⁵ and Margarida Arrobas^{1,2}

¹ Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal; almeidasawimbo4@gmail.com (A.S.); marrobas@ipb.pt (M.A.)

² Laboratório para a Sustentabilidade e Tecnologia em Regiões de Montanha, Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

³ Instituto Superior Politécnico do Cuanza Sul (ISPCS), Rua 12 de Novembro, Sumbe P.O. Box 82, Angola

⁴ Águas do Norte S.A., Rua Dom Pedro de Castro, n.º 1A, 5000-669 Vila Real, Portugal; julieta.silva@adp.pt

⁵ Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Trás-os-Montes and Alto Douro, 5001-801 Vila Real, Portugal; ccorreia@utad.pt

* Correspondence: angelor@ipb.pt

Abstract: Sewage sludge can be used as an organic amendment as long as it is ensured that there is no risk of environmental contamination or risk to public health. In this study, sewage sludge from two wastewater treatment plants (WWTPs) subjected to two disinfection and stabilization treatments [40% (mass/mass), calcium oxide, and calcium hydroxide] and their respective untreated sewage sludge were used. Three control treatments were also added: conventional farmyard manure (FYM), a nitrogen (N) mineral fertilizer (ammonium nitrate 34.5% N) applied at a rate of 50 kg N ha⁻¹ (N50) (the same rate of all organic amendments), and an unfertilized control (N0), totaling nine treatments. Lettuce (*Lactuca sativa* L.) was cultivated in pots for two growing cycles. The dry matter yield (DMY) was higher in the N50 treatment (13.5 and 10.6 g plant⁻¹ in the first and second growing cycles, respectively), followed by sewage sludge (10.8 to 12.4 and 8.4 to 8.7 g plant⁻¹), FYM (8.5 and 7.2 g plant⁻¹), and the control (7.7 and 6.0 g plant⁻¹). The DMY was related to the N provided by the different treatments, assessed by the N and nitrate concentrations in tissues, N uptake, and apparent N recovery (ANR). Sewage sludge, due to its high N concentration and low carbon (C)/N ratio, mineralized rapidly, providing a significant amount of N to plants, as well as other nutrients, such as phosphorus (P) and boron (B). FYM, with a higher C/N ratio, provided less N to plants, also due to the short duration of the lettuce growing cycle. Alkalinized sewage sludge increased soil pH and calcium (Ca) availability for plants. Fertilizer treatments minimally influenced cationic micronutrients. Heavy metals in the initial sewage sludge were below the threshold values established in international legislation, and the levels in soil and lettuce tissues were generally not higher than those in other treatments. Both of the sewage sludges used in this study showed high fertilizing value and very reactive behavior, making nutrients available much more quickly than FYM. This information is relevant to consider in defining their agricultural use.

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



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1. Introduction

The swift rise in the world population, projected to reach 9.7 billion people by 2050 [1], presents substantial challenges in the realm of food provision for upcoming societies. Over the past few years, a multitude of projection and quantitative scenario studies on global food security have been conducted. The outcomes of these studies range from mildly

concerning to highly alarming, and this variation hinges on the variables integrated into the projection models. Notably, the issue of climate change plays a pivotal role in shaping these diverse results [2]. According to the most common estimates, the projected increase in world population is expected to result in a 50 to 60% greater demand for food and agricultural products [3,4].

With the increase in the world population and the imperative to produce more food, there is a pressing need for profound changes in agricultural systems. Intensive agriculture has been implicated in soil degradation, water contamination, and air pollution, all of which have adverse effects on humans, animals, and ecosystems [5,6]. As the demand for increased food production persists, a shift in conventional agricultural practices is anticipated, moving progressively toward production systems often referred to as sustainable intensification [7,8].

Crop fertilization is a practice that must be rationalized, and the use of industrially synthesized fertilizers has raised significant concerns. N fertilizers, in particular, pose high risks of environmental contamination due to the leaching of nitrates into waterways [9,10] and the emission of greenhouse gases into the atmosphere, especially N oxides [11,12]. Additionally, there is an energy issue associated with fertilization. The Haber–Bosch process, commonly used for synthesizing N fertilizers, is known for being energy-intensive [13,14]. Any disruption to global stability, as recently witnessed with events like the war in Ukraine and the COVID-19 pandemic, poses serious challenges in the manufacturing and trade of fertilizers, leading to a surge in prices [15,16]. Moreover, sustainability issues surround phosphate fertilizers, as the phosphate rocks used in their production are depleting [14,17].

The utilization of organic amendments stands as one of the pillars of sustainable agriculture, fostering soil fertility and potentially reducing reliance on industrial synthetic fertilizers. However, the availability of manure is limited, and with the emergence of more specialized agricultural areas that do not raise animals, these resources become scarce [18,19]. It is imperative to consider all organic resources generated by human activities at local scales. Embracing the principles of the circular economy, where all resources are valued, agriculture emerges as a fitting destination for most organic waste [19–22]. In recent decades, the agricultural use of sewage sludge from wastewater treatment has gained significant visibility due to the vast quantities in which it is produced [21,23].

The global production of sewage sludge has experienced a significant increase, attributed to the ongoing migration of people from small villages to urban areas. Presently, the estimated annual global production of sewage sludge stands at 45 million tons [24,25]. Despite the ongoing debate surrounding the utilization of sewage sludge in agriculture, stemming from concerns about the potential contamination of food and the environment with substances such as heavy metals, pathogenic microorganisms, and/or toxic organic compounds [21,23,26], international legislation aims to establish appropriate guidelines to ensure safe use [27]. Sewage sludge is a material rich in organic C and other nutrients, including N and P [21,22,28,29]. Its application in agriculture has the potential to enhance soil properties and boost crop growth and yield [22,30,31].

While regulatory bodies strive to establish the safest practices for utilizing sewage sludge in agriculture, continuous assessment of its agronomic value is essential to formulating rational usage protocols. These protocols should encompass its impact on soil properties, plant productivity, and the ongoing monitoring of potential contamination risks to both soil and plants. Thus, this study aimed to assess both the agronomic value and the potential risks of soil and food contamination associated with the application of sewage sludge sourced from two distinct WWTPs. The sewage sludge underwent disinfection and stabilization treatment with calcium oxide and calcium hydroxide (40% mass/mass). Additionally, untreated sewage sludge was included. The experiment also involved three controls to better understand the availability of nutrients from the sewage sludge: conventional farmyard manure (FYM), mineral N fertilizer applied at the same N rate, and an unfertilized control. The primary objective of this study is to compare the fertilizing effect of sewage sludge sourced from two different WWTPs with those of FYM, mineral

N fertilization, and an unfertilized control. Additionally, we aim to ascertain the optimal placement of sewage sludge within fertilization recommendation programs. Furthermore, we seek to evaluate whether the levels of heavy metals present in sewage sludge, plants, or soil render its use as a fertilizer inadvisable.

2. Materials and Methods

2.1. Experimental Conditions

This study was conducted as a pot experiment with lettuce (*L. sativa* var. *capitata*) and took place in Bragança, in the northeast of Portugal. The region has a Mediterranean climate, characterized by a hot and dry summer and a winter with low temperatures and high precipitation. The average annual temperature in Bragança is 12.7 °C, and the total annual precipitation is 772.8 mm. Lettuce was cultivated in two growing cycles during the year 2022. The average air temperature and precipitation during the experimental period are shown in Figure 1, along with the values of the climatological normal. Crisp-leaved head lettuce (cv. Wonder of Summer), known for its high heat tolerance, was cultivated as the test plant. It is considered a hyperaccumulator of nitrates and heavy metals, serving as a good indicator of the release of nutrients and heavy metals from sewage sludge. Lettuce was grown in pots filled with 3 kg of dry soil sieved through a 2 mm mesh. The soil is a Eutric Regosol [32] with a sandy clay loam texture and slightly acidic properties. These and other soil properties are presented in Table 1.

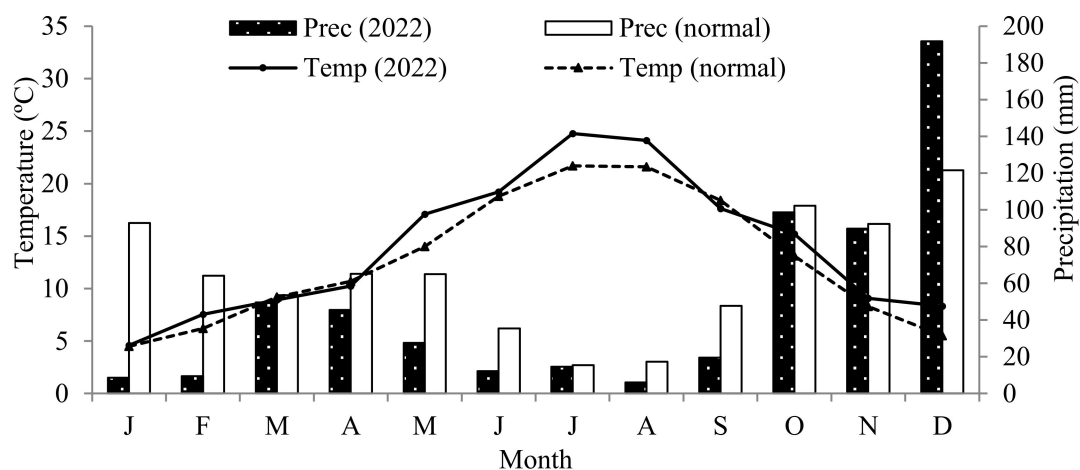


Figure 1. Climatological normal and average monthly temperature and precipitation during 2022 at the Santa Apolónia farm weather station in Bragança.

Table 1. Selected properties of the soil (average \pm standard deviation, $n = 3$) used in the pot experiment.

Soil Properties		Soil Properties	
¹ Organic carbon (g kg^{-1})	22.5 ± 0.71	⁷ Exch. calcium ($\text{cmol}^+ \text{kg}^{-1}$)	15.2 ± 0.73
² pH(H_2O)	6.1 ± 0.01	⁷ Exch. magnesium ($\text{cmol}^+ \text{kg}^{-1}$)	5.0 ± 0.04
³ Electrical conductivity (mS cm^{-1})	0.07 ± 0.01	⁷ Exch. potassium ($\text{cmol}^+ \text{kg}^{-1}$)	0.2 ± 0.01
⁴ Extrac. phosphorus (g kg^{-1} , P_2O_5)	109.4 ± 5.95	⁷ Exch. sodium ($\text{cmol}^+ \text{kg}^{-1}$)	0.3 ± 0.07
⁴ Extr. potassium (g kg^{-1} , K_2O)	180.0 ± 22.52	⁸ Exch. acidity ($\text{cmol}^+ \text{kg}^{-1}$)	0.2 ± 0.00
⁵ Extr. boron (mg kg^{-1})	1.6 ± 0.15	Cation exch. capacity ($\text{cmol}^+ \text{kg}^{-1}$)	20.9 ± 0.76
⁶ Ext. iron (mg kg^{-1})	196.2 ± 3.71	⁹ Sand (g kg^{-1})	543.7 ± 16.17
⁶ Extr. zinc (mg kg^{-1})	13.0 ± 0.32	⁹ Silt (g kg^{-1})	238.3 ± 8.08
⁶ Extr. copper (mg kg^{-1})	74.6 ± 3.46	⁹ Clay (g kg^{-1})	218.0 ± 8.72
⁶ Extr. manganese (mg kg^{-1})	254.5 ± 9.54	¹⁰ Texture	Sandy Clay Loam

¹ Walkley–Black; ² potentiometry; ³ soil/water suspension, 1/5; ⁴ Egner–Riehm; ⁵ hot water, azomethine-H; ⁶ ammonium acetate and EDTA (ethylenediaminetetraacetic acid); ⁷ ammonium acetate; ⁸ potassium chloride; ⁹ Robinson pipette method; ¹⁰ USDA (the United States Department of Agriculture).

2.2. Characterization of Sewage Sludge and Farmyard Manure and Experimental Design

The experiment was arranged as a completely randomized design with nine treatments and three replications. Sewage sludge from two WWTPs, one located in Lousada (L) and another in Amarante (A), was used. Thus, in total, 54 pots were used. The sludge treatment system in these WWTPs involves primary settling and medium-load activation in a liquid line, thickening, hot digestion (35 °C), and mechanical dewatering. At the outlet of the WWTP, the sewage sludge appears as a solid material with approximately 80% humidity. The average composition of the sewage sludge used in the study is presented in Table 2.

Table 2. Composition of untreated (Untr) and 40% (m/m) calcium hydroxide (CH)- or calcium oxide (CO)-treated sewage sludge from Amarante and Lousada and farmyard manure (FYM) (average \pm standard deviation, $n = 3$).

	Amarante			Lousada			
	Untr	CH	CO	Untr	CH	CO	FYM
Dry matter (%)	19.1 \pm 1.5	12.0 \pm 1.9	10.8 \pm 2.6	24.7 \pm 4.0	14.4 \pm 2.3	15.6 \pm 2.5	37.1 \pm 5.7
pH (H ₂ O)	7.5 \pm 0.1	9.8 \pm 0.6	9.7 \pm 0.7	8.0 \pm 0.1	9.9 \pm 1.1	10.0 \pm 0.8	8.8 \pm 0.2
El. cond. (mS cm ⁻¹)	2.2 \pm 0.1	1.8 \pm 0.1	1.8 \pm 0.1	2.3 \pm 0.1	2.0 \pm 0.1	1.9 \pm 0.2	2.9 \pm 0.3
Carbon (g kg ⁻¹)	235.7 \pm 24.3	176.0 \pm 25.5	185.7 \pm 22.1	358.1 \pm 10.3	267.7 \pm 25.5	243.2 \pm 16.0	349.5 \pm 32.2
Nitrogen (g kg ⁻¹)	46.7 \pm 4.6	24.2 \pm 2.5	24.6 \pm 1.9	49.7 \pm 1.2	33.1 \pm 4.8	28.8 \pm 3.6	22.7 \pm 0.6
Carbon/nitrogen	5.0 \pm 0.0	7.1 \pm 0.5	7.5 \pm 0.4	7.2 \pm 0.01	8.1 \pm 0.4	8.5 \pm 0.5	15.4 \pm 1.1
Phosphorus (g kg ⁻¹)	23.0 \pm 1.5	11.1 \pm 0.7	12.1 \pm 0.3	16.8 \pm 1.3	10.4 \pm 0.4	10.2 \pm 1.0	7.2 \pm 0.4
Potassium (g kg ⁻¹)	3.3 \pm 0.2	1.8 \pm 0.7	2.0 \pm 0.5	2.5 \pm 0.5	1.8 \pm 0.5	1.9 \pm 0.1	25.5 \pm 0.8
Calcium (g kg ⁻¹)	14.1 \pm 1.3	124.4 \pm 90.6	120.9 \pm 28.7	20.2 \pm 3.0	85.9 \pm 9.0	141.6 \pm 16.0	11.3 \pm 0.7
Magnesium (g kg ⁻¹)	4.2 \pm 0.7	3.4 \pm 1.0	3.3 \pm 0.2	4.5 \pm 1.0	3.3 \pm 0.2	3.6 \pm 0.5	17.3 \pm 2.8
Boron (mg kg ⁻¹)	47.7 \pm 1.4	21.3 \pm 3.2	22.6 \pm 0.5	20.2 \pm 2.0	16.9 \pm 2.2	13.3 \pm 3.3	50.6 \pm 12.4
Iron (mg kg ⁻¹)	32.0 \pm 2.4	9.1 \pm 10.9	18.9 \pm 1.5	11.6 \pm 3.1	7.7 \pm 1.8	7.5 \pm 0.6	9.4 \pm 1.1
Manganese (mg kg ⁻¹)	232.7 \pm 11.3	133.6 \pm 35.7	128.6 \pm 20.9	240.5 \pm 26.4	204.7 \pm 13.5	159.9 \pm 6.3	162.4 \pm 30.5
Zinc (mg kg ⁻¹)	574.3 \pm 22.8	324.7 \pm 95.4	333.4 \pm 57.1	790.8 \pm 115.0	553.6 \pm 90.1	538.8 \pm 19.8	45.0 \pm 1.5
Copper (mg kg ⁻¹)	197.2 \pm 6.9	114.6 \pm 40.3	115.5 \pm 19.5	250.6 \pm 21.2	126.4 \pm 18.4	116.5 \pm 4.2	423.3 \pm 25.7
Nickel (mg kg ⁻¹)	34.1 \pm 7.4	16.3 \pm 4.4	15.8 \pm 2.2	15.8 \pm 3.9	11.3 \pm 2.2	9.7 \pm 2.5	187.8 \pm 25.8
Cadmium (mg kg ⁻¹)	0.5 \pm 0.1	0.3 \pm 0.1	0.3 \pm 0.1	0.7 \pm 0.2	0.6 \pm 0.1	0.5 \pm 0.1	4.4 \pm 0.1
Chromium (mg kg ⁻¹)	49.1 \pm 5.8	27.0 \pm 6.1	30.6 \pm 3.5	80.8 \pm 3.3	54.6 \pm 2.6	49.7 \pm 12.3	159.3 \pm 51.4
Lead (mg kg ⁻¹)	15.9 \pm 2.9	8.2 \pm 0.7	8.0 \pm 0.9	23.0 \pm 5.6	15.9 \pm 2.4	13.2 \pm 4.7	8.6 \pm 1.9

Both sewage sludges underwent two stabilization and sanitization treatments with the application of calcium oxide (CO) or calcium hydroxide (CH) at 40% (mass/mass), and both methods are in use in the WWTPs that provided the sewage sludge for the study. Untreated sludge (Untr) from both WWTPs was also used in the experiments for comparison. Additionally, FYM (cow manure containing the excrement and urine of the animals, as well as residues from their feed and bedding, usually made from rye straw), a mineral N fertilizer (ammonium nitrate, 34.5% N) at a rate of 50 kg N ha⁻¹ (N50), and a control treatment (N0) were included. Sewage sludge and FYM were also applied at a rate of 50 kg N ha⁻¹. Thus, the nine treatments were A.CO, A.CH, A.Untr, L.CO, L.CH, L.Untr, FYM, N50, and N0.

Each lettuce plant received a fertilizer quantity equivalent to the application of 50 kg N ha⁻¹, considering a planting density in a commercial field of 140,000 lettuce plants ha⁻¹. Thus, each pot (with one lettuce plant) received the corresponding fraction of N (0.36 g N, 1.04 g ammonium nitrate, 34.5% N). The quantity of applied FYM was estimated by considering its N concentration and moisture content (Table 2). Additionally, the sewage sludge quantities were adjusted to account also for the dilution effect resulting from disinfection treatments. The applied amounts of fresh materials were 42.4, 66.7, and 48.5 g pot⁻¹ for FYM and sewage sludge from Amarante and Lousada, respectively.

2.3. Experimental Setup and Management

The first lettuce cycle began on 19 May 2022. The organic amendments and fertilizer were mixed with 3 kg of dry (40 °C) and sieved (2 mm mesh) soil, and the mixture was placed in pots. Subsequently, lettuce seedlings were planted at the “3rd true leaf unfolded”

phenological stage [33]. The pots were kept outdoors, but the sides of the pots were shielded from direct sunlight by a wooden structure to reduce the risk of overheating on hotter days. In the following days, weed emergence was monitored, and any weeds were promptly removed after germination. The pots were watered as needed. The amount of water applied varied substantially depending on environmental variables and lettuce size, which also varied considerably among treatments. It was ensured that there was no drought stress in the plants throughout the entire growing cycle.

The lettuce plants from this first growing cycle were harvested on 23 June 2022 at phenological stage 49, “typical size, form, and firmness of heads reached” [33]. On 31 August 2022, the second growing cycle began, with the application of a second dose of sewage sludge, FYM, and mineral fertilizer to the pots, repeating all of the procedures described for the first growing cycle. The harvest took place on 13 October 2022.

2.4. Leaf Gas Exchange Measurements

CO₂ and water exchange measurements were made 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 $1470 \pm 30 \mu\text{mol m}^{-2} \text{s}^{-1}$, an air temperature of $26.3 \pm 0.6 \text{ }^\circ\text{C}$, and a CO₂ concentration of $410 \pm 2.3 \mu\text{mol CO}_2 \text{ mol}^{-1}$. Measurements were made around midday with a portable photosynthesis system (LCpro+, Analytical Development Co., Hoddesdon, UK), operating in the open mode. The net photosynthetic rate (A), stomatal conductance to water vapor (g_s), and the ratio of intercellular to atmospheric CO₂ concentration (C_i/C_a) were calculated using the equations of von Caemmerer and Farquhar [34].

2.5. Plant and Soil Sampling and Laboratory Determinations

At the end of the growing season, the total leaf area of the lettuce was estimated by selecting two circles with a diameter of 3.7 cm from the blades of leaves in the middle part of the rosette. These circles were immediately placed in hermetically sealed screw-capped tubes, followed by weighing when fresh and then oven-drying at 70 °C. The remaining lettuce, cut with a knife at ground level, was gently washed in water to remove any dirt from rain splashes. The lettuce plants were subsequently dried at 70 °C and weighed. The total leaf area of the lettuce was calculated by establishing the ratio between the area and dry mass of the circles and the mass of the entire lettuce using a simple three-rule. For greater accuracy in the total dry mass determinations, the dry mass of the circles was added to the initially obtained dry mass of the lettuce. The lettuce tissues were then ground in a mill (1 mm mesh) for laboratory analysis.

In the dry matter of lettuce, the concentration of nitrates was determined. A sample of 1 g was shaken for 1 h in 50 mL of water. The extract was filtered with Whatman #42 paper and analyzed with a UV-Vis molecular absorption spectrophotometer [35].

The Kjeldahl method was employed for N determination. Prior to the analysis, samples were digested with sulfuric acid, using selenium as a catalyst. The B concentration in tissues was determined through colorimetry using the azomethine-H method. For other nutrients, samples were digested with nitric acid in a microwave. Subsequently, P was determined by colorimetry using the blue ammonium molybdate method with ascorbic acid as a reducing agent. Cations [potassium (K), Ca, magnesium (Mg), iron (Fe), copper (Cu), zinc (Zn), manganese (Mn), cadmium (Cd), chromium (Cr), lead (Pb), and nickel (Ni)] were determined by atomic absorption spectrophotometry after extraction with ammonium acetate and EDTA. For more detailed information on these analytical procedures, readers are referred to Temminghoff and Houba [36]. The results of the initial sewage sludge and manure samples, as shown in Table 2, were also obtained using the aforementioned analytical procedures.

At the end of the second growing cycle, the soil of the pots was thoroughly homogenized, and samples of approximately 250 g were taken. These samples were oven-dried at 40 °C and sieved again through a 2 mm mesh before undergoing laboratory analysis. Pre-

viously, samples of the initial soil had already been analyzed using the methods described below (Table 1).

In the initial samples only, soil separates were determined using the Robinson pipette method. For all samples, soil pH (H₂O) (soil/solution, 1:2.5) was determined by potentiometry and electrical conductivity in a soil/water suspension of 1/5. Soil organic C was determined using the Walkley–Black method. Extractable P and K were determined using the Egner–Riehm method, and soil B was extracted by hot water and determined by the azomethine-H method. Exchangeable bases were extracted by ammonium acetate at pH 7.0 and determined by atomic absorption spectrometry. Exchangeable acidity was extracted using KCl and quantified through titration with sodium hydroxide. For more details on the preceding procedures, readers are referred to Van Reeuwijk [37]. Metal micronutrients (Fe, Cu, Zn, Mn, Ni) and other trace elements (Cd, Cr, Pb) were extracted with diethylenetriaminepentaacetic acid (DTPA) buffered at pH 7.3 and determined by atomic absorption spectrometry [38].

2.6. Data Analysis

The normality and homogeneity of variance in the results were verified using the Shapiro–Wilk and Bartlett tests, respectively. One-way ANOVA was applied to the results to identify significant differences between treatments, utilizing the statistical 29.0.10 software SPSS Statistics (IBM SPSS, Chicago, IL, USA). In cases where significant differences were observed, mean separation was performed using the post hoc Tukey HSD test ($\alpha = 0.05$).

The data were also examined to verify the extent to which nutrient concentrations in the tissues (independent variables) are related to DMY (dependent variable). To combine the results from the two growing cycles into a single figure, DMY was expressed in relative terms, using the average DMY of the most productive treatment as 100%.

Apparent N recovery (%) = (N recovery in fertilized or amended plants – N recovery in unfertilized plants)/N applied × 100.

Nutrient uptake (mg plant⁻¹) = DMY (g plant⁻¹) × tissue nutrient concentration (g kg⁻¹)

3. Results

3.1. Lettuce Dry Matter Yield and Leaf Surface

The dry matter yield (DMY) of lettuce was higher in the first growing cycle than in the second (Figure 2). Considering the average of all treatments, the DMY in the second cycle was 77.1% relative to the first. In the first growing cycle, the DMY ranged from 7.7 (control) to 13.5 (N50) g plant⁻¹, and in the second cycle, it ranged from 6.0 (control) to 10.6 (N50) g plant⁻¹. The average DMY was higher in the N50 treatment, with the result from the second cycle significantly higher than any other treatment. When comparing treatments with sewage sludge, those receiving 40% calcium oxide showed values tending to be higher, especially in the first growing cycle, although without significant differences from the others. The control treatment produced the lowest average values, with the values in the second cycle being significantly lower than those of the other treatments. FYM showed values that were tendentially higher than the control treatment but lower than treatments receiving sewage sludge and N50.

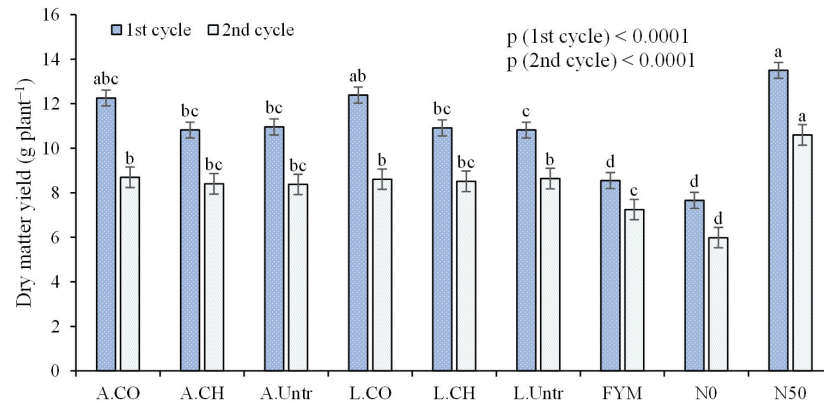


Figure 2. Lettuce dry matter yield in two growing cycles assessed in pots amended with sewage sludge from Amarante (A) or Lousada (L) and treated with 40% calcium oxide (CO) or 40% calcium hydroxide (CH) or left untreated (Untr). Additionally, farmyard manure (FYM), inorganic nitrogen (N50), and an untreated control (N0) were included in the study. Means followed by the same letter are not significantly different according to the Tukey HSD test ($\alpha = 0.05$). Error bars represent standard errors.

The total leaf area of the plant followed a similar pattern to the DMY (Figure 3). In the second growing cycle, the leaf area was only 67.0% of that in the first growing cycle. The N0 treatment showed the lowest average values, although without significant differences from FYM. FYM, in turn, had average values lower than treatments with sewage sludge. Considering the two growth cycles, the N50 treatment showed the highest average values among all treatments.

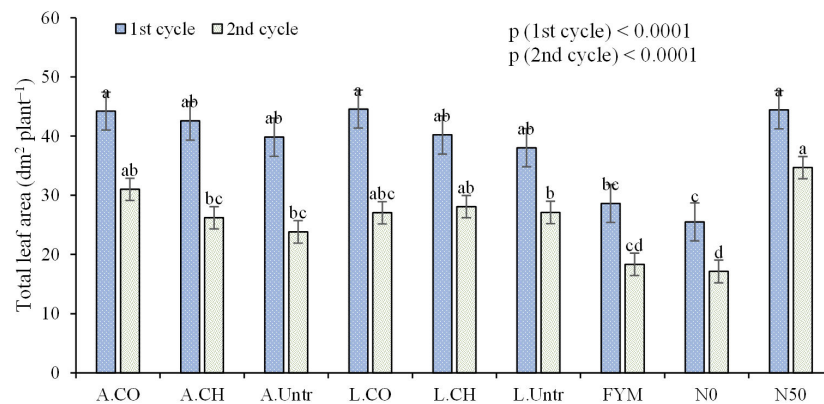


Figure 3. The total leaf area of lettuce from two growing cycles assessed in pots amended with sewage sludge from Amarante (A) or Lousada (L) and treated with 40% calcium oxide (CO) or 40% calcium hydroxide (CH) or left untreated (Untr). Additionally, farmyard manure (FYM), inorganic nitrogen (N50), and an untreated control (N0) were included in the study. Means followed by the same letter are not significantly different according to the Tukey HSD test ($\alpha = 0.05$). Error bars represent standard errors.

3.2. Leaf Gas Exchange

The applied treatments significantly affected leaf gas exchange variables (Table 3). The CO₂ assimilation rates of the control and FYM-treated plants were lower than those of plants receiving sewage sludge and especially lower than those treated with N50, in close association with g_s values. Nevertheless, non-stomatal limitations to photosynthesis were also evident, as supported by the greater C_i/C_a in control and FYM treatments.

Table 3. The net photosynthetic rate (A), stomatal conductance (g_s), and the ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a) (average \pm standard deviation) in lettuce leaves from the second growing cycle assessed in pots amended with sewage sludge from Amarante (A) or Lousada (L) and treated with 40% calcium oxide (CO) or 40% calcium hydroxide (CH) or left untreated (Untr). Additionally, farmyard manure (FYM), inorganic nitrogen (N50), and an untreated control (N0) were included in the study.

	A	g_s	C_i/C_a
	$\mu\text{mol m}^{-2} \text{s}^{-1}$	$\text{mmol m}^{-2} \text{s}^{-1}$	
A.CO	11.1 \pm 0.25 ^b	134.1 \pm 9.4 ^b	0.570 \pm 0.017 ^c
A.CH	10.9 \pm 0.11 ^b	128.7 \pm 2.2 ^b	0.573 \pm 0.004 ^c
A.Untr	10.8 \pm 0.28 ^b	132.4 \pm 8.3 ^b	0.576 \pm 0.025 ^c
L.CO	11.4 \pm 0.14 ^b	131.4 \pm 4.9 ^b	0.562 \pm 0.010 ^c
L.CH	11.1 \pm 0.24 ^b	142.3 \pm 5.0 ^b	0.579 \pm 0.009 ^c
L.Untr	11.2 \pm 0.26 ^b	138.8 \pm 2.6 ^b	0.572 \pm 0.011 ^c
FYM	8.3 \pm 0.42 ^c	132.1 \pm 6.7 ^c	0.677 \pm 0.027 ^a
N0	7.4 \pm 0.25 ^c	109.2 \pm 2.7 ^c	0.673 \pm 0.011 ^a
N50	12.6 \pm 0.31 ^a	173.4 \pm 6.3 ^a	0.620 \pm 0.015 ^b
Prob.	<0.0001	<0.0001	<0.0001

Means followed by the same letter are not significantly different according to the Tukey HSD test ($\alpha = 0.05$).

3.3. Nutrients in Plant Tissues and Relationship with Dry Matter Yield

The N concentration in lettuce tissues varied significantly between treatments in the two growing cycles (Table 4). In the first growing cycle, the average values ranged from 14.9 (N0) to 30.0 (N50) g kg^{-1} , and in the second cycle, they ranged from 17.2 (N0) to 35.1 (N50) g kg^{-1} . The values of the N50 treatment were significantly higher than those of any other treatment. Treatments with sewage sludge formed a group where no significant differences occurred. The average values of the FYM treatment were higher than those of the N0 treatment but without significant differences. However, significant differences were observed between the average values of the FYM treatment and those of treatments with sewage sludge. The N uptake accentuated differences between the average values of different treatments but had little influence on statistical differences, maintaining the pattern described for N concentration in the tissues.

Table 4. Nitrogen (N) concentration, N uptake, and apparent N recovery (average \pm standard deviation) in lettuce tissues from two growing cycles assessed in pots amended with sewage sludge from Amarante (A) or Lousada (L) and treated with 40% calcium oxide (CO) or 40% calcium hydroxide (CH) or left untreated (Untr). Additionally, farmyard manure (FYM), inorganic nitrogen (N50), and an untreated control (N0) were included in the study.

	N Concentration (g kg^{-1})		N Uptake (mg plant^{-1}) *		Apparent N Recovery (%) **	
	1st Cycle	2nd Cycle	1st Cycle	2nd Cycle	1st Cycle	2nd Cycle
A.CO	23.4 \pm 0.4 ^b	25.1 \pm 2.0 ^b	287.0 \pm 21.3 ^b	217.7 \pm 8.2 ^b	48.5 \pm 5.9	32.1 \pm 2.3
A.CH	23.4 \pm 1.2 ^b	25.7 \pm 2.6 ^b	252.3 \pm 14.9 ^{bc}	215.2 \pm 16.7 ^b	38.7 \pm 4.1	31.4 \pm 4.6
A.Untr	23.1 \pm 2.5 ^b	25.2 \pm 1.0 ^b	251.9 \pm 19.0 ^{bc}	211.3 \pm 21.2 ^b	38.6 \pm 5.3	30.3 \pm 5.9
L.CO	22.6 \pm 0.8 ^b	24.7 \pm 1.5 ^b	280.7 \pm 18.3 ^b	212.7 \pm 14.0 ^b	46.7 \pm 5.1	30.7 \pm 3.9
L.CH	21.4 \pm 1.7 ^b	24.6 \pm 0.9 ^b	233.8 \pm 23.5 ^c	209.6 \pm 20.1 ^b	33.5 \pm 6.5	29.8 \pm 5.6
L.Untr	21.4 \pm 1.1 ^b	23.4 \pm 0.7 ^b	230.9 \pm 11.2 ^c	202.6 \pm 25.0 ^b	32.7 \pm 3.2	27.8 \pm 6.9
FYM	16.1 \pm 0.8 ^c	19.4 \pm 0.7 ^c	137.4 \pm 7.6 ^d	140.6 \pm 9.8 ^c	6.6 \pm 2.1	10.5 \pm 2.7
N0	14.9 \pm 0.6 ^c	17.2 \pm 0.8 ^c	114.0 \pm 7.2 ^d	103.2 \pm 19.6 ^c	---	---
N50	30.0 \pm 0.9 ^a	35.1 \pm 0.9 ^a	418.0 \pm 20.1 ^a	372.1 \pm 30.3 ^a	81.3 \pm 5.6	75.3 \pm 8.4
Prob.	<0.0001	<0.0001	<0.0001	<0.0001		

* N uptake (mg plant^{-1}) = DMY (g plant^{-1}) \times tissue N concentration (g kg^{-1}). ** Apparent N recovery (%) = (N recovery in fertilized or amended plants – N recovery in unfertilized plants)/N applied \times 100. Means followed by the same letter are not significantly different according to the Tukey HSD test ($\alpha = 0.05$).

The ANR reached very high values in the N50 treatment, 81.3% and 75.3% in the first and second growing cycles, respectively (Table 4). In the first growing cycle, treatments with 40% calcium oxide showed average values above 45%, and the other treatments with sewage sludge showed values between 30 and 40%. In the second growing cycle, treatments with sewage sludge showed very similar values ranging from 27.85 to 32.1%. FYM showed much lower ANR when compared to sewage sludge, being 6.6% in the first growing cycle and 10.5% in the second.

The concentration of P in lettuce tissues did not vary significantly between treatments in either the first or second growing cycle (Table 5). In the first cycle, the average values ranged between 3.4 and 3.9 g kg⁻¹, and in the second cycle, they ranged between 2.9 and 3.9 g kg⁻¹. However, P uptake values, which incorporate the effect of DMY, differed significantly between treatments. There was a similar trend to that observed for N, with the N50 treatment resulting in the highest average values and the N0 treatment the lowest. FYM produced values similar to the N0 treatment and tended to be lower than treatments with sewage sludge.

Table 5. Phosphorus (P) concentration and P uptake (average ± standard deviation) in lettuce tissues from two growing cycles assessed in pots amended with sewage sludge from Amarante (A) or Lousada (L) and treated with 40% calcium oxide (CO) or 40% calcium hydroxide (CH) or left untreated (Untr). Additionally, farmyard manure (FYM), inorganic nitrogen (N50), and an untreated control (N0) were included in the study.

	P Concentration (g kg ⁻¹)		P Uptake (mg plant ⁻¹)	
	1st Cycle	2nd Cycle	1st Cycle	2nd Cycle
A.CO	3.7 ± 0.3 ^a	3.7 ± 0.6 ^a	44.8 ± 2.6 ^{ab}	31.9 ± 4.3 ^{ab}
A.CH	3.8 ± 0.2 ^a	3.9 ± 0.3 ^a	41.2 ± 3.9 ^b	33.0 ± 3.8 ^{ab}
A.Untr	3.9 ± 0.4 ^a	3.8 ± 0.2 ^a	43.0 ± 3.8 ^b	32.0 ± 4.1 ^{ab}
L.CO	3.5 ± 0.3 ^a	3.3 ± 0.3 ^a	43.4 ± 4.4 ^{ab}	28.3 ± 3.2 ^{ab}
L.CH	3.6 ± 0.3 ^a	3.9 ± 0.9 ^a	39.3 ± 4.1 ^{bc}	33.5 ± 9.3 ^{ab}
L.Untr	3.8 ± 0.5 ^a	3.6 ± 0.3 ^a	41.5 ± 5.8 ^b	30.8 ± 4.8 ^{ab}
FYM	3.4 ± 0.1 ^a	2.9 ± 0.1 ^a	29.1 ± 2.1 ^{cd}	21.4 ± 1.6 ^b
N0	3.5 ± 0.3 ^a	3.6 ± 0.4 ^a	26.9 ± 2.7 ^d	21.9 ± 5.2 ^b
N50	3.9 ± 0.2 ^a	3.8 ± 1.0 ^a	52.7 ± 4.4 ^a	40.1 ± 8.1 ^a
Prob.	0.1607	0.4112	<0.0001	0.0005

P uptake (mg plant⁻¹) = DMY (g plant⁻¹) × tissue P concentration (g kg⁻¹). Means followed by the same letter are not significantly different according to the Tukey HSD test ($\alpha = 0.05$).

The K concentration in plant tissues varied significantly between treatments in both growing cycles of lettuce (Table 6). Most average values were found between 35 and 50 g kg⁻¹. There was a tendency for higher values for the N50 treatment and lower values for the FYM treatment and for the sewage sludge treatments from Lousada. The Ca levels also varied significantly between treatments in both growing cycles of lettuce. Average values were found between 7.0 and 8.9 g kg⁻¹ when both growing cycles were taken into account. For each of the WWTPs, there was a trend toward higher values in treatments where sewage sludge was treated with calcium oxide and calcium hydroxide. The values of the N50 and N0 treatments were not consistent between growth cycles, being the lowest in the first growing cycle and moderately high in the second. The concentration of Mg in tissues also varied significantly between treatments in both growing cycles. FYM showed a tendency for low values, and the N50 treatment showed relatively high values.

The B levels in tissues also varied significantly between treatments in both growth cycles (Table 6). The sewage sludge from Amarante tended to have higher values than other treatments. The Fe levels in tissues varied significantly between treatments in the first growing cycle, although without an apparent relationship with the types of amendments used. In the second growing cycle, no differences occurred between treatments. The concentration of Mn in tissues varied significantly between treatments in both growing cycles. There seemed to be a tendency for the N50 and N0 treatments to have higher average values. The concentrations of Zn and

Cu, on the other hand, did not vary significantly between treatments. Considering the two growing cycles, the Zn levels in tissues were found in the range of 100.2 to 322.7 mg kg⁻¹, and the Cu levels ranged between 8.4 and 11.1 mg kg⁻¹.

Among the 10 nutrients analyzed in lettuce tissues, only the N concentration showed a significant correlation with the DMY (Figure 4). The N concentration in tissues was linearly related to the DMY ($p < 0.0001$), with a relatively high coefficient of determination ($R^2 = 0.65$).

Table 6. Potassium (K), calcium (Ca), magnesium (Mg), boron (B), iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) concentrations (average ± standard deviation) in lettuce tissues from two growing cycles assessed in pots amended with sewage sludge from Amarante (A) or Lousada (L) and treated with 40% calcium oxide (CO) or 40% calcium hydroxide (CH) or left untreated (Untr). Additionally, farmyard manure (FYM), inorganic nitrogen (N50), and an untreated control (N0) were included in the study.

1st Cycle	Macronutrients (g kg ⁻¹)				Micronutrients (mg kg ⁻¹)			
	K	Ca	Mg	B	Fe	Mn	Zn	Cu
A.CO	41.6 ± 1.9 ab	8.1 ± 0.4 ab	3.0 ± 0.1 b	33.4 ± 1.8 ab	1174.0 ± 576.5 a	66.3 ± 9.4 abc	164.2 ± 95.0 a	10.1 ± 0.7 a
A.CH	43.1 ± 1.7 ab	8.0 ± 0.5 abc	3.1 ± 0.2 b	34.2 ± 1.2 ab	929.0 ± 66.2 ab	65.3 ± 5.2 abc	145.9 ± 53.0 a	10.5 ± 0.9 a
A.Untr	43.2 ± 2.5 ab	7.2 ± 0.3 bc	3.0 ± 0.2 b	33.3 ± 1.3 b	980.3 ± 498.7 ab	62.4 ± 6.6 abc	101.3 ± 41.3 a	10.0 ± 1.4 a
L.CO	35.0 ± 3.5 b	8.1 ± 0.4 abc	2.9 ± 0.3 b	30.4 ± 1.0 bc	725.0 ± 240.9 ab	55.7 ± 5.2 abc	127.2 ± 32.8 a	8.4 ± 1.5 a
L.CH	36.2 ± 2.1 b	8.3 ± 0.6 a	2.8 ± 0.1 b	30.6 ± 1.0 bc	526.3 ± 38.6 ab	54.7 ± 6.5 bc	136.1 ± 29.5 a	7.9 ± 2.7 a
L.Untr	38.3 ± 10.8 b	7.5 ± 0.5 abc	2.6 ± 0.2 b	32.5 ± 1.7 b	425.0 ± 156.3 b	46.5 ± 10.5 c	106.3 ± 14.3 a	7.8 ± 2.6 a
FYM	37.8 ± 3.1 b	7.5 ± 0.7 abc	1.8 ± 0.3 c	37.3 ± 1.7 a	823.2 ± 105.9 ab	64.0 ± 5.9 abc	133.8 ± 18.2 a	8.1 ± 1.7 a
N0	45.4 ± 2.7 ab	7.0 ± 0.4 c	2.7 ± 0.1 b	31.8 ± 2.5 bc	877.2 ± 197.4 ab	75.7 ± 7.0 a	117.8 ± 53.1 a	8.5 ± 0.8 a
N50	50.7 ± 2.3 a	7.1 ± 0.2 c	3.9 ± 0.5 a	28.7 ± 1.2 c	789.1 ± 165.1 ab	74.5 ± 12.8 ab	100.2 ± 12.2 a	10.1 ± 1.3 a
Prob.	0.0006	0.0007	<0.0001	<0.0001	0.0332	0.0002	0.4611	0.1175
2nd cycle								
A.CO	42.6 ± 2.3 abc	8.5 ± 0.6 a	4.1 ± 0.2 a	40.1 ± 1.2 ab	717.4 ± 70.8 a	58.3 ± 4.8 ab	131.0 ± 89.3 a	10.7 ± 1.4 a
A.CH	44.5 ± 1.8 abc	8.7 ± 0.3 a	4.1 ± 0.2 a	42.7 ± 0.8 a	660.0 ± 146.9 a	57.5 ± 4.1 ab	135.3 ± 28.6 a	10.9 ± 1.0 a
A.Untr	42.2 ± 1.0 abc	8.4 ± 0.5 a	4.0 ± 0.1 a	39.0 ± 1.2 ab	932.4 ± 632.6 a	57.4 ± 16.3 ab	322.7 ± 233.7 a	11.1 ± 1.7 a
L.CO	35.3 ± 4.2 bc	7.4 ± 0.3 ab	3.1 ± 0.2 abc	38.8 ± 0.3 ab	491.8 ± 78.1 a	51.0 ± 6.8 b	259.4 ± 288.0 a	8.8 ± 0.8 a
L.CH	37.6 ± 4.3 abc	8.0 ± 1.0 ab	3.2 ± 0.4 abc	38.2 ± 1.6 ab	498.8 ± 162.6 a	52.1 ± 5.0 ab	300.5 ± 273.4 a	8.5 ± 2.1 a
L.Untr	32.7 ± 1.9 c	6.7 ± 0.5 b	3.5 ± 0.3 ab	36.5 ± 2.5 b	773.8 ± 92.8 a	54.8 ± 4.1 ab	237.4 ± 131.3 a	9.0 ± 1.6 a
FYM	36.5 ± 3.6 abc	7.6 ± 0.1 ab	2.5 ± 0.5 c	36.3 ± 1.5 bc	559.2 ± 75.6 a	51.5 ± 1.7 ab	291.7 ± 298.2 a	9.2 ± 1.5 a
N0	44.5 ± 4.2 abc	8.2 ± 0.6 a	3.2 ± 0.2 bc	31.9 ± 2.9 c	645.0 ± 134.2 a	61.2 ± 3.4 ab	226.8 ± 181.6 a	9.3 ± 1.1 a
N50	48.4 ± 10.2 a	8.0 ± 1.2 ab	3.9 ± 0.7 a	32.3 ± 2.1 c	723.5 ± 140.4 a	69.3 ± 6.7 a	276.1 ± 201.0 a	10.8 ± 1.5 a
Prob.	0.0007	0.0097	<0.0001	<0.0001	0.3734	0.0252	0.9174	0.0768

For each growing cycle, means followed by the same letter are not significantly different according to the Tukey HSD test ($\alpha = 0.05$).

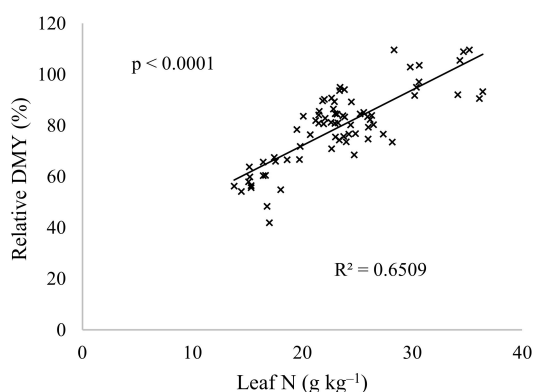


Figure 4. The relationship between the nitrogen (N) concentration in lettuce tissues and the dry matter yield (DMY) expressed relative to the average value of the most productive treatment in each growing cycle.

3.4. Heavy Metals in Plant Tissues

The Cd levels in tissues varied significantly between treatments in both growing cycles, although with little consistency from one growth cycle to another (Table 7). The highest average value was 0.18 mg kg^{-1} , and the lowest was 0.05 mg kg^{-1} . The Cr values did not vary significantly between treatments and generally remained below 10 mg kg^{-1} . Pb values varied extraordinarily between treatments (between 0.03 and 0.81 mg kg^{-1}), but without significant differences. There was, however, a tendency toward lower values in treatments that received Ca. The Ni concentrations in the tissues varied significantly between treatments in the second growing cycle. Considering the values observed in both growing cycles, there seems to be a tendency for low values in the N50 and N0 treatments.

Table 7. Cadmium (Cd), chromium (Cr), lead (Pb), and nickel (Ni) concentrations (average \pm standard deviation) in lettuce tissues from two growing cycles assessed in pots amended with sewage sludge from Amarante (A) or Lousada (L) and treated with 40% calcium oxide (CO) or 40% calcium hydroxide (CH) or left untreated (Untr). Additionally, farmyard manure (FYM), inorganic nitrogen (N50), and an untreated control (N0) were included in the study.

	Cd	1st Cycle Cr	Pb	Ni	Cd	2nd Cycle Cr	Pb	Ni
	mg kg^{-1}							
A.CO	0.10 ± 0.0 ab	9.18 ± 5.1 a	0.20 ± 0.2 a	14.87 ± 3.3 a	0.18 ± 0.1 a	6.47 ± 2.5 a	0.05 ± 0.0 a	9.34 ± 4.2 ab
A.CH	0.10 ± 0.0 a	4.95 ± 0.9 a	0.56 ± 0.5 a	13.51 ± 3.5 a	0.10 ± 0.0 ab	7.28 ± 0.4 a	0.03 ± 0.0 a	17.24 ± 11.9 a
A.Untr	0.08 ± 0.0 ab	5.44 ± 3.0 a	0.75 ± 0.7 a	13.23 ± 9.7 a	0.07 ± 0.0 b	8.72 ± 0.9 a	0.81 ± 1.7 a	14.50 ± 4.2 ab
L.CO	0.09 ± 0.0 ab	9.46 ± 3.4 a	0.03 ± 0.0 a	17.86 ± 5.1 a	0.09 ± 0.0 ab	7.13 ± 1.1 a	0.03 ± 0.0 a	12.41 ± 3.8 ab
L.CH	0.09 ± 0.0 ab	10.32 ± 4.1 a	0.55 ± 0.2 a	19.75 ± 2.3 a	0.08 ± 0.0 ab	5.63 ± 1.9 a	0.02 ± 0.0 a	10.13 ± 0.8 ab
L.Untr	0.08 ± 0.0 ab	9.35 ± 2.4 a	0.33 ± 0.2 a	11.85 ± 3.8 a	0.08 ± 0.0 b	6.26 ± 2.1 a	0.48 ± 0.5 a	10.66 ± 0.9 ab
FYM	0.07 ± 0.0 ab	9.67 ± 0.9 a	0.41 ± 0.3 a	17.83 ± 1.9 a	0.05 ± 0.0 b	5.31 ± 2.4 a	0.03 ± 0.0 a	10.50 ± 1.7 ab
N0	0.05 ± 0.0 b	5.24 ± 4.0 a	0.32 ± 0.3 a	8.38 ± 4.7 a	0.06 ± 0.0 b	4.81 ± 3.6 a	0.24 ± 0.5 a	7.13 ± 2.1 b
N50	0.09 ± 0.0 b	5.13 ± 3.6 a	0.23 ± 0.2 a	9.19 ± 8.2 a	0.12 ± 0.0 b	4.55 ± 3.3 a	0.54 ± 0.5 a	7.64 ± 2.2 ab
Prob.	0.0076	0.0892	0.1966	0.1489	0.0081	0.2401	0.6259	0.0314

Means followed by the same letter are not significantly different according to the Tukey HSD test ($\alpha = 0.05$).

3.5. Soil Properties

Organic C did not vary significantly between treatments (Table 8). The average values ranged between 15.7 and 18.1 g kg^{-1} . pH values varied significantly between treatments. The N0 and N50 treatments showed the lowest average values, followed by the values of sewage sludge that did not receive treatment with calcium oxide or calcium hydroxide. Soil P levels did not vary significantly between treatments, while K levels differed significantly between treatments, but with little coherence regarding the nature of the treatments included in the study. Exchangeable Ca varied significantly between treatments, with low values in the N0 and N50 treatments, FYM, and sewage sludge that was not treated with calcium oxide or calcium hydroxide. Exchangeable Mg levels in the soil did not vary significantly between treatments, while cation exchange capacity (CEC) values followed the trend of Ca, the quantitatively most relevant base in the exchangeable complex. The trend observed in exchangeable K followed that of extractable K. In addition, electrical conductivity, exchangeable Na, and exchangeable acidity did not exhibit significant variation with fertilizer treatments, nor did extractable B. Therefore, this set of variables is not included in Table 8.

Table 8. Organic carbon (C), pH, extractable phosphorus (P) and potassium (K), exchangeable calcium (Ca) and magnesium (Mg), and cation exchange capacity (CEC) in soil (average ± standard deviation) after a second growing cycle of lettuce assessed from pots amended with sewage sludge from Amarante (A) or Lousada (L) and treated with 40% calcium oxide (CO) or 40% calcium hydroxide (CH) or left untreated (Untr). Additionally, farmyard manure (FYM), inorganic nitrogen (N50), and an untreated control (N0) were included in the study.

	Organic C	pH _(H2O)	P	K	Ca	Mg	CEC
	g kg ⁻¹		mg kg ⁻¹ , P ₂ O ₅	mg kg ⁻¹ , K ₂ O		cmol _c kg ⁻¹	
A.CO	16.0 ± 0.4 ^a	7.5 ± 0.1 ^{ab}	123.0 ± 7.8 ^a	177.4 ± 3.5 ^{ab}	15.0 ± 2.0 ^{abc}	4.7 ± 0.7 ^a	20.4 ± 2.8 ^{abc}
A.CH	15.7 ± 0.9 ^a	7.4 ± 0.2 ^{bc}	117.2 ± 14.4 ^a	171.7 ± 6.1 ^b	15.4 ± 0.8 ^{abc}	4.9 ± 0.5 ^a	20.9 ± 1.0 ^{abc}
A.Untr	16.4 ± 1.4 ^a	6.8 ± 0.2 ^{def}	119.4 ± 21.4 ^a	194.4 ± 8.0 ^{ab}	11.3 ± 2.4 ^c	5.0 ± 0.5 ^a	17.0 ± 1.8 ^c
L.CO	17.9 ± 0.4 ^a	7.3 ± 0.1 ^{bc}	122.2 ± 3.6 ^a	196.6 ± 19.1 ^{ab}	17.0 ± 0.4 ^{ab}	5.3 ± 0.1 ^a	23.0 ± 0.4 ^{ab}
L.CH	16.5 ± 1.1 ^a	7.8 ± 0.1 ^a	111.6 ± 19.6 ^a	219.9 ± 13.7 ^{ab}	17.7 ± 2.4 ^a	5.4 ± 0.3 ^a	23.8 ± 2.6 ^a
L.Untr	16.7 ± 0.1 ^a	6.9 ± 0.1 ^{de}	108.6 ± 3.8 ^a	206.8 ± 10.0 ^{ab}	13.6 ± 0.7 ^{abc}	5.5 ± 0.2 ^a	19.9 ± 0.6 ^{abc}
FYM	18.1 ± 1.8 ^a	7.1 ± 0.1 ^{cd}	103.8 ± 9.5 ^a	227.8 ± 22.3 ^a	13.2 ± 0.2 ^{bc}	5.9 ± 0.1 ^a	20.0 ± 0.2 ^{abc}
N0	17.4 ± 1.1 ^a	6.5 ± 0.1 ^f	103.6 ± 5.4 ^a	208.5 ± 31.6 ^{ab}	13.3 ± 0.5 ^{bc}	5.1 ± 0.4 ^a	19.1 ± 0.3 ^{abc}
N50	17.6 ± 1.0 ^a	6.5 ± 0.2 ^{ef}	89.3 ± 8.2 ^a	209.7 ± 25.6 ^{ab}	12.5 ± 2.1 ^c	5.3 ± 0.4 ^a	18.4 ± 2.4 ^{bc}
Prob.	0.1223	<0.0001	0.0501	0.0207	0.0010	0.0720	0.0030

Means followed by the same letter are not significantly different according to the Tukey HSD test ($\alpha = 0.05$).

The Fe levels in the soil varied significantly between treatments, with the highest values recorded in the sewage sludge from Amarante (Table 9). Soil Zn levels also varied significantly between treatments, but the highest values tended to be in the sewage sludge from Lousada. The values of Cu and Mn also varied significantly between treatments, but without a coherent connection to different treatment groups. In the case of heavy metals, the significant differences between treatments observed for Cd, Cr, and Pb did not seem to have a coherent relationship with the origin of the sewage sludge or any of the other treatments. It may be worth noting that the average values of Cd, Cr, Pb, and Ni were below 0.03, 0.09, 1.00, and 2.64 mg kg⁻¹, respectively.

Table 9. Iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), cadmium (Cd), chromium (Cr), lead (Pb), and nickel (Ni) levels in soil (average ± standard deviation) after a second growing cycle of lettuce assessed from pots amended with sewage sludge from Amarante (A) or Lousada (L) and treated with 40% calcium oxide (CO) or 40% calcium hydroxide (CH) or left untreated (Untr). Additionally, farmyard manure (FYM), inorganic nitrogen (N50), and an untreated control (N0) were included in the study.

	Fe	Zn	Cu	Mn	Cd	Cr	Pb	Ni
	mg kg ⁻¹							
A.CO	231.1 ± 24.4 ^a	7.9 ± 0.3 ^{abc}	16.8 ± 0.7 ^{ab}	160.2 ± 3.1 ^{ab}	0.02 ± 0.0 ^b	0.09 ± 0.0 ^a	1.00 ± 0.2 ^a	2.14 ± 0.3 ^a
A.CH	182.1 ± 20.1 ^b	7.8 ± 1.0 ^{abc}	16.1 ± 0.9 ^b	166.4 ± 7.6 ^{ab}	0.02 ± 0.0 ^b	0.06 ± 0.0 ^{abc}	0.84 ± 0.1 ^{ab}	2.64 ± 0.5 ^a
A.Untr	156.4 ± 9.1 ^b	7.7 ± 1.2 ^{abc}	17.1 ± 1.0 ^{ab}	171.6 ± 22.6 ^a	0.02 ± 0.0 ^b	0.06 ± 0.0 ^{bc}	0.87 ± 0.1 ^{ab}	2.62 ± 0.5 ^a
L.CO	106.2 ± 9.6 ^c	9.3 ± 0.4 ^{abc}	18.7 ± 1.5 ^{ab}	139.7 ± 6.9 ^{ab}	0.02 ± 0.0 ^{ab}	0.08 ± 0.0 ^{ab}	0.64 ± 0.0 ^{bc}	2.23 ± 0.2 ^a
L.CH	113.8 ± 6.1 ^c	9.5 ± 1.8 ^{ab}	19.3 ± 1.7 ^{ab}	141.9 ± 6.5 ^{ab}	0.03 ± 0.0 ^{ab}	0.06 ± 0.0 ^{bc}	0.65 ± 0.0 ^{bc}	2.44 ± 0.1 ^a
L.Untr	103.9 ± 2.2 ^c	9.8 ± 0.6 ^a	19.4 ± 2.3 ^{ab}	151.3 ± 10.6 ^{ab}	0.03 ± 0.0 ^a	0.05 ± 0.0 ^c	0.60 ± 0.1 ^{bc}	2.50 ± 0.0 ^a
FYM	107.4 ± 7.4 ^c	6.5 ± 0.7 ^c	20.7 ± 1.5 ^a	161.6 ± 5.9 ^{ab}	0.03 ± 0.0 ^{ab}	0.06 ± 0.0 ^c	0.55 ± 0.1 ^c	2.64 ± 0.6 ^a
N0	92.2 ± 11.9 ^c	6.5 ± 1.0 ^c	19.7 ± 2.9 ^{ab}	137.8 ± 18.7 ^b	0.02 ± 0.0 ^b	0.04 ± 0.0 ^c	0.72 ± 0.2 ^{abc}	2.26 ± 0.1 ^a
N50	96.2 ± 4.6 ^c	6.9 ± 1.2 ^{bc}	17.1 ± 0.7 ^{ab}	145.4 ± 6.7 ^{ab}	0.02 ± 0.0 ^{ab}	0.04 ± 0.0 ^c	0.59 ± 0.1 ^{bc}	2.21 ± 0.1 ^a
Prob.	<0.0001	0.0029	0.0186	0.0137	0.0040	<0.0001	0.0003	0.4016

Means followed by the same letter are not significantly different according to the Tukey HSD test ($\alpha = 0.05$).

4. Discussion

4.1. Lettuce Dry Matter Yield, Photosynthesis, and Nitrogen Use Efficiency

The lettuce DMY responded to the fertilizer treatments in well-defined groups, showing a tendency to be lower in the N0 treatment, followed by the FYM treatment. The sewage sludge treatments followed, with a slight trend indicating that untreated sewage sludge displayed lower values. Notably, the N50 treatment resulted in the highest average lettuce

DMY. This response pattern held true for both sewage sludge types used in the study. The N concentration in the tissues and N uptake brought the FYM and N0 treatments closer, emphasizing the superiority of the N50 treatment compared to the sewage sludge treatments. It is worth noting that, overall, N concentrations in the tissues were below the sufficiency range established for the crop [39], indicating that N was a significant limiting factor for plant growth and likely the primary determinant of the plant's response to the treatments, as demonstrated by the photosynthesis in FYM and N0 plants, due to both stomatal and mesophyll limitations, key traits for the determination of DMY. A similar influence of N status on these morphological and physiological variables was reported in other studies [40]. Unsurprisingly, the N concentration in the tissues was linearly correlated with the DMY. The sewage sludges exhibited very high N concentrations and very low C/N ratios, conditions that have usually led to net N mineralization following organic substrate degradation by soil microorganisms [14,41,42]. In contrast, FYM had a lower N concentration and a higher C/N ratio, which, combined with the short lettuce growth cycle, likely resulted in low N release, contributing less to the N nutrition of the plants.

The ANR further supports the earlier hypothesis. The N50 treatment resulted in high ANR values (81.3% and 75.3% in the first and second growing cycles, respectively), surpassing values typically observed in the field, often below 50% [42,43]. This outcome is partially attributed to the cultivation in pots, where N losses from the system are low, at least by nitrate leaching. The sewage sludge exhibited a high N release, with ANR values much higher than those often recorded with organic amendments [44,45]. Conversely, FYM showed very low ANR, with only 6.6% and 10.5% in the first and second cycles, respectively. This result was influenced by the higher C/N ratio and the very short lettuce growth cycle, allowing little time for the net mineralization of organic N.

It is important to note that N content and the C/N ratio are the main predictors of the net result of the mineralization and immobilization process, but other variables also play a role, such as lignin, cellulose, and hemicellulose, which contain less available energy for soil heterotrophic microorganisms [41,42]. Considering their origins, sewage sludge is likely to have lower quantities of these compounds compared to FYM, which is probably one of the factors that makes sewage sludge so reactive, capable of releasing a high amount of N in such a short period. The application of calcium oxide and calcium hydroxide may have contributed to an even faster degradation of sewage sludge. Alkalinizing materials decrease the structural stability of organic matter, and the rise in pH increases its solubility, followed by increased decomposition of organic matter [46,47].

4.2. Macronutrients in Lettuce Tissues and Soil

The other analyzed macronutrients likely had less of an influence on lettuce performance. Although the concentrations of some macronutrients in the tissues differed between treatments, their values were poorly related to the lettuce DMY. For instance, the concentration of P in lettuce tissues did not vary significantly between treatments in either the first or second growing cycle. The P uptake appeared to be more related to the DMY than to the concentration of the nutrient in the tissues. It seems that the P contained in the fertilizers was less relevant compared to the P made available by the soil, which is consistent with findings from previous studies using similar soils, where a poor response of crops to applied P as a fertilizer was reported [48,49].

The K concentration in plant tissues varied significantly between treatments in both growing cycles of lettuce, with a tendency for higher values in the N50 treatment, despite the lack of K application in this treatment. The application of ammonium nitrate in the N50 treatment, which, due to nitrification, may have been taken up in nitrate form, could have favored the absorption of cations, leading to an increase in K in the tissues. Although a direct influence on the absorption of cations by anions is not common, as they are mediated by different transport proteins, the absorption of cations can indirectly benefit from the absorption of anions and vice versa. This is attributed to the necessity to maintain charge balance in plant tissue [50]. A similar, though less pronounced, tendency also appears to

be observed for the concentration of Mg in the tissues. Relatively high K values in the N0 treatment may be explained by concentration/dilution phenomena, which occur when there is a variation in the amount of DMY for an equivalent amount of a nutrient available in the soil [51,52].

The Ca levels in plant tissues also varied significantly between treatments in both growing cycles of lettuce. For each of the WWTPs, there was a trend toward higher values in treatments where sewage sludge was treated with calcium oxide and calcium hydroxide. These values aligned with the soil exchange Ca values and pH, suggesting a direct response to the Ca content in the treated sewage sludge. However, the Ca levels in plant tissues were not significantly related to the lettuce DMY.

4.3. Micronutrients in Lettuce Tissues and Soil

The B levels in tissues also varied significantly between treatments in both growth cycles. The sewage sludge from Amarante, with higher initial B levels, tended to have higher values in lettuce tissues, especially in comparison with the N0 and N50 treatments. The availability of B in the soil depends greatly on the quantity and type of clay and the organic matter content, particularly in alkaline soils, where B becomes adsorbed to both inorganic and organic colloids [14,42,53]. Thus, the intense mineralization of the organic substrate likely increased the availability of B from the sewage sludge, which had a higher initial concentration of B.

The Fe levels in plant tissues varied significantly between treatments in the first growing cycle but not in the second, and no relationship was found between Fe levels and the organic amendments used in the study. It appears that the initial concentration of Fe in the sewage sludge influenced soil Fe levels but had little impact on the nutrient uptake by plants. Several environmental variables, including pH and aeration, significantly influence the bioavailability of Fe and other metallic cations [14,54]. With an increase in pH, the ionic forms available to plants transform into hydroxide ions and subsequently into insoluble oxides, becoming less available to plants and potentially causing Fe chlorosis in alkaline pH soils [14,55]. However, in this study, the Fe levels in the tissues were high compared to the sufficiency range established for the crop by Bryson et al. [39], suggesting that pH may not have been the primary driver conditioning Fe availability to plants. On the other hand, reduction conditions increase the availability of metallic cations such as Fe due to the dissolution of Fe oxides [14,40,54]. Irrigation may have created conditions for high Fe availability. Although irrigation was carefully monitored, it is likely that cycles of wetting and drying created temporary reduction conditions that allowed for high Fe uptake by plants, possibly overriding the effects of the treatments.

The concentration of Mn in plant tissues varied significantly between treatments in both growing cycles. There seemed to be a subtle tendency for the N50 and N0 treatments to have higher average values, and they were the treatments showing lower pH values. The impact of soil pH on Mn concentration in plant tissues is generally pronounced at low pH values [48,55]. However, its influence on the results in this study was modest and likely not comparable to that of reduction conditions.

4.4. Heavy Metals in Lettuce Tissues, Soil, and Sewage Sludge

The levels of Cd in tissues varied significantly between treatments in both growing cycles, although with little consistency from one growth cycle to another, and the values were far below the safety threshold of the Codex Alimentarius established for leafy vegetables [56]. On the other hand, the initial values of Cd in the sewage sludge were very low, even lower than those in FYM, when compared to those specified in European legislation [27]. The Pb levels in plant tissue did not vary significantly between treatments. Like the Cd values, they were below the established threshold in the Codex Alimentarius for leafy vegetables [56]. For both elements, the values were not higher than those in the N50 and N0 treatments. The concentrations of these metals in the final soil samples were also well below the safety limits established in European legislation [27].

For Ni, safety limits for food are not established. Ni is currently considered an essential nutrient for higher plants [40]. Ni concentrations in tissues varied significantly between treatments in the second growing cycle. Considering the values observed in both growing cycles, there seems to be a tendency for lower values in the N50 and N0 treatments. However, the initial concentrations of Ni in the sewage sludge were even lower than in FYM and fell below the threshold values established in European legislation [27]. For Cr, safety limits for sewage sludge, soils, or plant tissues are not established. In this study, the values showed no significant variation between treatments and, in general, remained below 10 mg kg^{-1} .

5. Conclusions

Sewage sludge produced a higher DMY than conventional FYM, although less than the treatment with inorganic N (N50). The DMY from FYM was slightly higher than that from the unfertilized control (N0). The N and nitrate concentrations in tissues, N uptake, and ANR indicate that the N made available by the treatments was the major factor in determining lettuce productivity. ANR was considerably high for sewage sludge and quite modest for FYM. The high N concentration and low C/N ratio of sewage sludge likely led to extensive substrate mineralization, releasing a large portion of its N to the crop. In contrast, FYM released little N during the short lettuce growing cycle. Regarding other macronutrients, it is worth noting the P concentration in tissues, which was influenced by the rapid mineralization of sewage sludge, and the Ca concentration, which was related to soil pH and exchangeable Ca and increased in sewage sludge treated with calcium oxide and calcium hydroxide. The sewage sludge used in this study initially had low concentrations of heavy metals compared to the levels in international legislation. Heavy metal levels in the soil and plant tissues were not higher than those in the other treatments (FYM, N50, and N0). What stands out in this study is the high agronomic value of sewage sludge, which undergoes rapid mineralization, showing intermediate behavior between mineral fertilizers and traditional organic amendments. On the other hand, sewage sludge treated with calcium oxide or calcium hydroxide significantly increases the soil pH and should preferably be applied to acidic soils. Although lettuce was chosen for its suitability as a bioindicator plant to assess the availability of N and heavy metals, because of the risk of microbial contamination, it is recommended that sewage sludge be applied to taller crops, preferably ones not consumed as fresh vegetables.

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