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The potential of wastewater grown microalgae for agricultural purposes: Contaminants of emerging concern, heavy metals and pathogens assessment☆

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ABSTRACT

In the coming years, the use of microalgal biomass as agricultural biofertilizers has shown promising results. The use of wastewater as culture medium has resulted in the reduction of production costs, making microalgae-based fertilizers highly attractive for farmers. However, the occurrence of specific pollutants in wastewater, like pathogens, heavy metals and contaminants of emerging concern (CECs), such as pharmaceuticals and personal care products may pose a risk on human health. This study presents an holistic assessment of the production and use of microalgal biomass grown in municipal wastewater as biofertilizer in agriculture. Results showed that pathogens and heavy metals concentrations in the microalgal biomass were below the threshold established by the European regulation for fertilizing products, except for cadmium. Regarding CECs, 25 out of 29 compounds were found in wastewater. However, only three of them (hydrocinnamic acid, caffeine, and bisphenol A) were found in the microalgae biomass used as biofertilizer. Agronomic tests were performed for lettuce growth in greenhouse. Four treatments were studied, comparing the use of microalgae biofertilizer with a conventional mineral fertilizer, and also a combination of both of them. Results suggested that microalgae can help reducing the mineral nitrogen dose, since similar fresh shoot weights were obtained in the plants grown with the different assessed fertilizers. Lettuce samples revealed the presence of cadmium and CECs in all the treatments including both negative and positive controls, which suggests that their presence was not linked to the microalgae biomass. On the whole, this study revealed that wastewater grown microalgae can be used for agricultural purposes reducing mineral N need and guaranteeing health safety of the crops.

1. Introduction

In the circular bioeconomy approach, wastes are considered as a source of resources such as nutrients. For instance, it is estimated that 30% of phosphates imported into the EU could be recovered from sewage sludge, biodegradable wastes, meat and bone meal, or manure (EC, 2016). Microalgae-based wastewater treatment systems have been studied during the last decades as an alternative to conventional municipal wastewater treatment systems (Passos et al., 2017). These photosynthetic microorganisms perform wastewater treatment with high removal efficiencies and low costs in comparison with other treatments such as activated sludge. In addition, the microalgal biomass

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grown during the treatment process can be used for the production of bioproducts such as pigments, bioplastics, biofuel, or biogas (Arashiro et al., 2019; Arias et al., 2018).

Several studies have been conducted on biofertilizers, ranging from vegetal species to microalgae grown in numerous types of wastewater. Mulbry et al. (2005) studied the N release from microalgae grown in anaerobically digested dairy manure. They found that after 21 days, only 33% of total N was converted into plant available N, making microalgae a slow-release fertilizer. Coppens et al. (2016) used microalgae grown in aquaculture wastewater as a biofertilizer in tomato crops. They obtained comparable plant growth and even higher sugar and carotenoid content in the tomatoes fertilized with microalgae compared to those fertilized with a commercial organic fertilizer. Sharma et al. (2021) showed that *Chlorella* biomass obtained during the treatment of municipal wastewater provided similar yield (with spinach and baby corn) than a mineral fertilizer. Renuka et al. (2017) observed that both soil micronutrient availability and uptake in wheat crops were increased when microalgal biomass grown in wastewater was used as biofertilizer. Álvarez-González et al. (2022) studied the use of wastewater grown microalgae as biofertilizer in basil plants. They concluded that the combination of both mineral fertilizer and microalgal biomass was the best solution to reduce the use of fertilizers.

Although all these studies show the biofertilizer potential of microalgae grown in waste streams, more efforts should be focused to ensure crops quality and end users health safety. Wastewater is a source of nutrients, but it also contains contaminants, including pathogens, heavy metals and organic pollutants, among others. Traditionally, the use of sewage sludge in agriculture was restricted because of the presence of those contaminants. Markou et al. (2018) reviewed several studies about the capacity of microalgae to remove heavy metals from wastewater by different uptake pathways, such as cell surface sorption or intracellular accumulation. Indeed, Singh and Singh (2022) reported quite high efficiencies of heavy metals removals (50–94%) from municipal wastewater employing microalgae. Bacteria, viruses, and fungi are pathogens present in wastewater that can be transferred into the microalgal biomass (Markou et al., 2018). Indeed, microalgae are reported to reduce the abundance of pathogens in wastewater due to photosynthetic activity (Molinuevo-Salces et al., 2019). Another issue associated with wastewater is the presence contaminants of emerging concern (CECs), such as pharmaceuticals or self-care products. In this regard, microalgae-based treatments have been proven as an efficient technology to remove those contaminants from wastewater (García-Galán et al., 2021; Matamoros et al., 2016). Even if all those studies demonstrated that microalgae can remove pathogens, heavy metals, and CECs, there is still a lack of knowledge regarding the accumulation of contaminants into the biomass, which might compromise its safe use as biofertilizer. For this reason, this study proposes an exhaustive evaluation of wastewater, microalgae biomass and crops in order to track specific contaminants and verify the compliance of the European regulation about fertilizers. To the authors' knowledge, this is the first study assessing the presence of pathogens, heavy metals, and CEC in crops using microalgal biomass as biofertilizer.

2. Material and methods

2.1. Microalgal biomass characterization

Microalgal biomass (composed mainly by *Scenedemus* sp.) was obtained from a pilot wastewater treatment system, which is described in detail in (Álvarez-González et al., 2022). Briefly, municipal wastewater is collected from a sewer and pumped into a homogenization tank. Then, it is continuously fed to a primary settling tank for primary treatment, and subsequently to two High Rate Algal Ponds (HRAP) working in parallel for secondary treatment. Thereafter, the treated water is clarified and the microalgal biomass is harvested by gravity in a secondary settling tank and then thickened in Imhoff cones. Finally, biomass is centrifuged and freezed (−20 °C) until it is used. Before starting the agronomic experiment, all the biomass stored in the freezer was thawed and thoroughly mixed. After that, samples were taken for the determination of CECs, pathogens, nutrients and heavy metals. The rest was used for the agronomic trial.

Before employing the biomass as a biofertilizer, it was thawed and characterized in terms of macro- (nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), sulphur (S)) and micronutrients (iron (Fe), sodium (Na)) composition. These elements were measured as follows: N content was measured with N Kjeldahl (AOAC, 2005); K content was measured with flame spectrophotometer Corning 410C (Halstead, Essex, England), according to (AOAC, 2005); P was measured with spectrophotometer Agilent Cary 60 (Mulgrave, Victoria, Australia), according to Bray and Kurtz (1945); Ca, Mg and Fe were measured with atomic absorption Varian SpectrAA-110 (Mulgrave, Victoria, Australia), according to (AOAC, 2005); Na was measured with flame spectrophotometer Corning 410C (Halstead, Essex, England), according to (AOAC, 2005); S was measured with spectrophotometer Agilent Cary 60 (Mulgrave, Victoria, Australia), according to (AOAC, 2005). The organic matter was calculated as the difference between the dry biomass and the content of ashes. The ash content was measured according to AOC, 2005. Then, the organic carbon was calculated according to European regulation (EC, 2019/1009): organic carbon (C_{org}) = organic matter × 0.56.

The following pathogens were analysed: *Salmonella*, *Escherichia coli* (according to European Regulation EC, 2019), *Legionella* spp. and *Legionella pneumophila*. The measurements were performed following ISO 16140; ISO 16649-2; ISO 11731:2017 and OXOID Legionella Latex Test.

The content of heavy metals was also analysed. The following elements were measured in samples of the microalgal biomass: cadmium (Cd), cupper (Cu), chromium (Cr), hexavalent chromium (Cr(VI)), mercury (Hg), nickel (Ni), lead (Pb), zinc (Zn), aluminium (Al), arsenic (As). All the elements were measured with ICP-OES, except for chromium (VI), which was measured with UV-VIS.

2.1.1. Agronomic assay

The agronomic assay was performed in a greenhouse facility located in Castelldefels (Barcelona, Spain), using Lettuce (*Lactuca sativa* L. cv Maravilla). The greenhouse was equipped with an automated ventilation. During the experimental period (November–January), air temperature and relative humidity were measured (results shown in Supplementary material, Figure S1). Temperature and relative humidity were recorded every 30 min using a TESTO 174H data logger (testo SE & KGaA, Lenzkirch, Germany) placed both 1 m above and aside of the cultivation table within the greenhouse

In order to test if microalgae biomass could, at least partially, substitute inorganic N, four different treatments were tested:

(C-) nega

lgal biomass + inorganic PK (same dose as C+).

Fertilization rates were calculated by considering plant's extractions and an estimated final fresh weight of 250 g plant^{-1} which resulted in a total amount of 1 g of N plant^{-1}, 0.23 g P_2O_5 plant^{-1} and 1.84 g K_2O plant^{-1}. Mineral N supply was fractionated in two applications (i.e., at the beginning of the experiment and six weeks after crop planting) to avoid N losses in treatment C+ (Wu et al., 2019). In contrast, only half dose of N was applied six weeks after crop planting in treatment M1, in order to evaluate the potential of microalgae biomass to provide N. Microalgae biomass was expected to provide N at a slower rate than de mineral fertilizer, which might match the crop's nutrient needs, avoiding N loss (Rupawalla et al., 2021). Mineral N was provided as ammonium nitrate. Phosphorus and potassium were provided at the beginning of the experiment to all treatments as calcium superphosphate and potassium chloride, except in treatment C-, which did not receive any fertilizer.

In treatments M1 and M2, 16 g of microalgal biomass per pot were added in a single dose at the beginning of the experiment. The microalgal biomass was obtained after centrifugation, without drying it, with a solids content of 10.74%. According to the biomass characterization, this biomass incorporates 0.11 g of N plant^{-1} and 0.03 of P plant^{-1}. Therefore, the N doses of the treatments were: for C+ 100% of mineral N, for M1 50% of mineral N and 11% of microalgal N, for M2 11% of mineral N and for C- 0% of N. M1 presented 50% of mineral nitrogen + wastewater grown microalgae biomass. In the previous study Álvarez-González et al. (2022), the N content was hypothesized to be similar in all treatments (mineral fertilizer, microalgae fertilizer and a combination of both). Results showed that the combination of both types of fertilizer was the best alternative to reduce the use of mineral fertilizer. Considering the previous results, this study evaluates if the amount of applied biomass could be enough for replacing the mineral nitrogen, providing comparable growth results. With this strategy, the use of the mineral fertilizer can be reduced, which supposes a decrease of the cost and the contamination associated to nitrogen production. The experiment was set out in a completely randomized block design with four replicates. Each treatment had 4 replicates with a total of 30 pots per treatment (Fig. 1).

The substrate used in this experiment was a mixture of soil, sand, and nutrients. The soil was collected from the Parque UPC – Agrópolis, located in Barcelona, Spain (41°17′18″N and 2°02′42″E), their physicochemical properties are shown in Supplementary material (Table S3). Soil was mixed with sand (1:0.6; v/v); providing a homogenized nutrient content in the whole growth substrate. Nutrients (both inorganic and microalgal biomass) were added to the pots at 30 mm depth below the substrate surface, and then covered with the rest of soil. After 6 weeks, one lettuce seedling was transplanted to each individual 2L-pot. Automatic drippers were used for daily irrigation on each pot. The minimum amount of water was provided (the same for each treatment) to prevent nutrient losses through leaching (1 min/48h for the first month and 1 min/24h for the rest of experiment). The amount of irrigation water was the same for all the treatments studied and was set to achieve a minimum drain in order to prevent nutrient losses through leaching. The irrigation dose was increased when signs of plant wilting were observed.

Chlorophyll Content Index (CCI, relative units) was measured using a Chlorophyll Content Meter SPAD: Chlorophyll meter SPAD 502PLUS (Konica Minolta Inc.). Measurements were taken on the upper-most expanded mature leaf. The leaf chlorophyll content index was measured five times along the experiment. Nine weeks after crop planting, aboveground biomass was harvested and leaf fresh weight per plant were determined. Subsequently, plants were oven-dried at 80 °C for 48 h and then weighed to determine leaf dry weight per plant. Macro- and micronutrients from leaf samples were measured as previously described in section 2.1; prior to the measurements, the dried leaves were pooled for each replicate and were finely ground to a powder. Finally, heavy metals and CECs were also analysed in lettuce samples. For this, four lettuce replicates (combining three lettuce samples in each replicate) from each treatment were obtained in the end of the experiment and they were lyophilized.

3. Contaminants of emerging concern analysis

The occurrence of CECs was analysed in the wastewater used to produce microalgal biomass, in the microalgal biomass and in the lettuces. For wastewater, two samples were collected from the supernatant in the primary settling tank during the period and filtered through a 0.7 μm pore GF/F filter (Whatman). For the analysis, water samples (100 mL) were adjusted to pH 2–3 (with 37% HCl) and loaded in preconditioned Solid Phase Extraction (SPE) cartridges (6 mL of methanol and 6 mL of ultrapure water at pH 2–3). For microalgal biomass, two samples were collected at the beginning of the experiment from the centrifuged biomass after the harvested step. For the analysis of both, microalgal biomass or lettuce, 10 mg of lyophilized material were spiked with 25 μL of a 400 μg/L solution containing four isotopically labeled surrogates (caffeine, ibuprofen and atrazine).

Then, samples were extracted with 10 mL of methanol (15 min of in ultrasonic bath). Afterwards, samples were centrifuged for 15 min (4000 rpm) and the supernatant was recovered. This process was repeated twice and the supernatants were mixed. Finally, supernatants were evaporated to approximately 1 mL and reconstituted in 20 mL of

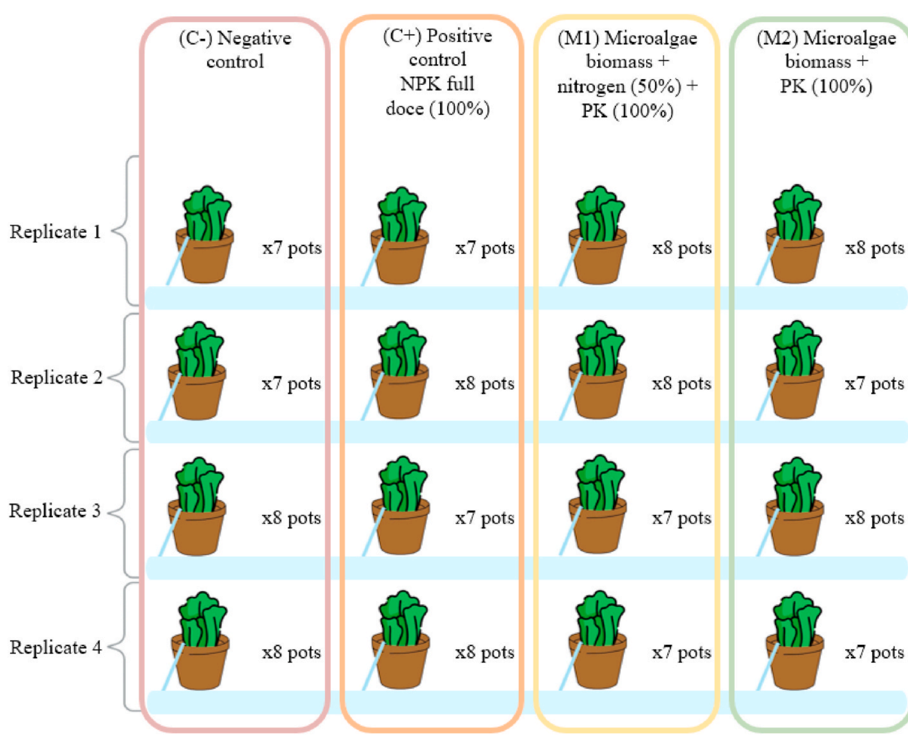

Fig. 1. Graphical scheme of the setup of the agronomic tests, consisting of four treatments: (C-) negative control without fertilizer; (C+) Positive control with inorganic fertilizer, NPK full dose (100%); (M1) microalgae biomass + inorganic nitrogen (50%) + PK (100%); (M2) microalgae biomass + PK (100%).

ultrapure water, which was loaded through pre-conditioned SPE cartridges (6 mL of each, ethyl acetate, methanol and ultrapure water at pH 2–3).

After being loaded, all SPE cartridges (water samples, biomass, and pigment-rich extracts) were cleaned with 1 mL of methanol:water (5:95) and dried. Then, cartridges were eluted with 10 mL of ethyl acetate and evaporated to 100 μL while transferring the sample to a GC vial. Then, triphenylamine was added as internal standard and a sample aliquot of 50 μL was transferred to a GC-vial with insert. Finally, 10 μL of TMSH (Trimethylsulfonium hydroxide) were added to the sample and 2 μL of sample were injected on a GC-Orbitrap (see instrument specifications on Table S1). Limit of Detection (LOD) and quantification ranged from 0.001 to 0.31 μg L-1 or from 0.030 to 3.281 $\mu g/g_{DryWeight}$ for water and biomass extraction samples respectively (Table S2).

3.1. Leachate assay

Once the plants are harvested, nutrient leaching due to an over-fertilization is one of the major causes of nitrate pollution in surface and groundwaters. In order to evaluate the extent of nutrient leaching of each treatment in this study, a leaching monitoring test was performed, adapted from (Sogn et al., 2018). For this purpose, after the lettuce was harvested, 250 mL of tap water was poured into the pots every 15 min during 1 h (1L of tap water in total). The experiment was carried out in 4 pots per each treatment. The leachate was recovered in 1 L beaker placed under each pot. A composite sample (1:1; v:v) consisting in the leachate from two pots per each treatment was analysed. Nutrients concentration was measured in the resulting composite samples: nitrate (NO_3^--N) and phosphate (PO_4^{3-}P) through isocratic mode with carbonate-based eluents at a temperature of 30 °C and a flow of 1 mL/min (ICS-1000, Dionex Corporation, USA) (limits of detection (LOD) were 0.9 mg/L of NO_2^--N, 1.12 of NO_3^--N, and 0.8 mg/L of PO_4^{3-}P). Ammonium-N (NH_4^+-N) was measured according to Solórzano method (Solórzano, 1969).

3.2. Statistical analysis

Statistical analysis of the agronomic assay data was performed with R software, version 4.1.0. The one-way analysis of variance (ANOVA) and Tukey's post hoc test ($\alpha = 0.05$) were used to study differences among treatments. The graphs represent the mean value of the different parameters and the Standard Error of the Mean (SEM) is indicated with the bars.

4. Results and discussion

4.1. Characterization of the microalgal biomass

The chemical characterization of the microalgal biomass after harvesting from the wastewater treatment system is shown in the Supplementary material (Table S4). Nitrogen was the nutrient with a highest concentration (6.69 $\%_{DM}$), as expected, and the NPK ratio (1:0.25:0.05) was similar to the one obtained in a previous study conducted in the same pilot plant (Álvarez-González et al., 2022). After N, Ca was the second most abundant nutrient (5.99 $\%_{DM}$). The microalgal biomass also contained micronutrients, i.e., S (0.86 $\%_{DM}$), Fe (0.46 $\%_{DM}$) and Mg (0.42 $\%_{DM}$). These results are in line with the composition measured in compost from different primary feedstocks, such as leaf and yard trimmings, food waste, manure or biosolids which presented a concentration of N, P, K, Ca, Mg and S in the range of 1.5–2.2; 0.2–0.95; 0.37–1.00; 1.77–2.98; 0.35–0.68; 0.18–0.48%, respectively (Rynk, 2022).

An evaluation of the pathogens and heavy metals content was performed to assess the fulfilment of the European regulation (EC, 2019/1009) about fertilizing products. Although the regulation does not include wastewater grown microalgae as a fertilizer, our results suggest that they could be considered as fertilizers. According to the carbon content of the microalgal biomass produced and used in this study (4.5% in total weight basis), it would fit within the category of organo-mineral fertilizers (more than 3% of carbon content, according to the European regulation). Moreover, a previous study Álvarez-González et al. (2022) demonstrated that the best growth results were obtained mixing microalgae biomass and mineral N, which is a combination of organic and mineral fertilizers.

Legionella ss and *Salmonella* spp. were not detected, while 400 CFU/g were reported for *Escherichia coli*, value below the threshold (1000 CFU/g). In general, heavy metals contents in microalgal biomass were below the European regulation limits (Table 1). Only Cadmium slightly exceed the regulation (3.10 mg/kg_{DM} when the limit for organo-mineral fertilizers is 3 mg/kg_{DM}).

Microalgae have the capacity of bioremediate wastewater with the presence of heavy metals by different mechanisms (Leong and Chang, 2020), allowing microalgae to accumulate these elements intracellularly. Markou et al. (2018) summarized the uptake of heavy metals in different microalgae species, reporting values of 13.5–44.5 mg/g_{DW}, 226–333 mg/g_{DW}, 9.2–15.1 mg/g_{DW} for cadmium, chromium and mercury, respectively, manifesting the capacity of microalgae to accumulate the abovementioned heavy metals. However, the authors highlighted the need for studies evaluating the accumulation of heavy metals in microalgal biomass using real wastewater. Considering the above results, using wastewater effluents with low heavy metals concentration (as municipal wastewater) seems the best approach to produce microalgal biofertilizer.

5. Effects of microalgae based biofertilizer in lettuce crops

5.1. Plant growth

A comparison between the lettuce shoot fresh and dry weight obtained in the different treatments is presented in Fig. 2. As expected, all fertilized treatments presented higher lettuce shoot fresh and dry weight than the negative control (without fertilizer). The highest shoot dry weight (Fig. 2A) was obtained in C+, which recorded 7.17 g/plant, which is 18.4% and 36.8% higher than M1 and M2, respectively. Conversely, C+ and M1 showed no significant difference ($p < 0.05$) in the shoot fresh weight (95.41 g/plant and 93.45 g/plant, respectively) (Fig. 2B). On the other hand, M1 provided a shoot fresh weight 53.2% higher than the treatment without addition of inorganic nitrogen (M2). These results suggest that the combination of inorganic fertilizer and microalgal biomass proposed in M1 obtained a successful result in spite of the lower amount of inorganic N provided.

Álvarez- González et al. (2022) compared inorganic fertilizers with a mixture of inorganic fertilizer and microalgae biomass to grow Basil crop. They obtained the best growth results when microalgal biomass was mixed with inorganic fertilizer. However, in that study, comparable plants growth were obtained when the amount of N from microalgae

Table 1
Comparation of heavy metals content between wastewater grown microalgal biomass and the European regulation limits (EC, 2019/1009) for mineral-organic fertilizers. The microalgal biomass is classified in mineral-organic fertilizer due to organic carbon content.

Heavy metal	Microalgal biomass	European regulation limits
Cadmiun (mg/kgDM)	3.10	3
Hexavalent chromium (mg/kg_{DM})	1.31	2
Mercury (mg/kg_{DM})	0.52	1
Nickel (mg/kg_{DM})	<46.5	50
Lead (mg/kg_{DM})	<46.5	120
Arsenic (mg/kg_{DM})	<18.6	40
Copper (mg/kg_{DM})	279	600
Zinc (mg/kg_{DM})	437	1500

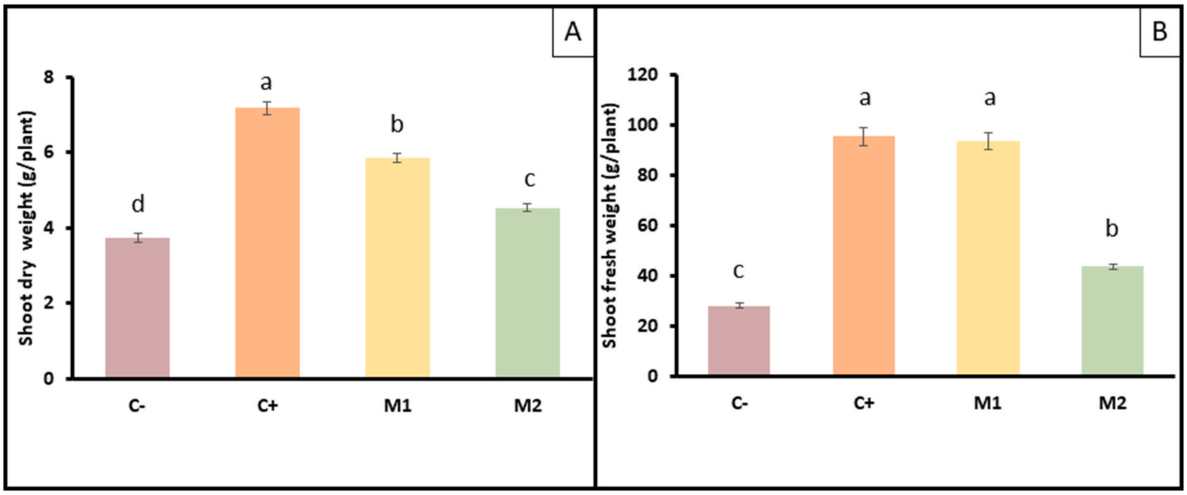

Fig. 2. Lettuce shoot dry and fresh weight for different treatments: C- (negative control), C+ (positive control), M1 (microalgae biomass with N supplement), M2 (microalgae biomass). A) Shoot dry weight (g/plant); B Shoot fresh weight (g/plant). The error bars represent the Standard Error of the Mean. Different letters indicate significant difference ($p < 0.05$).

was 2 times higher than the N provided from inorganic fertilizer, which is the opposite from this study. Rupawalla et al. (2021) also obtained similar growth yields when evaluating inorganic fertilizer with microalgae fertilizer, which needed to provided twice N than the synthetic one to obtain comparable growths. In fact, microalgae biomass provides N in organic form, that requires much time to be plant-available than mineral fertilizers. For this reason, microalgae biomass can be considered as a slow-release fertilizer preventing nutrients losses and groundwater pollution from leachate.

In the present study, in M2, a small dose of microalgae biomass (11% of N) in combination with inorganic P and K have enhanced plant growth in contrast to the negative control. On the other hand, in M1, the microalgal biomass combined with a half dose of inorganic N than the positive control, provided similar fresh weight (comparing M1 and C+). This result highlights that a small dose of microalgae would be sufficient to replace 50% of the inorganic N.

Microalgae are a natural source of biostimulant substances that can enhance nutrient uptake by altering the rhizosphere and plant metabolism (Kapoore et al., 2021). C+ despite having almost twice N dose than M1, reported comparable fresh weight. Garcia-Gonzalez and Sommerfeld (2016) observed a correlation between the number of lateral roots and the microalgae concentration, which may improve the nutrient uptake. Barone et al. (2018) showed how microalgae extracts modified the expression of genes related to primary and secondary plant metabolism, described to be involved in nutrient plant uptake.

All fertilizers treatments reported higher chlorophyll content than the negative control (Fig. 3). C+ presented the highest values along the whole experiment. Furthermore, all fertilizer treatments presented similar values and behaviours until inorganic N was supplemented to M1 and C+. After N supplementation, the chlorophyll content in C+ rapidly increased, while in M1 the chlorophyll content took longer to increase.

Chlorophyll synthesis is an indicator of foliar nitrogen content. As it can be seen in Fig. 3, the chlorophyll content increases as the dose of N in the treatments increases (C- < M2 < M1 < C+). On the other hand, microalgae are a source of biostimulants that can enhance the chlorophyll content by stimulating its biosynthesis and preventing its degradation (Kapoore et al., 2021). Therefore, it could have been expected that M1 and M2, despite having less N content, could have a chlorophyll content comparable to C+. More research is needed to clarify the role of microalgae fertilizer on chlorophyll synthesis and its upsides.

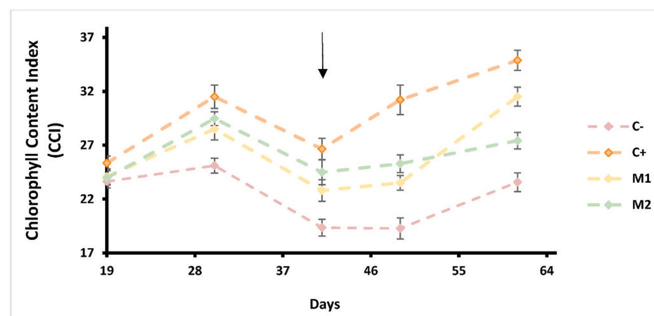

Fig. 3. Chlorophyll leaf content evolution during the whole experiment. Chlorophyll is measured as Chlorophyll Content Index (CCI) in each treatment: C- (negative control), C+ (positive control), M1 (microalgae biomass with N supplement), M2 (microalgae biomass). The error bars represent the Standard Error of the Mean. Black arrow indicates the day when inorganic nitrogen (ammonium nitrate) was added in both, C+ and M1.

5.1.1. Leaf nutrient content

The nutritional status analysis (Fig. 4) revealed that M1 presented similar nutrient content to C+. Moreover, P and S were significantly higher ($p < 0.05$) in M1 than in C+, with an increment of 37% and 20%, respectively. On the contrary, M2 did not show values significantly higher than C- for most of the tested nutrients; significantly higher concentrations were found for P and K (30% and 34%, respetively), althought these elements were provided with mineral fertilizer. Only S presented significantly higher content, with an increment of 25%. In comparison to C+, only P, Mg and Fe were similar in M2. A small dose of microalgae in combination with inorganic fertilizer, therefore, helps to increase the accumulation of P and S in leaves and allowed similar N accumulation as the positive control while reducing the need for inorganic N by 50%. These results suggest that microalgae might improve nutrient availability and, thus, improve nutrient uptake and stimulate plant growth.

The microalgae effect on foliar nutrient balance is still unclear. Plaza et al. (2018) observed that *Scenedesmus* extract increased the foliar content of some nutrients, such as P, K, Ca and Mg. Conversely, Dias et al. (2016) found that a low concentration of microalgae biofertilizer enhanced fruit yield, but did not modify nutrient content in leaves. Gemin et al. (2019) reported that microalgae extract improved onion bulb caliber and yield, but it was not related to modifications in nutrient content. The variability of results might be influenced by the different

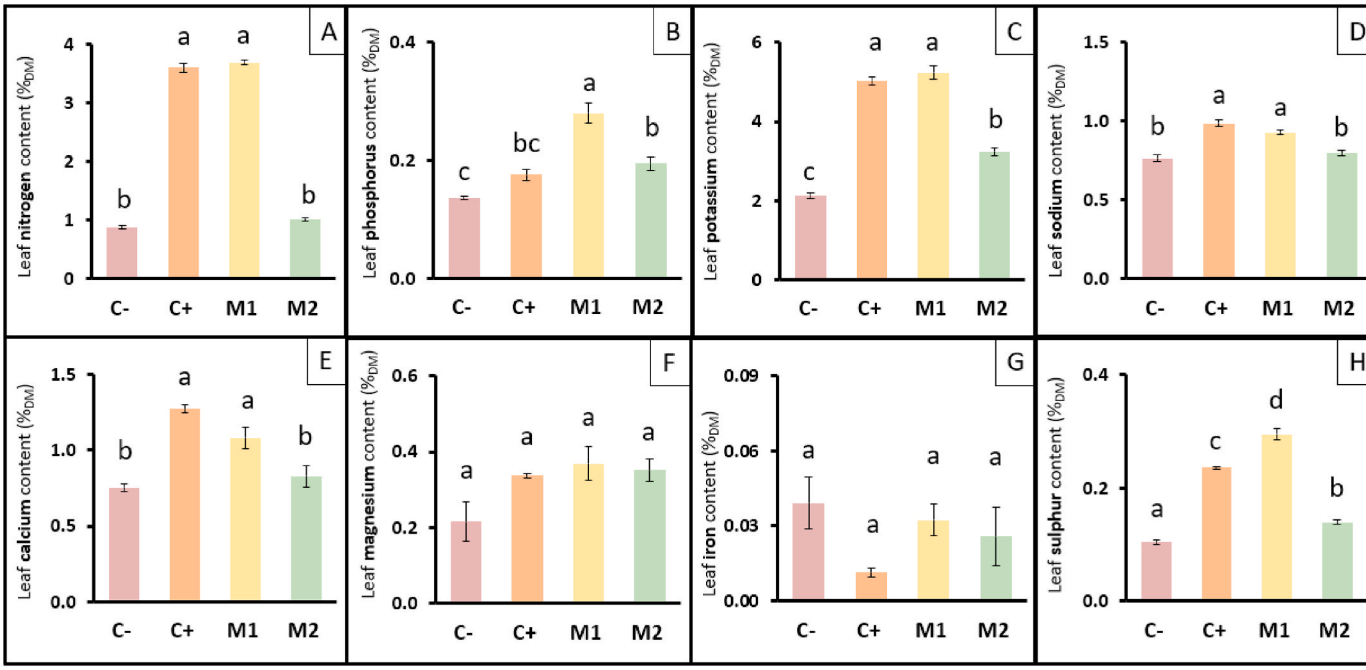

Fig. 4. Leaf nutrient content ($\%_{DM}$) in lettuce in different treatments: C- (negative control), C+ (positive control), M1 (microalgae biomass with N supplement), M2 (microalgae biomass). The error bars represent the Standard Error of the Mean. Different letters indicate significant difference ($p < 0.05$).

species of microalgae, the way it can be applied or biomass pre-treatments. For instance, Morillas-España et al. (2022) reviewed different studies about the use of microalgae as fertilizer. It highlights the great variability of results obtained with different species of microalgae, cultivated under diverse conditions and depending on the way of application (which can be directly on the soil or in foliar application, including a previous extraction step). Moreover, applying microalgae as fresh biomass or after a drying step might also affect the results (Alvarez et al., 2021).

In short, plant growth could be related to the presence of phytohormones in the microalgae biomass instead of to increasing nutrient uptake (Kapoore et al., 2021; Plaza et al., 2018). On the other hand, microalgae and cyanobacteria fertilizers might present beneficial effects for soil. For instance, cyanobacteria fertilizers help to preserve soil structure, which is fundamental for maintaining water balance (Rossi et al., 2017). Moreover, Alvarez et al. (2021) suggested that microalgal fertilizers might increase the microbial biomass carbon which improves soil quality.

5.1.2. *Leaf heavy metals content*

As cadmium was the only heavy metal which exceed the European limit, its content was measured in lettuce leaf at the end of the experiment, to guarantee the safety of the crop. Results are given in Fig. 5. Although the fertilizers treatments presented a significantly higher concentration of Cd ($p < 0.05$) than the negative control, the microalgae treatments (M1 and M2) presented a similar concentration to the positive control (C+). This demonstrates that the microalgal biomass used in this study was not the main source of this metal in lettuce leaves. In this case, the soil used for this experiment presented a Cd concentration of 0.5 mg/kgDM, (supplementary material, Table S3), which could be transferred from soil into the lettuce tissue. It is well known that plants are capable of uptaking heavy metals from contaminated soil which is a public health concern. Indeed, Khan et al. (2008) found Cd concentrations in lettuce samples ranging from 0.4 to 0.9 mg/kg due to the presence of that heavy metal in the soil used for the experiment.

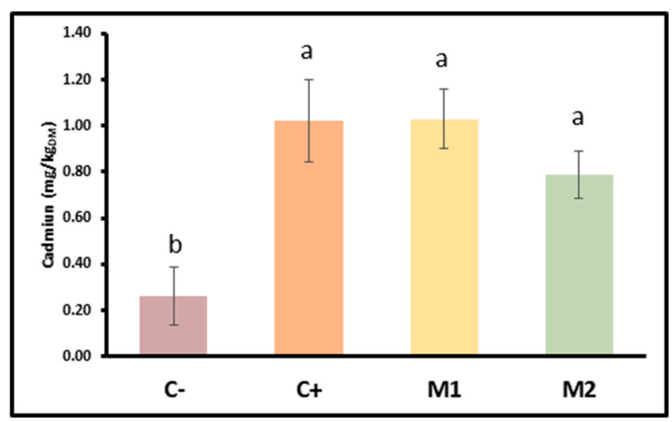

Fig. 5. Concentrations of cadmium in lettuce leaves in each treatment: C- (negative control), C+ (positive control), M1 (microalgae biomass with N supplement), M2 (microalgae biomass). Results are given as the average (Standard Error of the Mean) of four replicates per treatment. Different letters indicate significant difference ($p < 0.05$).

5.1.2.1. Tracking the content of contaminants of emerging concern: from wastewater to plant leaves. Most of the analysed CECs were found in wastewater (25 of the 29 analysed compounds). The ones that showed the highest concentration levels (over 3 μg/L) were caffeine, diclofenac, hydrocynamic acid, methyl dihydrojasmonate, and naproxen (Fig. 6 and Table S1). These findings are in agreement with previous studies. For instance, caffeine, despite being identified as easily removed by WWTPs (over 80%), has been observed at high concentrations in secondary WWTP effluents due to its very high consumption and its resulting elevated concentration in the raw wastewater (Buerge et al., 2003). Furthermore, diclofenac and naproxen, two commonly used pharmaceuticals, have been reported to be present at high concentrations in NE Spain WWTP effluents due to their low removal (Gros et al., 2007; Matamoros et al., 2017). Out of the 25 CECs identified in wastewater samples, only three compounds (hydrocinnamic acid, caffeine, and bisphenol A) were found to be over the LOQ in the microalgae biomass with concentration ranging from 0.1 to 25 μg/g DM (Table S2). This indicates that some CECs which were present at the greatest

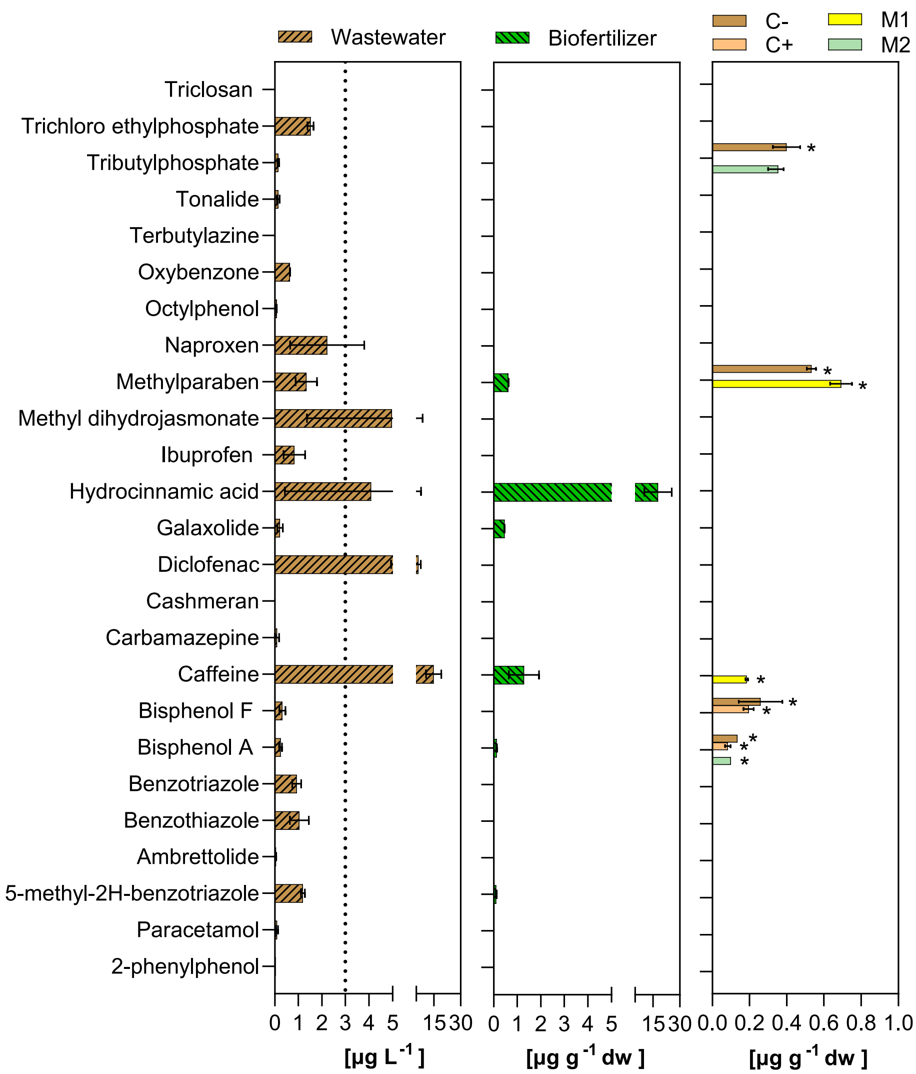

Fig. 6. Mean concentrations of the detected CECs (25 out of 29) in the wastewater (n = 2), biomass (n = 2) and lettuce leaves of each treatment (C-, C+, M1 and M2) (n = 4). Values are given in μg • L^{-1} for water and μg • g_{DW}^{-1} for biomass or lettuce. Compounds < LOD in all samples are not plotted. Only replicates with values > LOD were used to calculate means. Error bars show the range of the measurements. *Compounds were detected on two or less replicates. All values are found on Tables S1 and S2 of the supplementary materials.

concentration levels in the wastewater (i.e. hydrocinnamic acid and caffeine), were also those being incorporated into the microalgal biomass.

In contrast, bisphenol A was also quantified in the microbial biomass despite being found at low concentrations in wastewater. This could be due to the fact that bisphenol A can accumulate in microalgae, as it has been reported in the literature (Ji et al., 2014). García-Galán et al. (2020) observed that not only bisphenol A, but also the pharmaceutical venlafaxine was accumulated in the microalgal biomass. In another study, García-Galán et al. (2021) found that CECs were removed from irrigation and rural drainage water in a tubular photobioreactor by microalgae with a wide range of efficiencies, higher than 70%, moderate between 35 and 50% and others with efficiencies lower than 25%. According to the authors, these compounds could be degraded due to different mechanisms: photodegradation, adsorption onto the microalgae biomass, aerobic biodegradation or bioaccumulation.

Regarding lettuces, only some CECs were found in some sample replicates with concentrations ranging from 0.1 to 0.8 μg/g DM. Their occurrence could not be linked to the usage of microalgal biomass (Table S2). For example, Bisphenol A was found on one replicate of M2 but also on one replicate of C+ and in one of C-. Also, tributylphosphate was detected (<LOQ) on all C+ and in two replicates of C-, and was not present in the samples fertilized with microalgal biomass. These are very ubiquitous compounds, and their identification in lettuce crops could be due to their presence in soil or irrigating water. For instance, Margenat et al. (2019) observed the occurrence of some of these CECs in lettuces irrigated with groundwater, and suggested that their presence could be due to their presence in soil. Moreover, Margenat et al. (2018) suggested that the presence in lettuce leaves of some CECs, such as bisphenol, could be due to the plastic tubing for watering crops."

All in all, concentration levels observed in lettuce edible parts are very low and similar to those observed in other studies with no impact of wastewater, indicating that the usage of microalgae as biofertilizers is safe in terms of the lettuce uptake of CECs. However, future research should study the accumulation of CECs on the soil as well as the effects on lettuce after repeated applications of microalgal biomass on the same farming plot.

5.1.2.2. Influence of microalgae fertilizer on leachate quality. After lettuce plants harvesting, the water-soluble nutrients (ammonium, nitrate and phosphate) were measured in the leachate monitoring test, as an indicator of the potential groundwater contamination (Table 2). Since the assay was performed after the harvest, these nutrients are the left-over pool.

M2 showed a very low N-loss through leaching (4 mg/L N–NO_3^-). M1 presented nitrate-loss (204 mg/L N–NO_3^-), whereas C+ presented both, nitrate (128 mg/L N–NO_3^-) and ammonium (3 mg/L N–NH_4^+) in the leachate. However, M1 presented a lower concentration of NH_4^+ (0.2 mg/L) in the leachate, whereas the concentration of NO_3^- was higher than the one in C+. It should also be taken into account that mineral N

Table 2
Soluble inorganic nutrients concentrations (Ammonium; Nitrate; Phosphate) in the leachate in each treatment: C- (negative control), C+ (positive control), M1 (microalgae biomass with N supplement), M2 (microalgae biomass). Standard Error of the Mean is given in brackets (n = 4).

Treatment	N–NH_4^+ (mg/L)	N–NO_3^- (mg/L)	P-PO_4^- (mg/L)
C-	<0.02	3.0 (0.3)	<0.08
C+	2.7 (0.6)	127.8 (5.5)	5.4 (1.7)
M1	<0.2	204.1 (14.4)	<0.08
M2	<0.02	4.4 (0.6)	<0.08

supply was fractioned in two applications (one at the beginning and six weeks after crop planting) whereas only half dose of N was applied six weeks after crop planting in treatment M1. The nitrification process, which entails the oxidation of NH_4^+ to NO_3^-, is a rapid process in agricultural soils mediated by bacteria (Norton, 2008). Marks et al. (2017) demonstrated that the application of microalgae biomass into the soil enhanced the growth of microorganisms (both prokaryotic and eukaryotic) and their activity. So, these results suggest that the microalgae biomass provided some nitrifying bacteria or could have promoted the nitrifying activity of the bacterial community in the soil, transforming all the NH_4^+ into NO_3^-. Nevertheless, the N loss in M1 reveals that further research is needed to optimize the exact combination of microalgae and fertilizer in order to minimize the nutrients loss through leachate.

Regarding phosphorus, M1 nor M2 showed lower P-loss through leachate (<0.5 than mg/L P-PO_4^{3-}) C+ (5 mg/L P-PO_4^{3-}). Except for C-, all treatments were provided with the same dose of P at the beginning of the experiment. M1 and M2 were even expected to provide a 2% extra P coming from the microalgal biomass. Indeed, Rupawalla et al. (2021) found that P loss was higher in microalgae treatment, which was attributed to a higher P supplied in that treatment compared to the synthetic fertilizer. However, in this study only C+ showed phosphate leaching, suggesting that microalgal biomass can avoid the lixiviation of P. In conclusion, microalgal biomass application into the soil promoted nitrification and reduced the extent of phosphate leaching in the conditions of the present study.

6. Conclusion

Lettuce plants growth was tested in greenhouse using microalgal biomass cultivated in wastewater as biofertilizer with different treatments and doses. The agronomic assay revealed that microalgal-based fertilizer can reach similar results to those of inorganic fertilizers in terms of fresh shoot weight. Although pathogens and heavy metals are known to be present in wastewater, the biomass fulfilled the requirements of the European regulation regarding fertilizers, except for cadmium. Only three CECs were detected in the biomass fertilizer, out of 25 found in the wastewater. The cadmium content and CECs found in lettuce samples were not linked to the use of the microalgal biomass but to the soil composition. Even if more research is needed, this study suggests that wastewater grown microalgae can be safely used for agricultural purposes.

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Author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.121399.

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