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Multipurpose agricultural reuse of microalgal biomasses from different sources and their extracts

Emanuele La Bella

Advisor:
Prof.ssa Ivana Puglisi

Co-advisor:
Prof. Andrea Baglieri

Coordinators:
Prof. Antonio Biondi
Prof. Alessandro Priolo

Ph. D. attended during 2020/2023

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Research highlights

- Agricultural sector should be oriented towards sustainable management strategies for environmental pollution reduction.
- Microalgae are a source of high value metabolites that can enhance crop productivity.
- Microalgae can grow effectively on a wide range of substrates, including wastewater.
- This thesis is focused on the multifaceted applications of microalgae, such as their use in the agricultural sector as cellular extracts and/or living cells, as well as in phycoremediation processes.
- Lettuce seedlings treated with a microalgal cellular extract via foliar spray and root drenching showed higher yields.
- Phycoremediation of municipal wastewater treated decreased pollutant levels below the law limit for reuse in irrigation and allowed the growth of microalgae biomasses.
- Inoculation of living microalgae cells into the soil improved crop productivity, enhanced the biochemical fertility of the soil, and reduced nitrogen loses in groundwater.
- These results suggest that microalgae are promising microorganisms to address the sustainability challenges associated with increasing crop production demand.

Abstract

Under the current scenario, the use and development of new plant biostimulants has become a common practice in agriculture, providing a number of benefits in stimulating plant growth and potentially contributing to a more sustainable and resilient agriculture. Moreover, they offer an alternative to synthetic products, which have increasingly failed out of favour with consumers. A new emerging class of biostimulants is represented by microalgae-based products. However, the economic viability of microalgae production for agricultural purposes faces challenges. This being considered, the present thesis has been carried out to implement a methodological approach based on the use of microalgae to improve crop productivity while ensuring an easy and feasible approach for microalgae growth. As a starting base of the study, the research focused on the application of a cellular extract of *Chlorella vulgaris* as a biostimulant in lettuce cultivation. Lettuce plantlets underwent two different treatment modalities, foliar spray and root drenching. Both application methods successfully increased plant growth, stimulating some plant enzymes involved in primary and secondary metabolism. Subsequequently, a phycoremediation process was performed on a laboratory scale to evaluate the decontamination performance and growth potential of microalgae on waste substrate. The evidence showed that applying microalgae in wastewater treatment allows two main goals: water remediation and microalgae biomass production. Finally, microalgae biomasses cultivated on wastewater were investigated as soil inoculants. The results indicated that the addition of microalgae cells to the soil successfully improved plant growth by stimulating nitrogen assimilation through the enhancement of the key enzymes of this pathway and improving overall soil fertility. Finally, a considerable reduction in nitrogen losses in groundwater was also observed as a consequence of the addition of microalgae cells in the soil.

Keywords: microalgae, agriculture sustainable, plant

biostimulant, phycoremediation, soil fertility.

Riassunto

Nell'attuale scenario, l'uso di nuovi biostimolanti vegetali è diventato una pratica comune, fornendo numerosi vantaggi nel migliorare la crescita delle piante e contribuendo allo sviluppo di un'agricoltura più sostenibile e resiliente. Inoltre, offrono un'alternativa ai prodotti sintetici, che stanno perdendo consenso tra i consumatori. Una nuova classe emergente di biostimolanti è rappresentata dai prodotti a base di microalghe. Tuttavia, la produzione di microalghe per applicazioni in agricoltura non è economicamente vantaggiosa. La presente tesi è stata realizzata per implementare un approccio metodologico basato sull'uso di microalghe per migliorare la produttività delle colture e garantire allo stesso tempo una loro produzione economicamente sostenibile. In particolare, la ricerca si è concentrata sull'applicazione di un estratto cellulare di *Chlorella vulgaris* come biostimolante nella coltivazione della lattuga. Le plantule di lattuga sono state sottoposte a due diverse modalità di trattamento, irrorazione fogliare e somministrazione radicale. Entrambe le modalità di applicazione hanno aumentato la crescita delle piante, stimolando alcuni enzimi coinvolti nel metabolismo primario e secondario. Inoltre, è stato condotto uno studio su scala di laboratorio per valutare le prestazioni di decontaminazione di acque reflue (phycoremediation) e la capacità di crescita delle microalghe su un substrato di scarto. I risultati hanno mostrato che la phycoremediation consente di ottenere due importanti obiettivi: la bonifica dell'acqua e la produzione di biomasse. Quest'ultime, ottenute dalla phycoremediation, sono state studiate come biomasse direttamente aggiunte al suolo. I risultati hanno mostrato che l'aggiunta di cellule di microalghe è in grado di migliorare la crescita delle piante, stimolando l'assimilazione dell'azoto, e contemporaneamente migliorare la fertilità biochimica del suolo stesso. Infine, è stata osservata una considerevole riduzione delle perdite di azoto nelle acque sotterranee in seguito all'aggiunta di cellule di microalghe nel suolo.

Parole chiave: microalghe, agricoltura sostenibile,

biostimolanti, phycoremediation, fertilità del suolo.

Preface

The world population is projected to reach 9.7 billion people by 2050 (United Nations, 2017). The rapid growth of the human population exerts significant pressure on Earth's systems, leading to potential abrupt environmental changes and threaten the achievement of global sustainability goals, which are becoming a mere utopia.

Currently, there is no available food solution that can adequately address the expected increase in demand for food and clean water. Handling the interactions between social and environmental systems poses considerable challenges and involves making trade-offs (Dell'Angelo, D'Odorico, and Rulli, 2017). In particular, the interdependencies among food, energy, and water systems are central to achieve the global sustainability (Fuso Nerini et al., 2017).

In this context, agriculture remains the most important and stable sector, providing raw materials for the food and feed industries. Given the limitations of natural resources, there is a need for economically advanced, environmentally friendly, and efficient agricultural development. Therefore, it is imperative to adopt new technologies that are decidedly focused on enhancing agricultural production (Yunlong and Smit, 1994).

To achieve global sustainability, it will be essential to find innovative ways to balance the demands of the growing population with the need to preserve the environment and natural resources. Integrated approaches that take into account the interdependencies of food, energy, and water systems will play a significant role in addressing these complex challenges. Furthermore, advances in agricultural technologies and practises will be crucial to ensure sufficient food production without exacerbating environmental degradation.

To obtain a sustainable agricultural vision, crops must be mainly equipped with better nutritional value, as well as tolerance to biotic and abiotic stress conditions. One possible approach to achieve

the crop properties described above is to use beneficial microorganisms, such as bacteria, fungi, algae and microalgae, which can improve nutrient uptake and water use efficiency (Armada et al., 2014).

Increasing food demands and changing environmental conditions lead agriculture to increase yields without further harming ecosystems in the process (Godfray, 2010, Sudheer et al., 2020).

Among the new sustainable solutions in agricultural practises, biostimulants have acquired increasing interest. They are compounds derived from organic materials that increase germination, yield, or growth in plants through mechanisms other than nutrition (Del Buono, 2021). However, over the last decade, there has been intense discussion concerning the definition of a plant biostimulant. The most recent definition, which is currently being discussed in the context of the review of the EU fertilizer legislation (2009), "plant biostimulant is any microorganism or substance derived from natural resources, in the form in which it is supplied to the user, that is applied to plants, seeds, soil, or any other substrate with the intention of stimulating natural processes in plants to improve their nutrient use efficiency and/or stress tolerance, regardless of the nutrients content, or any stress tolerance, regardless of the nutrient content, or any combination of such substances and/or microorganisms intended for this purpose" (Regulation, 2019).

Microalgae are particularly promising as they are valuable sources of high-value chemicals, including carotenoids, long-chain polyunsaturated fatty acids, and other useful metabolites (Borowitzka, 2010; Mendes et al., 2009). Their potential as new sources of valuable chemicals and other products has gained widespread interest in recent years, attracting the interest of industries and farmers on the basis of their promising properties for use in agricultural technology. Hormones that actively stimulate germination, growth, or fruit set of higher plants, such as cytokinin and abscisic acid, have been detected in several microalgae species (Tarakhovskaya et al., 2007; Do et al.,

2020). Integrating microalgae into agricultural practices can result in multiple benefits, such as improving the efficiency of nutrient uptake and minimising resource waste, presenting an innovative and potentially sustainable approach to address the challenges of food production, resource management, and environmental preservation.

Hence, microalgae possess the potential to have a major influence on essential agro-ecosystem services. However, microalgae production must overcome several barriers in order for them to become economically viable, especially for the production of agricultural products (Brennan and Owende, 2010; Mata et al., 2010). One way to make microalgae biomass production more economically feasible, given current technologies, is to find potential applications for microalgae biomass or its byproducts that enable producers to offset production costs, since they can be cultivated in wastewater and agricultural runoff, recovering excess nutrients and reclaiming water for further use, and can sequester carbon dioxide and nitrous oxides from industrial sources, reducing greenhouse gas emission (Brennan and Owende, 2010).

Therefore, to make the microalgae cultivation process sustainable, feasible and economically viable, it is necessary to develop successful cultivation technologies for targeted biomass production. A possible solution may be represented by using wastewater as a growth substrate. Microalgae have long been proven to be efficient in removing nitrogen, phosphorus, and toxic metals from a wide variety of wastewater (Zhou et al., 2012; Boelee et al., 2012; Sturm and Lamer, 2011), offering an additional important economic use.

Until now, microalgae-based biostimulants have been considered an emerging class of products in agriculture. Among the various microalgae species studied, the genera *Chlorella* and *Scenedesmus* have been widely studied for their biostimulant properties. These microalgae species have demonstrated the ability to enhance plant growth by improving water uptake, root and shoot growth, tolerance

to environmental stress, protein content in plant tissues, and the activity of various enzymes. However, in the current scenario of a desirable circular and bio-based economy, the utilization of autochthonous microalgae appears to be an optimal solution. When these microalgae are grown on wastewater as a substrate, they can serve a dual purpose: first, to remove nutrients from wastewater and second, to produce suitable biomass for agricultural applications. Nonetheless, further research and development are still required to optimize the production and application of microalgae-based biostimulants in practical agricultural settings.

Outline of the thesis

This thesis is focused on the multifunctionality of microalgae, including both autochthonous and widely distributed species, possessing biostimulant effects and adapted to grow on wastewater as a substrate. In detail, the aims of this study were: (i) to carry out a preliminary comparison of the effects of a cellular extract of *Chlorella vulgaris,* applied through root drenching or foliar spray, on lettuce seedlings. This study aimed to assess the biostimulant properties of the extract under different application methods; (ii) to evaluate the performance of phycoremediation and biomass accumulation of an indigenous strain of filamentous microalga, previously identified as *Klebsormidium* sp. K39, in treatment of urban wastewater. A comparative analysis was conducted with *C. vulgaris* and *Scenedesmus quadricauda*, two extensively studied species; (iii) to explore the potential reuse of microalgae biomass (*C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39) previously cultivated in urban wastewater. The study examined the impact of these microalgae biomasses on plant growth and soil fertility.

In detail, this thesis is composed by an Introduction and three Chapters describes as following:

Introduction: is an extensive literature review, providing an overview of the critical literature of the multipurpose agricultural applications of microalgae biomasses. This study highlights the need to create a circular production system that harnesses the potential of microalgae as agents for wastewater remediation and as biomass for further applications in agriculture, such as biostimulants, biofertilizers, and biopesticides.

The review presented here has already been published in Agronomy.

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Chapter 1: is a study article focused on the biostimulant effects of *C. vulgaris*. This experiment addressed the effects of two different application modalities, root drenching and foliar spray, of the micro-algal extract on lettuce yield, monitoring morpho-biometric parameters and biochemical effects at different sampling times along the whole experimental period.

The composition of *C. vulgaris*, previously described in detail by Barone et al. (2018), is briefly presented in Tables 1 and 2.

The work presented in Chapter 1 has already been published in Journal of Applied Phycology.

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Table 1. Distribution of carbon intensity of carbon-13 nuclear magnetic resonance (^{13}C NMR) of biomass of *C. vulgaris* (CV) and its extract (CVextr).

	Alkyl 0-45 ppm	N and O alkyl 45-90 ppm	Aromatic 95-160 ppm	Carboxyl 160-195 ppm	HB/HI
CV	40.91	33.66	10.85	14.58	1.1
CVextr	63.39	4.75	22.5	9.37	6.1

Table 2. Element composition (%) of biomass of *C. vulgaris* (CV) and its extract (CVextr).

	C	N	P	S	Mg	Ca	Fe	K	Na
CV	51.4	7.76	0.20	0.36	0.47	0.50	0.13	0.09	0.46
CVextr	62.2	1.37	0.24	0.37	0.51	0.05	0.01	0.52	3.87

Chapter 2: is a study article on the phycoremediation performance of *Klebsormidium* sp. K39, in treatment of urban wastewater. A comparative analysis was conducted, involving *C. vulgaris* and *S. quadricauda*, two species that have been extensively studied in this context. The main objective of the study was to assess and compare the efficiency of the three microalgae in removing pollutants and accumulating biomass during the treatment of urban wastewater.

The work presented in Chapter 2 has been already published in

Sustainability.
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Chapter 3: is a study article focused on the potential reuse of microalgae biomass (*C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39), which were previously cultivated in urban wastewater. Microalgae cells were tested for their effects on plant growth, focussing primarily on nitrogen metabolism in lettuce seedlings. Furthermore, the study aimed to assess the impact of the addition of *C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39 on the biochemical fertility of the soil, by analysing the principal enzymatic activities of the soil related to the microorganism metabolism. Additionally, the study assessed the effect of microalgae biomasses on the rate of nitrate leaching through the soil.
This work presented in Chapter 3 will be submitted in Journal of Soil Science and Plant Nutrition.

Other activities: is a collection of research articles, projects and conference participations carried out during the PhD cycle.

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1 Introduction

Multipurpose Agricultural Reuse of Microalgae Biomasses Employed for the Treatment of Urban Wastewater – Review Article

Emanuele La Bella[a], Andrea Baglieri[a], Ferdinando Fragalà[a], Ivana Puglisi[a]

[a] Department of Agricultural, Food and Environment, University of Catania, Italy.

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Abstract

The pollution of water caused by the excessive presence of organic and inorganic compounds, such as nitrates, phosphates, heavy metals, antibiotics, agrochemicals, etc., is one of the major environmental problems in many countries. Various approaches to remediate wastewater are available, and this review mainly provides the state of the art about the possible adoption of microalgae-based treatments (phycoremediation), which may represent a good alternative to conventional purification methods. Because of its composition, wastewater can provide several nutritional compounds (e.g., carbon, nitrogen, and phosphorus), which represent the essential nutrients for microalgae growth. Microalgae are also attracting the interest of worldwide researchers due to their multipurpose applications; in particular, microalgae cells can represent a useful feedstock for various sectors, among these, the agricultural sector. This review proposes a

detailed description of the possible application of microalgae in the process of remediation of wastewaters of different sources, highlighting their possible advantages. Moreover, the review aims to report the application of the microalgae biomasses and their extracts in agriculture, as microalgae-based products can represent a valid alternative to traditional agrochemicals, offering sustainable solutions to improve agricultural technologies. Therefore, since the recently developed wastewater depuration technology based on phycoremediation may directly provide valuable microalgae biomasses, it can be used as a powerful starting means to produce agricultural products able to improve yield and quality of crops (biostimulants, biofertilizers), as well as induce pest and disease resistance (biopesticides).

1.1 Introduction

The pollution of agricultural, industrial, and municipal wastewaters with many organic and inorganic compounds, such as nitrates, phosphates, heavy metals, etc., is one of the most critical and common environmental problems in the main industrialized countries. The excessive presence of pollutants, particularly nitrogen and phosphorus, causes ecosystem problems and subsequent eutrophication of waterbodies, producing alteration of water system health (Chai et al., 2021).

Wastewater treatment is an important issue, and it globally cannot be managed by a single technology because of the extremely variable scales, depending on different types of contaminants, different wastewater sources, as well as different regional conditions which are involved (Wollmann et al., 2019). Conventional wastewater treatment systems mainly focus on the elimination of solid suspension and the reduction of biological oxygen demand (BOD_5) by activated sludge (Bolognesi et al., 2021).

Therefore, the presence of a wide range of pollutants, continuously discharged in urban wastewater, such as pharmaceutically active

compounds, personal care products, pesticides, synthetic and natural hormones, and industrial chemicals, represents a serious problem to the environment and human health. These chemicals are also called contaminants of emerging concern (CECs) (Rizzo et al., 2019). However, the capability of conventional methods in the elimination of microcompounds and inorganic nutrients is not always effective towards a complete removal. Moreover, the management of municipal wastewater through the conventional methods, such as tricking filters, activated sludge process, or oxidation ponds, is often very expensive. To solve these problems, good alternatives seem to be represented by new technologies, such as magnetic field and biological membrane reactors, especially in the local contexts (Puzowski and Skoczko, 2020; Skoczko et al., 2020). The employ of the magnetic field is indicated for the remediation of urban wastewater, and it provides high efficiency in water treatment, especially in hardness, turbidity, and minerals removal (Puzowski and Skoczko, 2020). The biological membrane reactors are based on pressure separation techniques and have many advantages over traditional methods, the most important of which are represented by very high phase separation efficiency, high quality of treated wastewater, and the possibility to remove specific pollutants (Skoczko et al., 2020). In this contest, another new and sustainable biotechnology for wastewater treatment is epresented by phycoremediation.

1.2 Conventional purification methods of wastewater

Conventional purification methods of wastewater involve a combination of physical, chemical, and biological processes, and operations to remove insoluble particles and soluble contaminants from effluents. There is not a single method capable of adequate treatment, mainly due to the complex nature of effluents (Crini and Lichtfouse, 2019).

The conventional wastewater treatments usually consist of five

steps, described in Figure 1 (Crini and Lichtfouse, 2019):

1. Preliminary treatment (physical and mechanical) includes screening, grinding, grit removal, flotation, equalization, and flocculation. The primary objective of this treatment consists of the removal of solids and other large substances often present in raw wastewater (Sonune and Ghate, 2004). This step aims to remove or reduce, in size, the solids.
2. Primary treatment (physiochemical and chemical) involves the physical processes of screening, comminution, and sedimentation. This stage is aimed to remove solid substances, both organic and inorganic, from wastewater (Zinicovscaia, 2016). Some forms of organic nitrogen, organic phosphorous, and heavy metals associated with solids are also removed during this process (Crini and Lichtfouse, 2019).
3. Secondary treatment or purification (chemical and biological) is based on the use of microorganisms to remove the contaminants. Several aerobic biological processes are used in the way in which the oxygen is supplied to the microorganisms, and in the rate at which organisms metabolize the organic matter (Sonune and Ghate, 2004). The main purpose of these treatments is the removal of fine suspended and dispersed solids, and dissolved organics.
4. Tertiary or final treatment (physical and chemical) is the final process that enhances the quality of wastewater before it is reused or discharged to the environment, and treatment of the sludge formed.
5. Treatment of the sludge (supervised tipping, recycling, incineration) consists of the sustainable management of the sludge in order to reduce the impact on the environment.

The number of stages adopted depends on the extent of pollutant removal and the mechanisms through which pollutants are removed (Zinicovscaia, 2016).

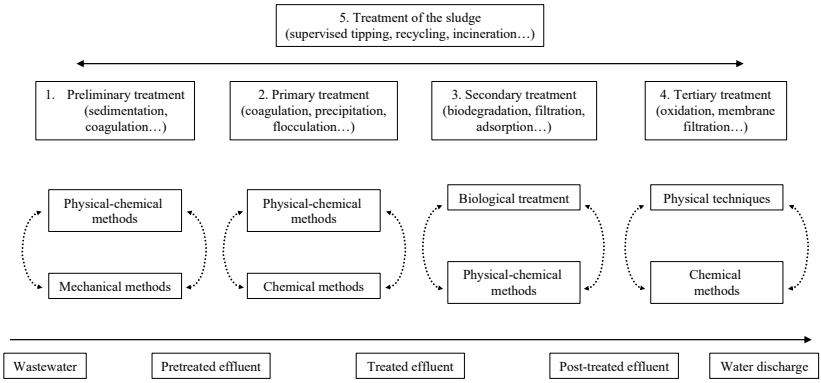

Figure 1. Main processes for the decontamination of wastewater (adapted by Crini and Lichtfouse (2019)).

1.3 Phytoremediation

An environmentally friendly and cost-effective technology for conventional treatments is represented by phytoremediation. Some plants are able to accumulate pollutants through their roots, and then translocate these compounds to the aboveground part of their body (Sharma et al., 2015). This method seems to be particularly indicated for the management of urban wastewater, containing a great variety of contaminants along with higher contents of biodegradable organic matter (Dar et al., 2011).

Phytoremediation is based on the application of vegetation and microorganisms for recovery of many pollutants and environmental decontamination. In this process, a crucial role is played by aquatic plants, which are able to absorb different compounds, such as organic and inorganic contaminants, heavy metals, and pharmaceutical pollutants present in agricultural, domestic, and industrial wastewaters.

Phytoremediation follows different mechanisms, such as phytoextraction, phytostabilization, phytovolatilization, and rhizofiltration (Rahman and Hasegawa, 2011).

The efficiency of the phytoremediation systems in the removal of different pollutants, such as nutrients, heavy metals, organic matter,

agrochemicals, and polycyclic aromatic hydrocarbons, are reported in literature (Malaviya and Singh, 2012). Physical, chemical, and biological processes, such as volatilization, sorption, sedimentation, photodegradation, plant uptake, and microbial degradation, may occur simultaneously, contributing to remove many types of compounds (D. Zhang et al., 2014).

Table 1 reports the advantages and disadvantages of phytoremediation approaches in wastewater treatment (Ahmad et al., 2017).

Table 1. Advantages and disadvantages of phytoremediation of wastewater (adapted by Ahmad et al. (2017)).

Advantages	Disadvantages
Low capital requirement	Limited to shallow contaminants
Low energy requirement	Phytotoxicity of contaminants
Environmental friendliness	Slower than conventional methods
Utilizes natural and renewable source	Unknown effects of biodegradation products
Less secondary waste generation	
Less carbon footprint	
Reclamation of wastewater and nutrient recovery	
Generation of feedstock for different applications	
Cost effectiveness and the possibility of harvesting the plants for the extraction of absorbed and accumulated contaminants such as toxic heavy metals for recycling	

1.4 Phycoremediation

The microalgae-based wastewater treatment process is one of the most promising technologies for the treatment and nutrient recovery of wastewaters from various sources (industrial, municipal, and agricultural): microalgae could be adapted to a variety of water bodies, can be extensively used to treat effluents (Luo et al., 2016), and could provide a tertiary biotreatment coupled with the production of potentially valuable biomass (Abdel-Raouf et al., 2012).

Therefore, this technology offers a good solution for their ability in the fixation of inorganic compounds, including carbon dioxide and heavy metals (Chen et al., 2018; Koppel et al., 2018; Li et al., 2020; Suganya et al., 2016). This method has two main aims: direct uptake of water contaminants (Figure 2), and the improvement of the purification performance of microalgae–bacteria aggregates by providing additional oxygen from photosynthesis (Figure 3), thus reducing the total energy costs of direct oxygen supply (Quijano et al., 2017). This advantage is made possible by the metabolic flexibility of microalgae, since they can be as follows:

- Autotrophic: microalgae grow by obtaining energy through the absorption of light energy for the reduction of CO_2 by oxidation of the substrates with the release of O_2.
- Heterotrophic: microalgae grow using organic carbon in the dark, solving problems related to the presence and distribution of light and CO_2.
- Mixotrophic: microalgae grow depending on the environmental conditions in their regime, during which CO_2 and organic carbon may be assimilated, depending on their availability, under either autotrophic or heterotrophic conditions.

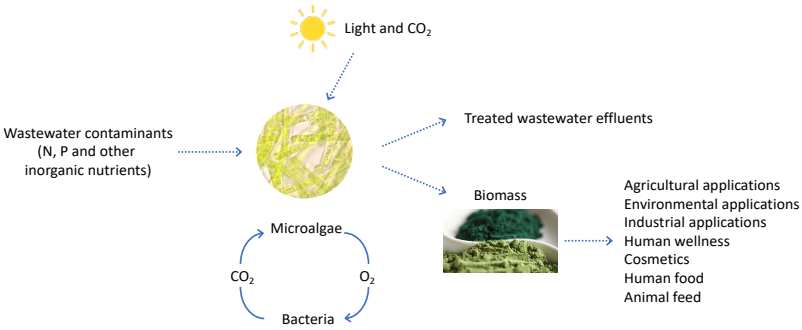

Figure 2. Uptake mechanism of nutrient and interactions among bacteria and microalgae.

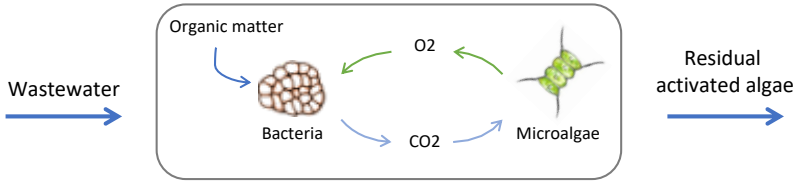

Figure 3. Aerobic treatment step.

In the actual context of a desirable circular and bio-based economy, microalgae treatment is considered an optimal option for its capability of treating wastewater in a single step. Meanwhile, the production of microalgae has also attracted the attention of researchers for their further multipurpose uses: in fact, they can be used to produce biochar, biofertilizers, biofuels, and biomaterials for the food and feed sectors (Rawat et al., 2011).

Until now, the research on microalgae-based wastewater treatment has focused on the most common species, such as *Chlorella* sp., *Ankistrodesmus* sp., and *Scenedesmus* sp.; however, their efficiencies are different. The removal efficiency rates of pollutants in term of nitrate ($NO3^-$), nitrite ($NO2^-$), ammonium ($NH4^+$), total nitrogen (N), phosphorus (P), biochemical oxygen demand (BOD5), and chemical oxygen demand (COD) in wastewaters of different sources treated with microalgae are reported in Table 2.

Table 2. Removal rates by microalgae of pollutants in wastewater of different sources.

Microalgae Species	Wastewater Type	Treatment Efficiency (%)	Reference
Anabaena flos-aquae	Ammonium form nitrogen group	N: 94.9 P: 96.8	(Zhu et al., 2018)
Anabaena flos-aquae	Orthophosphate form phosphorous group	P: 97.7	(Zhu et al., 2018)
Ankistrodesmus falcatus	Aquaculture wastewater	NO_3^-: 80.85 NO_2^-: 99.73 NH_4^+: 86.45 P: 98.52 COD: 61	(Ahmad et al., 2017)

Arthrospira platensis	Dairy farm wastewater	NO_3–N: 99.6 NH_4–N: ~100 PO_4–P: 98.8 COD: 98.4	(Hena et al., 2018)
Calothrix sp.	Sewage water	N: 57 P: 74	(Renuka et al., 2013)
Chlamydomonas sp. (YG04)	Municipal wastewater	N: 77.57 P: 100	(Rasoul-Amini et al., 2014)
Chlamydomonas sp. (YG05)	Municipal wastewater	N: 74.49 P: 100	(Rasoul-Amini et al., 2014)
Chlorella sp.	Domestic wastewater	N: 50.2 P: 85.7 BOD_5: 68.4 COD: 67.2	(Colak and Kaya, 1988)
Chlorella sp.	Municipal wastewater before primary settling	NH_4–N: 82.4 P: 83.2 COD: 50.9	(Wang et al., 2010)
Chlorella sp.	Municipal wastewater after primary settling	NH_4–N: 74.7 P: 90.6 COD: 56.5	(Wang et al., 2010)
Chlorella sp.	Municipal wastewater after activated sludge tank	NH_4–N: 62.5 P: 4.7	(Wang et al., 2010)
Chlorella sp.	Municipal wastewater generated in sludge centrifuge	NH_4–N: 78.3 P: 85.6 COD: 83	(Wang et al., 2010)
Chlorella sp.	Sewage water	N: 78 P: 45	(Hena et al., 2018)
Chlorella sp. (YG01)	Municipal wastewater	N: 84.11 P: 82.36	(Rasoul-Amini et al., 2014)
Chlorella sp. (YG02)	Municipal wastewater	N: 68.23 P: 99	(Rasoul-Amini et al., 2014)
Chlorella vulgaris	Wastewater from the Shatin sewage treat.	N: 86 P: 78	(Lau et al., 1996)
Chlorella vulgaris	Agricultural wastewater	NH_4–N: 99 NO_3–N:83 P: 88	(Baglieri et al., 2016)
Lyngbya sp.	Sewage water	N: 59 P: 92	(Renuka et al., 2013)
Oocystis sp. (YG03)	Municipal wastewater	N: 83.32 P: 99.01	(Rasoul-Amini et al., 2014)
Scenedesmus obliquus	Secondary effluent—without stirring (20 °C)	N: 94 P: 97	(Martínez et al., 2000)

Scenedesmus obliquus	Secondary effluent—without stirring (25 °C)	N: 99 P: 98	(Martínez et al., 2000)
Scenedesmus obliquus	Secondary effluent—without stirring (30 °C)	N: 99 P: 94	(Martínez et al., 2000)
Scenedesmus obliquus	Secondary effluent—without stirring (35 °C)	N: 79 P: 54	(Martínez et al., 2000)
Scenedesmus obliquus	Secondary effluent—with stirring (20 °C)	N: 80 P: 98	(Martínez et al., 2000)
Scenedesmus obliquus	Secondary effluent—with stirring (25 °C)	N: 100 P: 98	(Martínez et al., 2000)
Scenedesmus obliquus	Secondary effluent—with stirring (30 °C)	N: 99 P: 97	(Martínez et al., 2000)
Scenedesmus obliquus	Secondary effluent—with stirring (35 °C)	N: 82 P: 62	(Martínez et al., 2000)
Scenedesmus quadricauda	Agricultural wastewater	NH_4–N: 99 NO_3–N: 5 P: 94	(Baglieri et al., 2016)
Scenedesmus sp. LX1	Secondary effluent	N: 98 P: 98	(Xin et al., 2010)
Ulothrix sp.	Sewage water	N: 67 P: 85	(Renuka et al., 2013)

1.4.1 *Chlorella* sp.

Chlorella sp. is widely used for wastewater treatment, and has proven abilities of removing nitrogen, phosphorus, and COD, mixing with bacteria or not, which show their potentiality as tertiary biotreatment step (Figure 2) (Wang et al., 2010). Microalgae of the genus *Chlorella* can be grown both in autotrophic and mixotrophic cultivation conditions, reaching high growth rates.

Lau et al. (1996) reported that *Chlorella vulgaris* can reduce 86% of the inorganic nitrogen and 78% of the inorganic phosphates in primary settled wastewater. Instead, Colak and Kaya (1988) reported that *Chlorella* sp. can remove 50.2% and 85.7% of these two elements from industrial wastewater.

Wang et al. (2010) evaluated the ability of *Chlorella* sp. to remove nitrogen, phosphorus, COD, and metals on wastewaters sampled from four different points of the treatment process flow of a local municipal

wastewater treatment plant: wastewater before primary settling, wastewater after primary settling, wastewater after activated sludge tank, and wastewater generated in sludge centrifuge. The results, reported in Table 2, demonstrate the efficiency in nutrient removal of *Chlorella* sp.

Baglieri et al. (2013) tested the ability of *C. vulgaris* to remove contaminants from agricultural wastewater, considering two case studies: (i) the first on the growth rate of the species in wastewater from a hydroponic greenhouse cultivation, in order to evaluate the degree of removal of the main inorganic compounds; (ii) the second on microalgae ability to degrade five different active ingredients commonly used in agricultural practices (pyrimethanil, metalaxyl, iprodione, fenhexamid, and triclopyr). *C. vulgaris* demonstrated a good aptitude for the decontamination, removing about 99% of nitric nitrogen, 83% of the ammonia nitrogen, and 88% of phosphates. A reduction in the contents of other elements, such as iron, potassium, and total organic carbon, was also observed. The microalgae also showed ability to grow in the presence of all five active ingredients used in the trials, although in some cases, signals of suffering from a slightly toxic effect were observed. The dissipation of metalaxyl and fenhexamid provided the most interesting results, occurring faster in the presence of microalgae (Baglieri et al., 2013). With regard to iprodione and triclopyr, the dissipation was less evident. Pyrimethanil showed a different behavior with respect to the other pesticides, resulting in more resistance to dissipation, although in the presence of *C. vulgaris* (Baglieri et al., 2013).

Rasoul-Amini et al. (2014) tested two strains of *Chlorella* sp. (YG01 and YG02) for removal of nitrogen and phosphorus from municipal wastewater. The experiment confirmed that *Chlorella* sp. (YG01) can be considered an efficient nutrient remover in wastewaters of different origin, while in the other strains, a minor efficiency in the purification process was shown. All this evidence is summarized in Table 2.

1.4.2 *Ankistrodesmus* sp.

Ankistrodesmus sp. is a green phototrophic microalga that has a long crescent shape with a slight curve at both ends (Lananan et al., 2016). Mixotrophic conditions of growth of *Ankistrodesmus* sp. have shown the highest specific growth.

The phycoremediation ability of *Ankistrodesmus* sp. is reported only in a few studies available in literature. Among these, Ahmad Ansari et al. (Ahmad et al., 2017) focused on the potential strains, biomass-enhancement strategy, nutrient removal potential, and biochemical composition of the microalgae. In this study, *Ankistrodesmus falcatus* was grown using aquaculture wastewater. With regard to the removal efficiency, *A. falcatus* showed good performance (e.g., 80.85% of NO^{3-}, 98.52% of P, and 61% of COD), and the results are reported in detail in Table 2.

Also available in literature are some studies on the possible use of *Ankistrodesmus* sp. as an autoflocculating microalga with a shape, and zeta potential that could have the ability to coagulate other microalgae species, as *Chlorella* sp., and so act as bioflocculant in harvesting biomass (Lananan et al., 2016).

1.4.3 Scenedesmus sp.

Scenedesmus sp. is one of the microalgae genera particularly interesting for wastewater treatment due to its efficiency of nutrient removal, rapid growth rate, and high biomass productivity (Martínez et al., 2000; Ruiz-Marin et al., 2010; E. Zhang et al., 2008). *Scenedesmus* sp. can be grown under autotrophic, heterotrophic, and mixotrophic cultivation conditions.

Xin et al. (2010) studied the properties of lipid accumulation and nutrient removal of *Scenedesmus* sp. LX1 in secondary effluent. With regard to the total nitrogen and total phosphorus contents, the results showed a notable removal efficiency, for both nutrients, of over 98% (Table 2).

Martinez et al. (2000) studied the kinetics of N and P elimination as well as simultaneous growth of *S. obliquus* in the effluent from a secondary-sewage-treatment facility, under different conditions of stirring and temperature. The researchers chose as experimental conditions 20, 25, 30, and 35°C, representing the range of average temperatures of wastewater in different seasons of a warm climate, and two levels of mixing: maximum (magnetic stirring and air bubbling in the culture medium) and minimum (absence of magnetic stirring), as reported in Table 2.

Many works are also available in literature about the cultivation process of microalgae to promote the degradation of inorganic compounds and pesticides in water.

Baglieri et al. (2016), as above reported on *C. vulgaris* in the same case studies, also evaluated *Scenedesmus quadricauda* removal efficiency, showing in the wastewater of hydroponic greenhouse cultivation a consumption of about 99% nitric nitrogen, but only 5% of the ammonia nitrogen, and a remotion of 94% phosphates. *S. quadricauda* also showed to be able to grow in the presence of all five active ingredients (pyrimethanil, metalaxyl, iprodione, fenhexamid, and triclopyr) used in the trials, determining a reduction in their contents, and providing similar results to those above reported for *C. vulgaris* (Baglieri et al., 2016). Another study in which the removal ability of active ingredients from agricultural wastewater by microalgae was conducted by Kurade et al. (2016). The researchers screened *S. obliquus* for the removal of diazinon, an organophosphorus insecticide. The removal efficiency was evaluated in Erlenmeyer flasks containing 100 mL of BBM added to 20 mg diazinon L^{-1}. However, *S. obliquus* did not show high removal capacity of diazinon.

Although microalgae-based wastewater treatment is oriented towards efficient removal of nitrogen and phosphorus, not all contaminants can be eradicated (Chai et al., 2021).

1.4.4 Other species

In literature, other studies about microalgae species and cyanobacteria able to remove organic and inorganic compounds from wastewaters, of different origins, are reported.

Rasoul-Amini et al. (2014) evaluated the removal efficiency of nitrogen and phosphorus from municipal wastewater of the following species: two strains of *Chlamydomonas* sp. (YG04 and YG05), and one strain of *Oocystis* sp. (YG03). The results showed that *Chlamydomonas* sp. (YG04 and YG05) can act as efficient nutrient removers from wastewaters of different origin, while *Oocystis* sp. (YG03) showed a minor efficiency in the purification process, as reported in Table 2.

Zhu et al. (2018) studied the nitrogen and phosphorus removal during the *Anabaena flos-aquae* biofilm growth in two nutrient mediums, containing different nitrogen and phosphorus compounds. The results demonstrated that the nitrogen and phosphorus removal reached 94.9 and 96.8%, respectively, in the form of ammonium nitrogen, while 97.7% of phosphorus were removed in the form of orthophosphate phosphorous (Table 2).

Renuka et al. (2013) tested the phycoremediation ability of four microalgae strains: *Calothrix* sp., *Lyngbya* sp., *Chlorella* sp., and *Ulothrix* sp. The researchers observed a different behavior of the strains, obtaining in all the cases a significant removal of NO_3–N (ranging from 57–78%) and PO_4–P (44–91%), as reported in detail in Table 2.

Hena et al. (2018) evaluated the removal ability of *Arthrospira platensis* cultivated in dairy farm wastewater for biodiesel production. The results showed a good aptitude of *A. platensis* to remove the main pollutants.

1.5 Employ of microalgae in agriculture

In the last decades, the increase in worldwide population has caused an additional demand for food supplies, which may be obtained through an improvement of agricultural productivity. At the same time, the development of eco-friendly alternative methods of production to reduce the use of chemicals in agriculture appears necessary for the attenuation of their environmental effects (Puglisi et al., 2019; Tilman et al., 2002). In this context, a lot of attention has been focused on the development of bio-based products, among them microalgae products, to improve plant growth, yield, and quality by enhancing plant nutrition, and reducing abiotic and biotic stresses impacts (du Jardin, 2015; Kocira et al., 2018; Yakhin et al., 2017).

However, until now, the use of microalgae for applications in agriculture is an undergoing initiation, and the production of microalgae is only an emerging activity, due to its potential economic and commercial opportunities, but shows high costs of cultivation (Hultberg et al., 2013; Mata et al., 2010). An interesting solution to increase the cost-effectiveness of this process may be represented by the application of low-cost resources, such as nutrient-rich wastewaters and agricultural byproducts (Baglieri et al., 2016; Gong and Jiang, 2011; Mata et al., 2010).

To this aim, Barone et al. (2019) proposed the cocultivation of tomato plants and microalgae (*C. vulgaris* and *S. quadricauda*) in a hydroponic system, in which a biostimulant effect of agro-industrial waste both on tomato and microalgae (*C. vulgaris* and *S. quadricauda*) was proved (Puglisi et al., 2018). Even Zhang et al. (2017) suggested the simultaneous cultivation of *Chlorella infusionum* and tomato plants by using a hydroponic system, with the input only for crop production. These cultivation systems may represent a good opportunity to both reduce the costs for microalgae cultivation and provide a benefit for plant growth.

Microalgae and cyanobacteria represent an important source of

biologically active compounds, such as hormone-like substances, proteins, and polysaccharides, known for their benefits as antioxidant agents, plant-growth promotors, etc. These biological compounds may improve the agricultural productivity by different modes of action: soils' improvement, crops' protection, and direct plant growth stimulation (Gonçalves, 2021).

Considering these roles, microalgae-based products used in agriculture could be classified into three major categories: biostimulants, biofertilizers, and biopesticides.

Biostimulants, usually applied as extracts, may improve crops' productivity by acting directly on the plant, enhancing plant's metabolism, and thus plant's growth (Gonçalves, 2021). These products can exert stimulatory activity under both optimal and adverse conditions, improving plant resistance and tolerance against stress conditions (Chiaiese et al., 2018; Ronga et al., 2019).

Biofertilizers are biologically-based compounds that promote an improvement in crops' yields through their activity at the soil level, providing macro- and micronutrients for plant growth (Kusvuran and Kusvuran, 2019; Reddy and Saravanan, 2013; Ronga et al., 2019; Win et al., 2018). Typically, these products are applied as biomass.

Biopesticides are known for their activity against pests and plant pathogens (Gonçalves, 2021). These compounds have antimicrobial, antioxidant, antiviral, or antifungal properties and promote crops' development by protecting plants from pathogenic organisms. However, agronomic, physiological, chemical, biochemical, and molecular studies are required to better understand the changes induced by the microalgal products in crop productions.

1.5.1 Biostimulants

Plant biostimulants, according to the European Union regulation (2019/1009), are "products able to stimulate plant nutrition processes independently of the product's nutrient content with the sole

aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: nutrient use efficiency; tolerance to abiotic stress; quality traits; availability of confined nutrients in soil or rhizosphere". In other words, biostimulants can be defined as products able to promote the growth and quality of food crops, vegetables, and fruits when applied in small quantities to the soil or on the foliar surface directly. They may positively affect plant growth by enhancing water uptake, root and shoot growth, tolerance to abiotic stress, protein content in plant tissues, and the activity of several enzymes (Alam et al., 2014; Baglieri et al., 2014; Bulgari et al., 2015; Ertani et al., 2013; Parrado et al., 2008). However, from a legislative point of view, the inclusion of biostimulants in fertilizer legislation has always presented a series of disputes, which, until now, have not been completely clear.

Plant biostimulants include a wide range of natural substances, such as humic acids, protein hydrolysates, seaweed extracts, and beneficial microorganisms, such as mycorrhizal fungi and plant growth-promoting rhizobacteria (Rouphael et al., 2018). According to Colla et al. (2015) and Battacharyya et al. (2015), protein hydrolysates, along with macroalgae seaweed extracts, may be considered natural plant biostimulants. However, an emerging class of compounds, able to stimulate primary and secondary metabolism in plants, is represented by microalgae products. Indeed, different studies have demonstrated the biostimulant effects both of microalgae biomasses and their extracts.

Microalgae biomasses were shown to contain micro- and macronutrients, particularly N, P, and K, and different plant growth-promoting substances, such as auxins, cytokinins, betaines, etc. (Spolaore et al., 2006; Stirk et al., 2013; Tate et al., 2013).

Ronga et al. (2019) reported that the main species of microalgae showing biostimulant effects that are commercially available are *Scenedesmus* spp., *Chlorella* spp., *Acutodesmus* spp., *Isochrysis* spp., *Chaetoceros* spp., *Arthrospira* spp., and *Dunaliella* spp.

Barone et al. (2018) showed that different concentrations of extracts from microalgae *C. vulgaris* or *S. quadricauda* may act as biostimulant in the early stages of sugar beet cultivation by improving root and plant growth, by modulating gene expression related to the nutrient acquisition in sugar beet. Moreover, the effects of *C. vulgaris* and *S. quadricauda* microalgae extracts showed that their application, especially the one of *C. vulgaris*, improve the germination rates of *Beta vulgaris* seeds cv. Shannon and root development, promoting further nutrient acquisition and plant growth (Puglisi, Barone, et al., 2020). Furthermore, Barone et al. (2019) evaluated the response of soil enzymatic activities to the application of living cells of *C. vulgaris* and *S. quadricauda* and their extracts. The authors, in order to evaluate the biostimulant effects of the microalgae, monitored the main enzymatic activities of the soils: fluorescein diacetate hydrolysis, dehydrogenases, acid and alkaline phosphomonoesterase, and urease activities. The microalgal extracts were added to the soil at two different concentrations, 0.5 and 1 mg of dry organic matter of the extracts per kg of soil (w/w); while the living cells of *C. vulgaris* and *S. quadricauda* were added to the soil corresponding to the amount necessary to obtain an extract concentration of 1.5 mg Corg L^{-1}. The results showed that both the microalgae and their extracts positively affected soil biological activity by increasing values of the biochemical index of potential soil fertility.

Puglisi et al. (2020) investigated the potential use of a *S. quadricauda* extract as biostimulant on lettuce seedlings, grown on pumice substrate. The researchers carried out two radical treatments, using a concentration of the microalgal extract corresponding to 1 mg Corg L^{-1}, and evaluated the physiological parameters, chlorophylls, carotenoid, and total protein contents, as well as several plant enzymatic activities involved in primary and secondary metabolisms. The results showed that the *S. quadricauda* extract positively affected the growth of lettuce seedlings, mainly acting at the shoot level, determining an

increase in dry matter, chlorophylls, carotenoids, proteins, and enhancing the activities of several activities (glutamate synthase - GOGAT; glutamine synthase - GS; citrate synthase - CS; malate dehydrogenase - MDH; phenylalanine ammonia lyase - PAL).

La Bella et al. (2021) studied the effects of the foliar application of a *C. vulgaris* extract in lettuce plants, monitoring the morphobiometric parameters, chlorophylls, carotenoids, total protein contents, and several enzymatic activities involved in different biosynthetic pathways. The researchers performed three foliar applications of the microalgal extract, using a concentration of 1 mg Corg L^{-1}, one week apart. The results showed that the *C. vulgaris* extract positively influenced the growth of lettuce seedlings, increasing all the parameters tested, and from a biochemical point of view, primary and secondary metabolisms of shoots, in particular nitrogen metabolism, were positively influenced.

Puglisi et al. (2022) investigated the effects of two different methods of application of a *C. vulgaris* extract, foliar spray and root drenching, on lettuce seedlings, monitoring their morphobiometric parameters and chlorophyll, carotenoid, and total protein contents.

The authors also tested several enzymatic activities involved in primary and secondary metabolisms. In this study two consecutive applications, 1 week apart, of the microalgal extract (1 mg Corg L^{-1}) were performed, and the samples at different times (1, 4, and 7 days after the first treatment and at 7 days after the second treatment) were collected. The results demonstrated that both application methods positively affected the growth of lettuce seedlings, increasing the dry matter, chlorophyll, carotenoid, and protein contents in the edible portion of the plant. From a biochemical point of view, the extract application methods influenced the primary and secondary metabolism by coordinated regulation of C and N metabolic pathways, which may represent the key point in the mechanism of action.

Garcia-Gonzalez and Sommerfeld (2016) evaluated

biostimulant properties of the microalga *Acutodesmus dimorphus* on Roma tomato plants. The researchers tested the influence of the cellular extracts, at different concentrations, growth medium, and culture of *A. dimorphus* on the seed germination. They also evaluated the effects of foliar spray applications of the aqueous extracts, applied in various concentrations. The results showed a positive influence of all treatments on the seed germination: germination energy calculations demonstrated a relationship between increasing extract concentrations and increasing germination energy. The most interesting result regarding germination energy, an increase of 40% compared with the untreated control, was obtained with the extract applied at 100% concentration; while the fastest germination speed at 63% was observed on seeds treated with *A. dimorphus* living culture. With regard to foliar applications, all treatments positively influenced plant growth, leading also to greater flowering. However, foliar spray application at higher concentrations showed a smaller increase in the development of the plants, compared to the other treatments.

Plaza et al. (2018) studied the effects of foliar spray applications with extracts of *Scenedesmus almeriensis* and *A. platensis* hydrolysates on *Petunia x hybrida* plant development and leaf nutrients status. The researchers performed three treatments: foliar application with water; foliar application with *A. platensis* (10 g L^{-1} of biomass); foliar application with *S. almeriensis* (10 g L^{-1} of biomass). The treatments were applied five times. The results of these trials demonstrated positive influences of both microalgae extracts. With regard to biometric parameters, the application of *Arthrospira* and *Scenedesmus* increased root dry weight and flower dry and fresh weight compared with the control. The results also showed that microalgae hydrolysate extracts supply can improve the plant nutrition status, particularly for P, K, Ca, and Mg.

Mutale-joan et al. (2020) investigated the effects of 18 crude bio-extracts (CBEs), obtained by acid hydrolysis, from microalgae and

cyanobacteria on tomato plants (*Solanum lycopersicum* L.) at three different biomass concentrations: 0.1, 0.5, and 1 g L^{-1} under laboratory conditions. The evaluated species were: *Aphanothese* sp., *Arthrospira maxima*, *A. platensis*, *Chlorella pyrenoidosa*, *C. vulgaris*, *Chlorella ellipsoidae*, *C. sorokiniana*, *Chlorella marina*, *Scenedesmus dimorphus*, *S. obliquus*, *Chlamydomonas reinhardtii*, *Dunaliella salina*, *Tetraselmis marina*, *Tetraselmis* sp., *Tetraselmis suecica*, *Porphyridium* sp., *Isochrysis galbana* and *Nannochloropsis gaditana*. The results showed that the application of CBEs to tomato plants improved chlorophyll contents, nutrient uptake, and, in many cases, the root and shoot length and dry weight.

Specifically, *Aphanothese* sp. extracts enhanced root length (112.6%), root (34.8%), and shoot (58.7%) dry weights. The enhanced root lengths also improved nutrient uptake from the soil. With regard to the pigment contents, the maximum increase in chlorophyll b content (92.5%, 92.3%, and 83.9%) across all treatments were observed with *Aphanothece* sp., *A. maxima*, and *C. pyrenoidosa* extracts for freshwater species, respectively, and *Tetraselmis* sp. and *N. gaditana* extracts for seawater species, which increased by 93.3% and 83.9%, respectively, compared with control plants. In this study, the researchers also highlighted the potential of CBEs on many metabolic pathways.

Table 3 reported the microalgae species retrieved from recent literature, used as living cells or extracts, showing a plant biostimulant effect.

Table 3. Biostimulant effects of different microalgae species.

Microalgae Species	Extract/Biomass	Application	Effects	Reference
A. dimorphus	Cellular extracts, growth medium and culture	*Solanum lycopersicum* cv Roma	Improving seed germination. Increasing plant growth through foliar application	(Garcia-Gonzalez and Sommerfeld, 2016)

A. maxima	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)
A. platensis	Hydrolysate extracts	*Petunia x hybrida*	Increasing root dry weight, flower dry weight and fresh weight. Improving plant nutrition status	(Plaza et al., 2018)
A. platensis	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)
Aphanothese sp.	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)
C. ellipsoidae	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)
C. marina	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)
C. pyrenoidosa	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)
C. reinhardtii	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)
C. sorokiniana	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root	(Mutale-joan et al., 2020)

			and shoot length and dry weight	
C. vulgaris	Cellular extracts	*Beta vulgaris* cv Shannon	Improving germination rates and root development	(Puglisi et al., 2020)
C. vulgaris	Cellular extracts	Lettuce seedlings	Increasing dry matter, chlorophylls, carotenoids, proteins, and influencing the activities of several enzymes	(La Bella et al., 2021)
C. vulgaris	Cellular extracts	Lettuce seedlings	Increasing dry matter, chlorophylls, carotenoids, proteins, and influencing the activities of several enzymes	(Puglisi et al., 2022)
C. vulgaris	Cellular extracts and living cells	Application on soil	Increasing values of the biochemical index of potential soil fertility	(Barone et al., 2019)
C. vulgaris	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)
D. salina	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)
I. galbana	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)
N. gaditana	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)

Porphyridium sp.	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)
S. almeriensis	Hydrolysate extracts	*Petunia x hybrida*	Increasing root dry weight, flower dry weight and fresh weight. Improving plant nutrition status	(Plaza et al., 2018)
S. dimorphus	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)
S. obliquus	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)
S. quadricauda	Cellular extracts	*Beta vulgaris* cv Shannon	Improving germination rates and root development	(Puglisi et al., 2020)
S. quadricauda	Cellular extracts and living cells	Application on soil	Increasing values of the biochemical index of potential soil fertility	(Barone et al., 2019)
S. quadricauda	Cellular extracts	Lettuce seedlings	Increasing dry matter, chlorophylls, carotenoids, proteins, and influencing the activities of several enzymes	(Puglisi et al., 2020)
T. marina	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)
T. suecica	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root	(Mutale-joan et al., 2020)

			and shoot length and dry weight	
Tetraselmis sp.	Crude Bio-Extracts (CBEs)	*Solanum lycopersicum*	Improving chlorophyll contents, nutrient uptake, root and shoot length and dry weight	(Mutale-joan et al., 2020)

1.5.2 Biofertilizers

Fertilization is one of the most common agricultural practices used in order to obtain good crop yields. However, the massive use of synthetic fertilizers may cause serious environmental problems (Chien et al., 2009).

The European Parliament has recently launched a new regulation (EU—2019/1009) which defines the "fertilizing product" as "a substance, mixture, microorganism, or any other material, applied or intended to be applied on plants or their rhizosphere or on mushrooms or their mycosphere, or intended to constitute the rhizosphere or mycosphere, either on its own or mixed with another material, for the purpose of providing the plants or mushrooms with nutrient or improving their nutrition efficiency"; therefore the biofertilizers may be defined as products containing living or dormant microorganisms alone or in combination, which help in fixing atmospheric nitrogen or solubilizers soil nutrients in addition to the secretion of growth promoting substances for enhancing crop growth and yield (Dineshkumar et al., 2018).

In this regard, a valid option as biofertilizer may be represented by microalgae, with the potential function to prevent nutrient losses through a gradual release of nitrogen, phosphorous, and potassium (Coppens et al., 2016; Schreiber et al., 2018).

However, microalgal products are considered borderline, showing intermediate effects between biostimulants and fertilizers (Ronga et al., 2019).

Some studies, available in literature, highlight an association among greater nutrient uptake, higher biomass accumulation, and greater crop yields when microalgae are used as biofertilizers (Shaaban, 2001; Faheed and Abdel Fattah, 2008).

Wuang et al. (2016) assessed the applicability of the biomass of *Spirulina platensis* as agricultural fertilizer to leafy vegetables (*Eruca sativa*, *Amaranthus gangeticus*, *B. rapa* ssp. *chinensis*, and *Brassica oleracea alboglabra*). The results showed the ability of *Spirulina* based biofertilizers to enhance plant growth, influencing many biometric parameters and improving the germination process.

Elhafiz et al. (2015) evaluated the effects of the microalgae *C. vulgaris* and *C. pyrenoidosa* on vegetable crops (lettuce, rice, eggplant, and cucumber) in salt-affected soil. For each crop, the authors tested the influence of both microalgal species on seed germination in Petri dishes, and only *C. pyrenoidosa* as biofertilizer for plotted plants. The microalgae were not applied as biomass, but as solution. The results highlighted the possible use of *C. pyrenoidosa* live cells as biofertilizer to promote the growth of vegetable crops in salt soil; indeed, the treated seedlings of the four crops had a positive effect from the biofertilizer and had a major content of chlorophylls and were healthy.

Dineshkumar et al. (2018) analyzed rice growth at different concentrations of microalgae *C. vulgaris* and *S. platensis* and determined their potentiality as biofertilizer application in order to have maximum yield. Both microalgal biomasses positively influenced the main growth parameters of the plants, allowing a reduction of N fertilizer up to 50 or 75% of the recommended dose. The authors also analyzed the seed yield characters of the rice plants, the biological activity, and chemical properties of soil. With regard to the seed yield characters, data obtained showed significant improvements in rice yield parameters. With regard to the biological activity and chemical properties of the soil, the application of microalgae enhanced dehydrogenase activity and nitrogenase, reduced soil pH and electric conductivity,

and increased the availability of macronutrients in soil.

Another interesting example of microalgae soil application is reported for tomato. Coppens et al. (2016) evaluated the potentiality of two types of microalgal biomass, microalgal bacterial flocs, dominated by filamentous microalgae *Ulothrix* sp. and *Klebsormidium* sp. from a raceway pond treating aquaculture wastewater, and a marine culture of *Nannochloropsis* sp. as organic slow-release fertilizers for tomato cultivation. The authors assessed the growth rate of the tomato plants and the tomato yield for each fertilizer treatment, as well as the composition of the leaves and the fruits. The results showed both microalgal fertilizers improved the fruit quality through an increase in sugar and carotenoid content, although a lower tomato yield was obtained. In Table 4 are summarized the microalgae species retrieved from recent literature, used as living cells or extracts, showing a biofertilizer effect.

Table 4. Biofertilizer effects of different microalgae species.

Microalgae Species	Biomass/Solution	Application On	Effects	Reference
C. pyrenoidosa	Solution	*Lactuca sativa* (lettuce)	Improving germination process and salinity tolerance, and enhancing chlorophyll content	(Elhafiz et al., 2015)
C. pyrenoidosa	Solution	*Oryza sp.* (rice)	Improving germination process and salinity tolerance, and enhancing chlorophyll content	(Elhafiz et al., 2015)
C. pyrenoidosa	Solution	*Solanum melongena* (eggplant)	Improving germination process and salinity tolerance, and enhancing chlorophyll content	(Elhafiz et al., 2015)

C. pyrenoidosa	Solution	*Cucumis sativus* (cucumber)	Improving germination process and salinity tolerance, and enhancing chlorophyll content	(Elhafiz et al., 2015)
C. vulgaris	Biomass	*Oryza* sp.	Improving biological activity and chemical properties of the soil and increasing the availability of macronutrients	(Dineshkumar et al., 2018)
Microalgal bacterial flocs	Biomass	*Solanum lycopersicum*	Improving fruit quality through an increase in sugar and carotenoid content	(Coppens et al., 2016)
Nannochloropsis sp.	Biomass	*Solanum lycopersicum*	Improving fruit quality through an increase in sugar and carotenoid content	(Coppens et al., 2016)
S. platensis	Biomass	*Eruca sativa*	Enhancing plant growth and improving germination process	(Wuang et al., 2016)
S. platensis	Biomass	*Amaranthus gangeticus*	Enhancing plant growth and improving germination process	(Wuang et al., 2016)
S. platensis	Biomass	*Brassica rapa* spp. *chinensis*	Enhancing plant growth and improving germination process	(Wuang et al., 2016)
S. platensis	Biomass	*Brassica oleracea alboglabra*	Enhancing plant growth and improving germination process	(Wuang et al., 2016)
S. platensis	Biomass	*Oryza* sp.	Improving biological activity and chemical properties of the soil and increasing the	(Dineshkumar et al., 2018)

	availability of macronutrients

1.5.3 Biopestidices

The implementation of biopesticides in agriculture is an important goal for the development of sustainable agriculture practices (Costa et al., 2019). Pesticides of biological origin can act efficiently in pest control through a variety of mechanisms, such as by inhibiting the growth, nutrition, development, or reproduction of pests or pathogens (Mnif and Ghribi, 2015).

In relation to the production of biopesticides, microalgae and cyanobacteria may be considered as potential biocontrol agents: they exhibit antagonistic effects against many plant pathogens, e.g., bacteria and fungi, mainly as a result of production of hydrolytic enzymes and biocidal compounds such as benzoic acid, majusculonic acid, etc. (Prasanna et al., 2008; Chandel, 2009; Chaudhary et al., 2012; Gupta et al., 2013). Therefore, the addition of microalgae in plant crops may stimulate the response to pathogens through different metabolic processes of plants, such as the activation of enzymes with defense function (Gupta et al., 2013; Renuka et al., 2018).

With regard to the effects of cyanobacteria and microalgae on plant defense mechanisms, many studies have reported their ability to elucidate the antioxidant and pathogenesis related machinery of the plant (Renuka et al., 2018). For instance, Babu et al. (2015) studied the effects of the inoculation with different cyanobacteria (*Anabaena laxa* RPAN8 and *Calothrix* sp.) on the activity of plant defense enzymes in wheat plant. Highest activity of peroxidase, polyphenol oxidase, and phenylalanine ammonia lyase was obtained in the treatments inoculated with *Calothrix* sp.

Therefore, plant–microalgae/cyanobacteria interactions may contribute to improve plant tolerance to different stress conditions (Renuka et al., 2018). The efficient use of these microorganisms as

biochemicals for the control of plant diseases is often associated with the production of biocidal metabolites, which can suppress or kill pathogenic bacteria, fungi, or nematodes (Swain et al., 2017).

Specifically, the use of cyanobacteria has been associated with biocontrol by increasing the defense mechanisms in plants because it tends to stimulate the production and the action of antioxidant compounds (Renuka et al., 2018); while the microalgae can improve pest resistance, exerting nematocidal activity, antimicrobial activity against pathogenic bacteria and fungi, and insecticidal activity. Moreover, microalgae can produce allelochemicals for weed control (El-Mougy and Abdel-Kader, 2013).

In literature, many studies are available on the antifungal activity of microalgae.

Scaglioni et al. (2019) evaluated the ability of microalgae (*Spirulina* sp. and *Nannochloropsis* sp.) extracts to inhibit trichothecene production by *Fusarium* genus. The authors conducted the experiment in vitro in Petri dishes, containing potato dextrose agar (PDA) or PDA and whole grains, and performed different treatments: they evaluated the phenolic extract from each microalga at the concentration of 40 μg mL^{-1}, compared to a control, cultured with only sterile water, and a treatment with the fungicide tebuconazole (0.6 mg mL^{-1}). Each Petri dish was inoculated with an isolate of Fusarium. The results showed that both microalgae extracts have the capacity to inhibit the halo of fungal development in the substrate PDA or wheat grain, but they were less efficient compared to tebuconazole. However, with regard to the production of trichothecenes, the treatments with the phenolic extracts of *Spirulina* sp. and *Nannochloropsis* sp. were more efficient than the fungicide.

Ranglovà et al. (2021) analyzed biopesticide effects of the extracts of *C. vulgaris* MACC-1, growth in two nutrient sources (BG-11 and municipal wastewater), using various bioassays, such as determination of inhibition index. The biopesticide activity of the extracts was

tested against two fungi (*Fusarium oxysporum* f.sp *melonis* and *Rhizoctonia solani*), two oomycetes (*Phytophthora capsici* and *Phytium ultimum*), and four bacteria strains (*Clavibacter michiganensis* subsp. michiganensis, Xanthomonas campestris pv. *vesicatoria*, *Pseudomonas syringae* pv. tomato, and *Pectobacterium carotovorum*). The results showed that the antibacterial and antifungal activity were higher when *C. vulgaris* cultures were grown in urban wastewater as compared to those grown in BG-11; this could be associated with an accumulation of bioactive compounds responsible for antibacterial and even more for antifungal activity.

In Table 5 are summarized the microalgae species retrieved from recent literature, used as living cells or extracts, showing a plant biopesticides effect.

Table 5. Biopesticide effects of different microalgae species.

Microalgae Species	Application	Microorganism Target	Effects	Reference
Anabaena laxa RPAN8	In vivo on *Gossypium hirsutum* F1861 and *Gossypium arboretum* CISA 310	*Rhizoctonia* spp.	Enhancing the levels of defense enzyme activities, reducing mortality, and improving growth and yield	(Babu et al., 2015)
C. vulgaris MACC-1 (cultivated in BG-11)	In vitro	*Fusarium oxysporum* f.sp. *melonis*	Inhibiting microorganism development	(Ranglovà et al., 2021)
C. vulgaris MACC-1 (cultivated in BG-11)	In vitro	*Rhizoctonia solani*	Inhibiting microorganism development	(Ranglovà et al., 2021)
C. vulgaris MACC-1 (cultivated in BG-11)	In vitro	*Phytophthora capsici*	Inhibiting microorganism development	(Ranglovà et al., 2021)
C. vulgaris MACC-1 (cultivated in BG-11)	In vitro	*Clavibacter michiganensis* subsp. *michiganensis*	Inhibiting microorganism development	(Ranglovà et al., 2021)
C. vulgaris MACC-1	In vitro	*Fusarium oxysporum* f.sp. *melonis*	Inhibiting microorganism development	(Ranglovà et al., 2021)

(cultivated in urban wastewater)				
C. vulgaris MACC-1 (cultivated in urban wastewater)	In vitro	*Rhizoctonia solani*	Inhibiting microorganism development	(Ranglovà et al., 2021)
C. vulgaris MACC-1 (cultivated in urban wastewater)	In vitro	*Phytophthora capsici*	Inhibiting microorganism development	(Ranglovà et al., 2021)
C. vulgaris MACC-1 (cultivated in urban wastewater)	In vitro	*Phytium ultimum*	Inhibiting microorganism development	(Ranglovà et al., 2021)
C. vulgaris MACC-1 (cultivated in urban wastewater)	In vitro	*Clavibacter michiganensis* subsp. *michiganensis*	Inhibiting microorganism development	(Ranglovà et al., 2021)
C. vulgaris MACC-1 (cultivated in urban wastewater)	In vitro	*Xanthomonas campestris* pv. *vesicatoria*	Inhibiting microorganism development	(Ranglovà et al., 2021)
C. vulgaris MACC-1 (cultivated in urban wastewater)	In vitro	*Pseudomonas syringae* pv. *tomato*	Inhibiting microorganism development	(Ranglovà et al., 2021)
C. vulgaris MACC-1 (cultivated in urban wastewater)	In vitro	*Pectobacterium carotovorum*	Inhibiting microorganism development	(Ranglovà et al., 2021)
Calothrix sp.	In vivo on *Gossypium hirsutum* F1861 and *Gossypium arboretum* CISA 310	*Rhizoctonia* spp.	Enhancing the levels of defense enzyme activities, reducing mortality, and improving growth and yield	(Babu et al., 2015)
Nannochloropsis sp.	In vitro	*Fusarium graminearum* species complex	Reducing mycelial halo formation and ergosterol production, inhibiting the production of the acetylates and the	(Scaglioni et al., 2021)

			production of trichothecenes	
Spirulina sp.	In vitro	*Fusarium graminearum* species complex	Reducing mycelial halo formation and ergosterol production, inhibiting the production of the acetylates and the production of trichothecenes	(Scaglioni et al., 2021)

1.6 Future perspective

The multifunctionality of microalgae may offer an interesting perspective for the development of new technologies to remediate wastewater, due to their ability to remove organic and inorganic pollutants, meanwhile reducing the costs of production of microalgae biomasses, making the use of microalgae for treating wastewater possible, and reusing the residual biomasses for multipurpose agricultural applications. As a consequence of what is reported in the present review, a proposal for the future perspective may be summarized in Figure 4. The hypothesis to use phycoremediation as secondary or tertiary treatment for wastewater treatment, and the reuse of the produced microalgae biomass, should be confirmed by (i) selection of the microalgae species which guarantee the best depuration efficiency for typology of wastewaters; (ii) microalgae growth in the selected wastewater; (iii) collection of microalgae biomasses at their stationary phase, and separation from water. After these steps, the depurated water and previously performed analysis which confirms the agronomic suitability may be used for irrigation, whereas microalgae biomass may be further processed to obtain products with different characteristics which can define the best agricultural application. An aliquot of the living microalgae biomass may be used to inoculate wastewater again which needs to be depurated by the phycoremediation system. This results in

environmental and agriculture applications of microalgae, in accordance with an urgent worldwide request for an eco-sustainable agriculture.

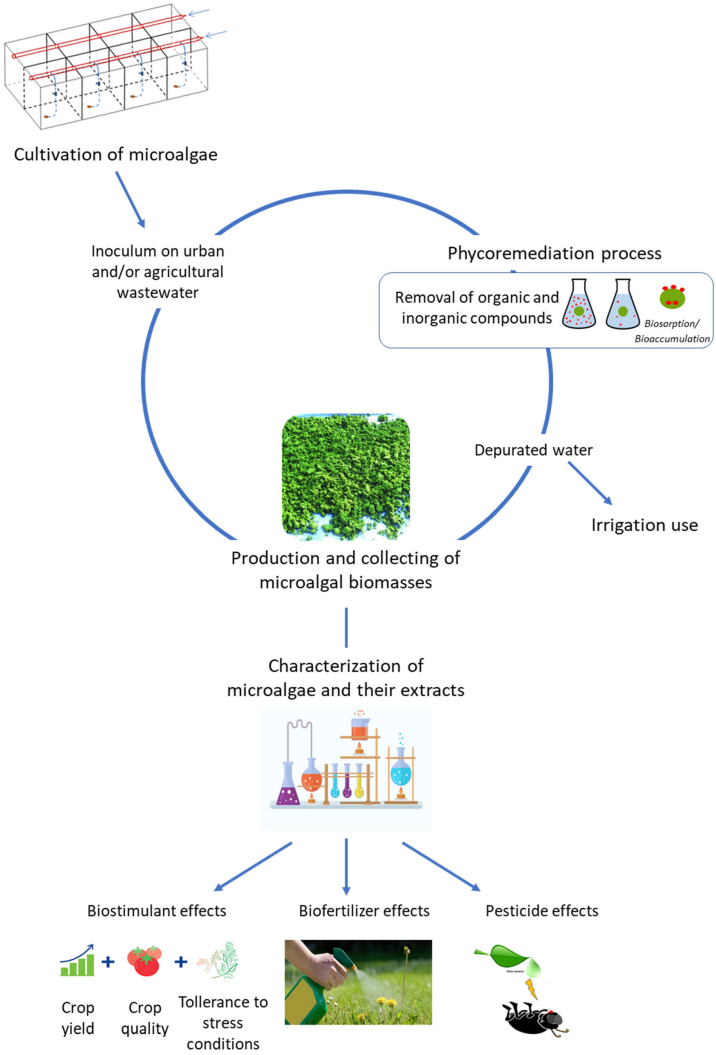

Figure 4. Ideal multipurpose applications of microalgae used for wastewater treatment.

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2 Chapter I

Morpho-biometric and biochemical responses in lettuce seedlings treated by different application methods of *Chlorella vulgaris* extract: foliar spray or root drench? – Experimental activity

Ivana Puglisi[a], Emanuele La Bella[a], Ermes Ivan Rovetto[a], Piergiorgio Stevanato[b], Giancarlo Fascella[c], Andrea Baglieri[a]

[a] Department of Agricultural, Food and Environment, University of Catania, Italy.
[b] Dipartimento di Agronomia, Animali, Alimenti, Risorse Naturali e Ambiente (DAFNAE), Università degli Studi di Padova, Italy.
[c] CREA Research Center for Plant Protection and Certification, Bagheria (Palermo), Italy.

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Abstract

Microalgae-based products applied as biostimulants have recently attracted the attention of researchers. The effect of two different methods of application of a *Chlorella vulgaris* extract, foliar spray and root drenching, was evaluated in lettuce seedlings by monitoring their morpho-biometric parameters and chlorophyll, carotenoid, and total protein contents. The biochemical response, through the study of enzymatic activities involved in primary and secondary metabolism, was also evaluated. Two consecutive applications, 1 week apart, of the *C.*

vulgaris extract were carried out on the lettuce seedlings grown on an inert substrate (pumice) irrigated with Hoagland solution. Lettuce seedlings were then collected at 1, 4, and 7 days after the first treatment and at 7 days after the second treatment. Both application methods positively affected the growth of lettuce seedlings, increasing the dry matter, chlorophyll, carotenoid, and protein contents in the edible portion of the plant. From a biochemical point of view, the extract application methods influenced the primary and secondary metabolism by coordinated regulation of C and N metabolic pathways, which may represent the key point in the mechanism of action. The foliar application mostly influenced the activities of enzymes involved in nitrogen primary metabolism, whereas the root drenching application mainly affected the enzymatic activities involved in carbon primary metabolism. These results are very promising since both application methods of *C. vulgaris* extract acted as a biostimulant on lettuce seedlings, although their mechanism of action seems to be quite different.

2.1 Introduction

Interest in microalgae has recently increased worldwide due to their relevance at the economic and commercial level, as well as for their great versatility (Mata et al., 2010; Hultberg et al., 2013; Ronga et al., 2019). Microalgae are photosynthetic organisms that can be grown under different conditions since they can be autotrophic, heterotrophic, or mixotrophic (Mata et al., 2010). Their biomass is cultivated for different purposes, such as the production of biofuels and biomaterials, and use in the human food and animal feed industries (Plaza et al., 2009; Maurya et al., 2016). Recently, the use of microalgae and their byproducts was also extended to the agricultural field to obtain natural biofertilizers, and biostimulants, as well as to improve the germination process, aiming to attain a sustainable and environmentally friendly agricultural systems (Faheed and Abd-El Fattah, 2008; Elhafiz et al., 2015; Zhang et al., 2017; Barone et al., 2018,

2019a, b; Ronga et al. 2019; Puglisi et al., 2020a, b; La Bella et al., 2021).

The function of green microalgae as bioactive agents in the soil and their ability to improve plant growth make them biofertilizers of considerable practical interest (Chiaiese et al., 2018). *Chlorella vulgaris* and *Chlorella pyrenoidosa* living cells, distributed through irrigation water, can represent a promising biofertilizer able to improve the dry weights and the chlorophyll content in rice, lettuce, cucumber, and eggplant crops (Elhafiz et al., 2015). The application of a mixture of microalgae (MaB-flocs and Nannochloropsis biomass) to the culture medium positively influenced the growth of tomato seedlings at a level comparable to that obtained by using a commercial organic fertilizer (Coppens et al., 2016).

Living microalgae may also exert a biostimulant effect on plants. Living cells of *Scenedesmus quadricauda* and *C. vulgaris* exerted a biostimulant effect on tomato seedlings, when growing them in a co-cultivation microalgae-plant system in hydroponic Hoagland solution (Barone et al., 2019a, b). Similarly, living cells of *C. vulgaris* and *S. quadricauda*, directly applied into the soil, increased the growth parameters of the cultivated tomato plants (Barone et al., 2019a, b).

Several studies have been carried out on the biostimulant effect of microalgal extracts (Faheed and Abd-El Fattah, 2008; Elhafiz et al., 2015; Zhang et al., 2017; Barone et al., 2018, 2019a, b; Chiaiese et al., 2018; Ronga et al. 2019; Puglisi et al., 2020a, b; La Bella et al., 2021). The extraction method used to obtain biologically active compounds from the microalgal biomass is mainly linked to the type of raw material, the molecules which are extracted, and often includes the use of organic solvents (Chiaiese et al., 2018). Seaweed extracts are well-known biostimulant compounds as they have shown to positively affect the physiology of the plant by influencing both the transcriptome and metabolome profiles of the treated plants (Nair et al., 2012; Jannin et al., 2013; Battacharyya et al., 2015). Similarly, a commercial brown

algal extract induced an increase in abundance of transcripts of regulatory enzymes, involved in the nitrogen metabolism and in the antioxidant regulatory system, and an increase in total protein, phenolic, and flavonoid contents in spinach (Fan et al., 2013). Methanolic extracts from the microalgae *C. vulgaris* and *S. quadricauda* exerted a biostimulant effect on sugar beet grown in Hoagland solution at their early stages of growth (Barone et al., 2018) and methanolic extracts of *C. vulgaris* and *S. quadricauda*, when applied directly into the soil, increased the growth parameters of tomato plants (Barone et al., 2019a, b).

Lettuce (*Lactuca sativa* L.) is an important vegetable crop growing in the Mediterranean area, and often the use of biostimulants is required for its cultivation since it is a moderately sensitive crop to salt (Lucini et al., 2015). A formulation composed of *C. vulgaris* and plant growth-promoting bacteria (*Bacillus licheniformis*, *Bacillus megatherium*, *Azotobacter* sp., *Azospirillum* sp., and *Herbaspirillum* sp.) positively affected fresh weight, total antioxidant capacity, and total carotenoid content in lettuce cultivated for spring and summer crop (Kopta et al., 2018). More recently, a methanolic extract of *C. vulgaris* applied on lettuce seedlings by foliar spray positively affected plant growth both at leaf and root levels and positively influenced the primary and secondary metabolism of shoots, in particular nitrogen metabolism (La Bella et al., 2021). A methanolic extract of *S. quadricauda* positively affected the growth of lettuce seedlings, mainly by acting at the shoot level, inducing an increase in dry matter, chlorophyll, carotenoid, and protein contents, and influencing the activities of several enzymes involved in the primary and secondary metabolism of the plant (Puglisi et al., 2020a, b).

Considering that the use of microalgal extracts as biostimulants is a very promising method to obtain sustainable cultivation and reduction in the use of chemicals, this work aimed to compare, in lettuce seedlings, the effects of a methanolic extract of *C. vulgaris* applied by

root drenching or by foliar spray, monitoring morpho-biometric and biochemical effects at different sampling times along the whole experimental period.

2.2 Materials and methods

2.2.1 Microalgae culture and extract preparation

Chlorella vulgaris (CCAP 211/11C) was obtained and maintained in the algal collection of the Department of Agriculture, Food and Environment (Di3A) (University of Catania, Italy). *Chlorella vulgaris* was grown for 46 days (when stationary phase was reached) in a growth chamber in standard BG11 medium (Stanier et al., 1971), bubbled with air and illuminated by a 3500-lx, average photon flux (PPF) 100 µmol photons m^{-2} s^{-1} light source (PHILIPS SON-T AGRO 400) with a 12-h photoperiod (Baglieri et al., 2016). The microalgal biomass was centrifuged and the pellet was washed several times with distilled water to reach a conductivity < 200 µS cm^{-1} (Puglisi et al., 2018).

Chlorella vulgaris extract was obtained as described in detail by Barone et al. (2018). Briefly, the microalgal biomass was harvested, after 46 days of growth, by centrifugation and freeze-dried. The biomass (3.5 g L^{-1} BG11) was then washed with distilled water to reach conductivity values lower than 200 µS cm^{-1}. The pellet (1 g DW) was treated with methanol (12 mL) to lyse the cell wall to obtain the intracellular contents. The lysate was centrifuged and the organic solvent was evaporated. Then, the extract was collected with distilled water to obtain the microalgal extract stock solution (extraction yield: 1.2% DW/DW).

The characterization of the biomass of *C. vulgaris* and its extract was reported in detail in Barone et al. (2018).

2.2.2 Experimental conditions

The experiments were carried out in a transparent container (40

× 20 × 10 cm), filled with pumice as inert substrate (Vanni et al., 2006). Substrate was wetted with 1 L Hoagland solution (Arnon and Hoagland, 1940). In a completely random design, 10 lettuce (*Lactuca sativa* L.) seedlings, at four true leaves, provided by a local nursery in Catania, were transplanted in each container. Experimental trials were composed of five replications for treatment and each replicate was made up of 30 seedlings (each treatment was replicated five times and each replicate included three containers). Lettuce seedlings were acclimatized by growing them for 6 days in a growth chamber at 25 ± 2 °C, with a 16-h photoperiod under natural light (light intensity about 150 µmol photons m^{-2} s^{-1}). Seedlings were irrigated every day with 100 mL distilled water and after the acclimation period, the first treatment was performed. A second treatment was performed 1 week later (Puglisi et al., 2020a, b). By randomly picking 5 plants for each treatment and replicate, 4 samplings were performed over the experimental period, both in treated (Drench and Spray) and untreated plants (control), as follows: T1 (I), 1 day after the first treatment; T4 (I), 4 days after the first treatment; T7 (I), 7 days after the first treatment; T7 (II), 7 days after the second treatment (Fig. 1). Leaf tissues were randomly picked at each sampling time and immediately frozen with liquid nitrogen and stored at − 80° C until further use.

Chlorella vulgaris extract, to a final concentration of 1 mg Corg L^{-1} (this concentration was chosen taking into account the results previously obtained in Barone et al. (2018, 2019a) and La Bella et al. (2021)), was applied following two different procedures: root drenching or foliar spray applications. The root drenching treatment (Drench) was performed by irrigating the substrate with a solution of Hoagland (500 mL) containing 1 mg Corg L^{-1} *C. vulgaris* extract, whereas the control plants received 500 mL Hoagland solution. The foliar spray application (Spray) was performed by spraying the seedlings with a solution of Hoagland (500 mL) containing 1 mg Corg L^{-1} *C. vulgaris* extract, whereas the control plants were sprayed with 500 mL

Hoagland solution. The two controls, as described above, were reported as only one control in all tables and figures, as mean of all values. The seedlings were then grown for 14 days in a growth chamber at 25 ± 2 °C, with a 16-h photoperiod, being irrigated every day with 100 mL distilled water, according to the experimental condition described in Puglisi et al. (2020a, b).

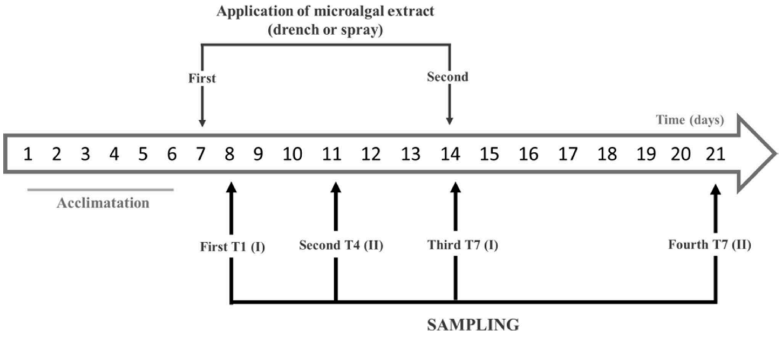

Figure 1. Timeline of the experimental trial.

2.2.3 *Morpho-biometric parameters in lettuce seedlings*

Lettuce seedlings were collected at each sampling time, immediately divided into roots and shoot, and the fresh weight (FW) was separately measured. Leaves and root length were measured by using a digital ruler to the nearest 0.5 mm and the leaf number for each seedling was recorded.

Dry weights (DW) were obtained for each seedling (leaves and root) by placing tissues in a drying oven at 105 °C until constant weight was reached, each sample was allowed to cool for 2 h inside a closed bell jar, and then the dry weight of leaves and roots were measured separately.

All parameters were determined on 5 seedlings for each treatment and replica.

2.2.4 *Chlorophyll and total carotenoid determination*

Chlorophyll *a*, chlorophyll *b*, and carotenoids were photometrically determined in lettuce leaves according to Sumanta et al. (2014). Briefly, leaf tissue samples (0.5 g FW) were homogenized using 10 mL 80% ace tone as extraction solvent. Samples were centrifuged at 10,000 rpm for 15 min at 4° C, and then an aliquot of supernatant (0.5 mL) was mixed with 4.5 mL 80% acetone. Samples were measured at three different wavelengths, 470 nm, 646.8 nm, and 663.2 nm (Jasco V-530 UV–vis spectrophotometer). Then, the relative amounts of chlorophyll *a* (Ch-*a*), chlorophyll *b* (Ch-*b*), and total carotenoids (C), expressed as mg g^{-1} leaf dry weight (DW), were calculate as follows:

$$\text{Ch-}a = 12.25A_{663.2} - 279A_{646.8}$$
$$\text{Ch-}b = 21.5A_{646.8} - 5.1A_{663.2}$$
$$C = (1000A_{470} - 1.82\text{Ch-}a - 85.02\text{Ch-}b)/198$$

2.2.5 Total protein extraction from lettuce leaves

Total protein extraction from leaves of lettuce was performed according to Puglisi et al. (2020a, b). Briefly, frozen lettuce tissues were homogenized using an extraction buffer containing 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM cysteine, and 5 mM HEPES–KOH pH 7.5, in a 1:1.25 w/v ratio. The homogenate was filtered and centrifuged at 13,000 rpm for 30 min at 4° C, the supernatant was then recovered, and total proteins were precipitated with solid (NH4)2SO4 at 55% of saturation. The total protein content, expressed as mg protein g^{-1} DW, was quantified according to the Bradford (1976) method.

2.2.6 Enzyme activities

All the enzymatic activities were performed by using an aliquot (1 mL) of the total protein extract containing the enzymes obtained as previously described. The enzymatic aliquot was centrifuged at 13,000 rpm for 30 min at 4° C, the supernatant was discarded, and the pellet

was dissolved in the lowest volume as possible with the appropriate buffer for each enzymatic activity.

Glutamate synthase (GOGAT) activity was performed according to Avila et al. (1987). Briefly, in a final volume of 1.1 mL, the assay mixture was made of 25 mM HEPES–NaOH (pH 7.5), 2 mM L-glutamine, 1 mM α-ketoglutaric acid, 0.1 mM NADH, 1 mM Na2EDTA, and 100 µL enzyme extract. GOGAT activity was determined by a spectrophotometer (Jasco V-530 UV–vis spectrophotometer), monitoring NADH oxidation at 340 nm and using a molar extinction coefficient of 6220 L mol^{-1} cm^{-1}. GOGAT activity was expressed as nmol NAD^+ mg^{-1} protein min^{-1}. Glutamine synthetase (GS) activity was performed as transferase activity according to Canovas et al. (1991). The assay mixture, in a final volume of 750 µL, contained 90 mM imidazole–HCl (pH 7.0), 60 mM hydroxylamine (neutralized), 20 mM KAsO4, 3 mM MnCl2, 0.4 mM ADP, 120 mM glutamine, and 100 µL enzyme extract. The enzymatic reaction was incubated for 15 min at 37 °C, and then added 250 µL of a mixture (1:1:1) of 10% (w/v) FeCl3·6H2O in 0.2 M HCl, 24% (w/v) trichloroacetic acid, and 50% (w/v) HCl. The γ-glutamyl hydroxamate produced during the reaction was spectrophotometrically quantified at 540 nm, using a standard curve of γ-glutamyl hydroxamate. GS activity was expressed as µmol γ-glutamyl hydroxamate mg^{-1} protein min^{-1}.

Citrate synthase (CS) activity was performed according to Schiavon et al. (2008). In a final volume of 3 mL, the assay mixture contained 50 µL 0.17 mM oxalacetic acid, 50 µL of 0.2 mM acetyl coenzyme A (acetyl-CoA), and 100 µL enzyme extract in 0.1 M Tris–HCl, pH 8.0. The activity was spectrophotometrically determined by following the reduction of acetyl-CoA to CoA, at 232 nm using a molar extinction coefficient of 5400 L mol^{-1} cm^{-1}. CS activity was expressed as nmol CoA mg^{-1} protein min^{-1}.

Malate dehydrogenase (MDH) activity was measured as described in Schiavon et al. (2008). In a final volume of 1 mL, the assay

mixture contained 94.6 mM phosphate buffer pH 6.7, 0.2 mM NADH, 0.5 mM oxalacetic acid, 1.67 mM $MgCl_2$, and 100 µL enzyme extract. The activity was spectrophotometrically measured by monitoring NADH oxidation at 340 nm using a molar extinction coefficient of 6220 L mol^{-1} cm^{-1}. MDH activity was expressed as nmol NAD^+ mg^{-1} protein min^{-1}.

Phenylalanine ammonia-lyase (PAL) activity was performed as described in Mori et al. (2001). In a final volume of 1 mL, the assay mixture was made of 0.4 mL 100 mM Tris–HCl buffer (pH 8.8), 0.2 mL of 40 mM phenylalanine, and 200 µL enzyme extract. The reaction was developed for 30 min at 37 °C and then stopped with 200 µL 25% (v/v) TCA. After centrifugation at 10,000 rpm for 15 min at 4 °C, the absorbance of the supernatant was measured at 280 nm. PAL activity was calculated by using a molar extinction coefficient of 16,890 L mol^{-1} cm^{-1} and was expressed as nmol cinnamic acid mg^{-1} protein min^{-1}.

All enzymatic activities were repeated using 3 replicates, by following separated extraction. Each extraction was performed on tissues sampled from 5 plants, for each treatment and replicate. Protein concentration in each enzyme aliquot was measured by using the Bradford method (1976).

2.2.7 Statistical analysis

Data were analyzed by one-way ANOVA ($P < 0.05$) followed by Tukey's test for multiple comparison procedures, using the Statistica package software (version 10; Statsoft Inc., USA). The arithmetic means were calculated by averaging the values determined for the single replicates of each treatment. Data of the two controls (water spray and drench) were previously statistically analyzed and they showed no significant difference among them (data not shown). Therefore, for each parameter, data are shown as the arithmetic means by averaging the values of the two controls.

2.3 Results

2.3.1 Morpho-biometric parameters of lettuce seedlings

Chlorella vulgaris extract positively affects all the morphological traits of lettuce seedlings, whatever the method of application, being the total plant weights of treated plants always significantly greater than controls (Table 1). In particular, at the shoot height level, both the treatment methods act after 1 day (T1 (I)), sampling time in which the highest increment with respect to the control (17% Drench and 13% Spray, respectively) was recorded, although at the end of the experimental period (T7 (II)), height values of treated plants were similar to those measured in the controls (Table 1). At T4 (I) and T7 (I) sampling times, the number of leaves was significantly higher than the control in both application methods. The root length of the seedlings treated by root drenching application, already at the first sampling time (T1 (I)), was significantly increased with respect to the control (around 24%) and higher values than controls were maintained all over the experimental period, to reach at T7 (I) the highest increment with respect to the control (around 55%) (Table 1). The effect of the foliar spray treatment on root length was lower and in treated plants values were significantly higher than the controls, starting from T4 (I) sampling time (Table 1).

Both application methods significantly increased leaf fresh and dry weights of seedlings from T4 (I) until the end of the experimental time (Fig. 2). Both treatment methods influenced the morphological traits of the aerial portion (corresponding to the edible part of the plant) mainly at the weight level (Fig. 2) rather than at the plant height degree (Table 1). At the shoot level, the spray resulted to be the best treatment, reaching at T7 (I) the highest increase as compared to the control of around 56% in fresh weight (Fig. 2A) and around 74% in dry weight (Fig. 2B).

The fresh weights of the root seedlings treated by root

drenching application (Fig. 2C), already at the first sampling time (T1 (I)), were significantly increased with respect to the control (around 30%), and they were maintained all over the experimental period to reach at T7 (I) the highest increments (around 53%). Although to a lesser extent than root treatment, in the foliar spray treatment, the weights of roots in treated plants were significantly higher than those in the controls starting from T4 (I) sampling time (Fig. 2C). The dry weights of the root seedlings of lettuce (Fig. 2D) were not significantly influenced by both the treatments, except that at T4 (I), in which the root dry weights of treated plants were higher than values recorded in the controls (Fig. 2D).

Table 1. Morphological traits of lettuce seedlings subjected to *Chlorella vulgaris* extract treatment by root drenching application (Drench) and foliar spray application (Spray) at each sampling time (T1 (I), 1 day after the first treatment; T4 (I), 4 days after the first treatment; T7 (I), 7 days after the first treatment; T7 (II), 7 days after the second treatment). Data are means ± SD. The values are means of data from five plants for each replica. Values in the same column for the same sampling time followed by different letters are significantly different ($P < 0.05$, Tukey's test). The absence of letters shows the lack of significant differences.

Sampling	Treatment	Plant weight (g)	Shoot height (cm)	Leaves (number)	Root length (cm)
T1 (I)	Control	7.3 ± 0.21 b	15 ± 1.10 b	7 ± 1	9.93 ± 0.40 b
	Drench	8.07 ± 0.27 a	17.5 ± 1.20 a	7 ± 1	12.33 ± 1.61 a
	Spray	8.49 ± 0.38 a	17 ± 0.80 a	7 ± 1	10.17 ± 1.26 b
T4 (I)	Control	7.95 ± 0.23 b	17.26 ± 0.25 b	8 ± 1 b	10.05 ± 1.40 b
	Drench	9.67 ± 0.31 a	19.23 ± 0.40 a	9.66 ± 0.58 a	13.10 ± 1.20 a
	Spray	9.89 ± 0.20 a	18.27 ± 0.40 a	10.67 ± 1.15 a	14.18 ± 1.30 a
7 (I)	Control	7.99 ± 0.27 c	18.05 ± 0.78 b	11.50 ± 0.71 b	10 ± 1.41 b
	Drench	10.40 ± 0.20 b	21.25 ± 1.06 a	13 ± 0 a	15.50 ± 0.71 a
	Spray	12.22 ± 0.22 a	20.30 ± 0.42 a	12 ± 0 a	12.8 ± 1.12 a
7 (II)	Control	11.63 ± 0.16 b	21.10 ± 0.54	12.50 ± 0.71	11.25 ± 1.06 c
	Drench	13.41 ± 0.15 a	20.50 ± 1.12	12.50 ± 0.71	15.25 ± 1.06 a
	Spray	13.47 ± 0.16 a	20.50 ± 0.71	13 ± 0	13.50 ± 0.42 b

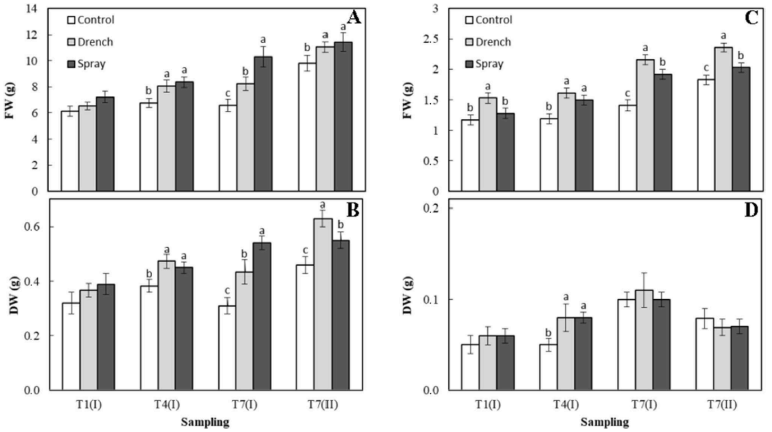

Figure 2. Fresh (A) and dry (B) weights of shoots of lettuce seedlings; fresh (C) and dry (D) weights of roots of lettuce seedlings. Error bars indicate standard deviation. The values are the means of data from five replications. Extract treatments were performed by drenching root application (Drench) and foliar spray application (Spray) at each sampling time (T1 (I), 1 day after the first treatment; T4 (I), 4 days after the first treatment; T7 (I), 7 days after the first treatment; T7 (II), 7 days after the second treatment). Values within each sampling time followed by different letters are significantly different ($P < 0.05$, Tukey's test). The absence of letters above the columns shows the lack of significant differences.

2.3.2 Protein and pigment contents

The total protein content (Fig. 3) was significantly influenced by the foliar spray treatment throughout the experiment, reaching an increase of around 49% with respect to the control soon after the first day of treatment (T1 (I)). On the contrary, the root treatment increased the values of total protein starting from T7 (I), reaching values similar to those measured in the foliar treatment, and maintaining them until the end of the experiment (Fig. 3).

All pigments, in particular chlorophyll *a* and total carotenoids, at all sampling times, showed values always significantly higher than the respective controls (Table 2), except chlorophyll *b* at T1 (I) in

seedlings treated by foliar spray and at T7 (I) in seedlings treated by root drenching, recording values always similar to the controls (Table 2).

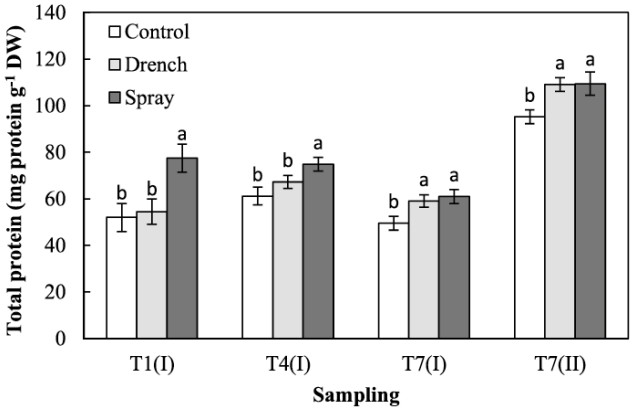

Figure 3. Total protein content in leaves of lettuce seedlings subjected to *Chlorella vulgaris* extract treatment. Error bars indicate standard deviation. The values are the means of data from five replications. Extract treatments were performed by root application (Drench) and foliar application (Spray) at each sampling time (T1 (I), after 1 day from the first treatment; T4 (I), after 4 days from the first treatment; T7 (I), after 7 days from the first treatment; T7 (II), after 7 days from the second treatment). Values within each sampling time followed by different letters are significantly different ($P < 0.05$, Tukey's test).

Table 2. Chlorophyll and total carotenoid contents in leaves of lettuce seedlings subjected to *Chlorella vulgaris* extract treatment by drenching root application (Drench) and spray foliar application (Spray) at each sampling time (T1 (I), 1 day after the first treatment; T4 (I), 4 days after the first treatment; T7 (I), 7 days after the first treatment; T7 (II), 7 days after the second treatment). Ch-*a*, chlorophyll *a*; Ch-*b*, chlorophyll *b*; C, total carotenoids. Data are means ± SD. The values are the means of data from five replications. Values in the same column for the same sampling time followed by different letters are significantly different (P < 0.05, Tukey's test).

Sampling	Treatment	Ch-*a* (mg g^{-1}DW)	Ch-*b* (mg g^{-1}DW)	C (mg g^{-1}DW)
T1 (I)	Control	0.323 ± 0.032 b	0.293 ± 0.025 b	0.098 ± 0.010 b

	Drench	0.467±0.030 a	0.363±0.021 a	0.130±0.015 a
	Spray	0.424±0.025 a	0.257±0.020 b	0.135±0.020 a
T4 (I)	Control	0.405±0.025 c	0.249±0.025 c	0.142±0.020 b
	Drench	0.919±0.031 a	0.520±0.032 a	0.234±0.025 a
	Spray	0.829±0.031 b	0.438±0.025 b	0.227±0.020 a
T7 (I)	Control	0.546±0.030 b	0.466±0.031 b	0.144±0.015 c
	Drench	0.765±0.040 a	0.489±0.025 b	0.194±0.020 b
	Spray	0.714±0.033 a	0.544±0.032 a	0.231±0.021 a
T7 (II)	Control	0.463±0.028 b	0.180±0.022 b	0.172±0.015 b
	Drench	0.632±0.032 a	0.255±0.030 a	0.222±0.020 a
	Spray	0.588±0.025 a	0.214±0.026 a	0.223±0.025 a

2.3.3 Enzyme activities in lettuce seedlings

At all the sampling times, enzyme activities in the treated plants were always significantly higher than those measured in the controls (Fig. 4). Both application strategies of *C. vulgaris* extract immediately induced the activation of GOGAT activity, reaching the highest increase with respect to untreated plants at T1 (I), showing values around 9 and 8 times greater than those recorded in the controls, for foliar and root application, respectively. Between the two application strategies, at the end of the experimental time (T7 (II)), foliar spray treatment reached GOGAT activity values greater than those measured in plants subjected to root drenching (Fig. 4A).

GS activity was always significantly higher in all treated plants than in the control (Fig. 4B), at all sampling times, except that at T7 (II), in which the GS activity measured in plants treated by root drenching was similar to the untreated plants.

As regards CS, both treatments with *C. vulgaris* extract significantly increased the activity compared to untreated plants at all monitoring times (Fig. 5A). Nevertheless, between the two application methods, some differences were detected. Root drenching treatment seems to act on CS activity to a greater extent soon after the first treatment, inducing an increase of around 2.5 times at T1 (I) compared to

the control. Conversely, foliar spray application induced the greatest increase, with respect to the control, at T4 (I) (around 4.1 times), maintaining values always significantly higher than those measured in plants treated by root drenching, also for all the other sampling times (Fig. 5A).

The root application positively affected MDH activity (Fig. 5B) soon after the first application (T1 (I)), reaching at this sampling time the highest increase with respect to the control (around 5.5 times), and maintaining values always greater than those measured in the controls and the treated plants by foliar spray, over all the experimental period. Conversely, the foliar application influenced the MDH activity (around 1.7 times higher than the control) only at the first sampling time (T1 (1)), whereas at all other sampling times, MDH activities were maintained at values always similar to those measured in the control plants.

PAL activity was always significantly higher than the control in all treated plants at all sampling times, except that at T1 (I), in which the activity measured in plants treated by root drenching was similar to the untreated plants (Fig. 6).

A hypothesis of mechanism for foliar spray and root drench applications is proposed and illustrated (Fig. 7).

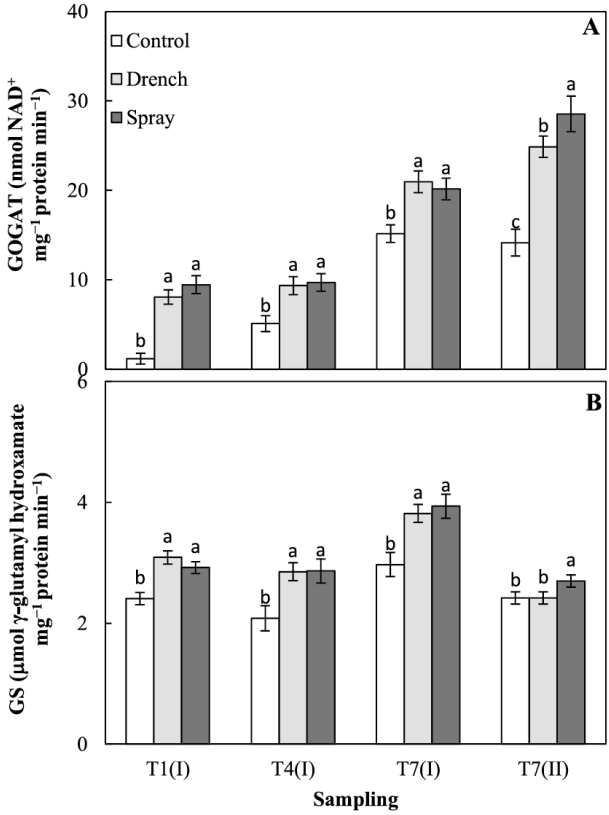

Figure 4. Glutamate synthase (GOGAT) activity (A) and glutamine synthase (GS) activity (B) in leaves of lettuce seedlings. Error bars indicate standard deviation. *Chlorella vulgaris* extract treatments were performed by root drenching application (Drench) and spray foliar application (Spray) at each sampling time (T1 (I), 1 day after the first treatment; T4 (I), 4 days after the first treatment; T7 (I), 7 days after the first treatment; T7 (II), 7 days after the second treatment). The values are the means of data from five replications. Values within each sampling time followed by different letters are significantly different ($P < 0.05$, Tukey's test).

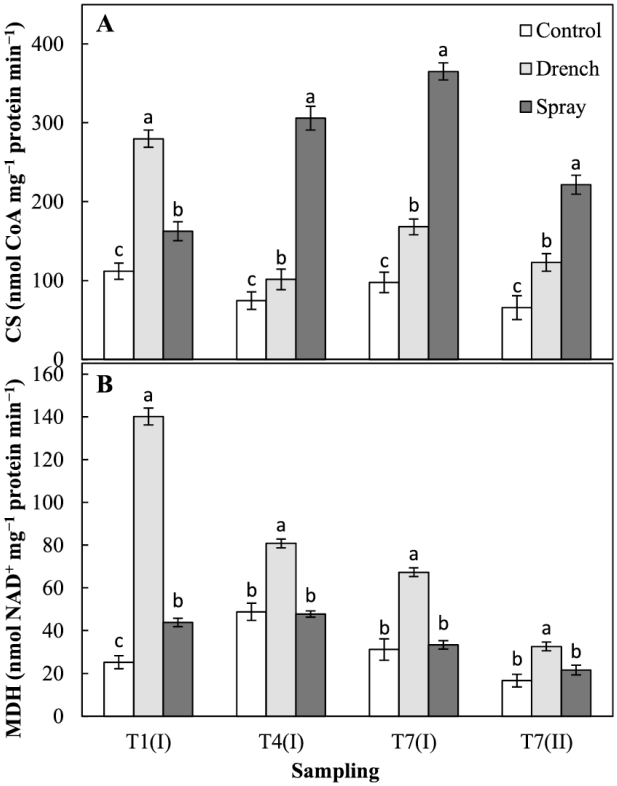

Figure 5. Citrate synthase (CS) activity (A) and malate dehydrogenase (MDH) activity (B) in leaves of lettuce seedlings. Error bars indicate standard deviation. *Chlorella vulgaris* extract treatments were performed by root drenching application (Drench) and foliar spray application (Spray) at each sampling time (T1 (I), 1 day after the first treatment; T4 (I), 4 days after the first treatment; T7 (I), 7 days after the first treatment; T7 (II), 7 days after the second treatment). The values are the means of data from five replications. Values within each sampling time followed by different letters are significantly different ($P < 0.05$, Tukey's test).

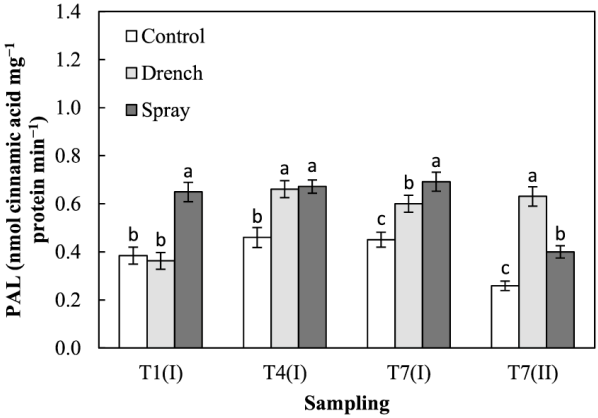

Figure 6. Phenylalanine ammonia-lyase (PAL) activity in leaves of lettuce seedlings. Error bars indicate standard deviation. Extract treatments were performed by root drenching application (Drench) and foliar spray application (Spray) at each sampling time (T1 (I), 1 day after the first treatment; T4 (I), 4 days after the first treatment; T7 (I), 7 days after the first treatment; T7 (II), 7 days after the second treatment). The values are the means of data from five replications. Values within each sampling time followed by different letters are significantly different ($P < 0.05$, Tukey's test).

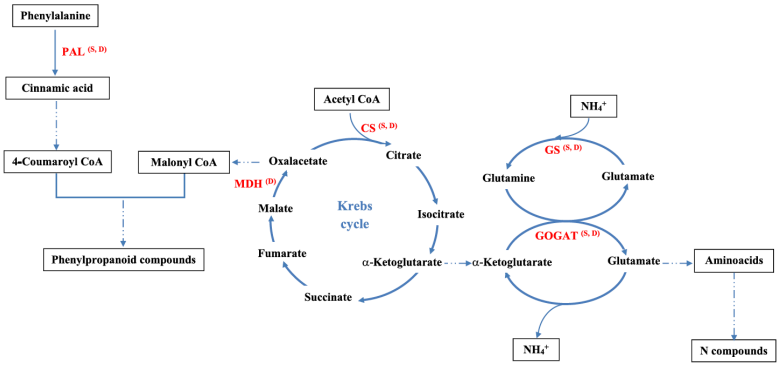

Figure 7. Schematic representation of the biochemical pathways induced by *C. vulgaris* extract treatments involving primary and secondary metabolism. In apex are reported the application methods involved in the increased activity of each enzyme (S, spray foliar application; D, drench root application).

2.4 Discussion

Several studies have evaluated the effect of microalgae extracts on a wide range of crops (Ronga et al., 2019). The foliar application of 60% *Nostoc* sp. water extracts (obtained by means of ultrasonic treatment) showed a biostimulant effect on lettuce seedlings treated every 7 days up to 9 weeks, increasing shoot and root length, fresh biomass, dry biomass, and chlorophyll and carotenoid contents with respect to control plants (Silambarasan et al., 2021). Lettuce seedlings, treated with an *S. quadricauda* extract at the root level with the concentration of 1 mg Corg L^{-1}, increased their leaf fresh and dry weights of around 22% and 27%, respectively (Puglisi et al., 2020a, b). Similarly, tomato plants, grown in pots of soil for 18 days, and treated by soil application of the *C. vulgaris* extract at the concentration 1 mg Corg L^{-1}, increased their leaf dry weight of around 33% with respect to the control (Barone et al., 2019a, b). In the early stages of plant growth in sugar beet, the addition of *C. vulgaris* extract (1 mg Corg L^{-1}) to the Hoagland solution significantly increased the total root length (Barone et al., 2018). The present results showed that the *C. vulgaris* applications (both spray and drench) were a promising treatment also in lettuce, with an increase in fresh and dry weights at the level of the edible part, already starting 4 days from the first treatment (Fig. 2A and B).

An increase in shoot height and number of leaves in tomato plants treated by foliar spray every 5 days with 40% cellular aqueous extract of an algal consortium (*Chlorella* sp., *Scenedesmus* sp., *Synechocystis* sp., and *Spirulina* sp.) occurred after 10 days of growth (Supraja et al., 2020). The present results showed better performances than those observed in tomato, as both the drench and foliar treatments with *C. vulgaris* extract showed a biostimulant effect on lettuce seedlings, having a positive influence on all their morphological traits, biostimulating the growth already after 1 day from the first treatment (Table 1) whereas at the root level (length and fresh weight), the

drench application resulted in more effectiveness than the foliar spray treatment, probably due to the direct addition near the roots (Table 1 and Fig. 2).

An increase in leaf protein content occurred in lettuce seedlings treated at the root level by performing two consecutive treatments with *S. quadricauda* extract (Puglisi et al., 2020a, b). Similarly, both the application methods positively affected the total protein content; however, the effect is quite different in terms of time, with the foliar spray affecting protein accumulation soon after the first day of application, probably due to the direct treatment on leaves, whereas protein increase in leaves started after 7 days from the first treatment in root drenching (Fig. 3). This increased protein content is probably necessary to support the increased growth of plants subjected to treatment (Taiz et al., 2018). On a wide range of crops (spinach, tomatoes, lettuce, etc.), an increase in chlorophyll content was observed in those plants treated with algae extracts (Spinelli et al., 2010; Fan et al., 2013; Ronga et al., 2019; Puglisi et al., 2020a, b; Supraja et al., 2020; La Bella et al., 2021). Chlorophyll content of plants is a measure of plant overall growth as it is essential for photosynthesis whereby plants derive energy for their growth, metabolism, and reproductive processes (Taiz et al., 2018). In accordance with previous studies, pigment content showed values significantly higher than the respective controls at all sampling times (Table 2). Both the strategies of application of *C. vulgaris* extract improved chlorophyll *a* content in a similar way, already after the first day of application. The pigments of light harvesting systems (chlorophyll *b* and carotenoids) were also increased (Table 2), thus enhancing the ability of the photosystems to intercept light and to transfer the absorbed energy to the reaction centers (Taiz et al., 2018). Carotenoids are also important antioxidant pigments, showing an essential role not only in photosynthesis, but also in plant defense against pathogens (Supraja et al., 2020). Pigments of the antenna complexes are mainly made up of chlorophyll *b*, xanthophylls, and

carotenoids, whereas chlorophyll *a* is the key chlorophyll molecule in the reaction center (Taiz et al., 2018; Yahia et al., 2018). The chlorophyll *a* and *b* ratio can be used as an indicator of N partitioning in leaves because this value should be positively correlated with the ratio of PSII cores to allow the light capture by chlorophyll-protein complex (Kitajima and Hogan, 2003). Accordingly, the chlorophyll *a* and *b* ratios of treated plants were rather constant within all the experimental period (data not shown), showing a good N partition in the leaves.

The evidence that the interception of solar radiation, as well as the improvement in carbon fixation, is strictly related to the increase in yield and biomass in the most important crops (Murchie et al., 2009) suggests that the increase in fresh and dry weights of leaves in treated lettuce seedlings was probably linked to the increased content of soluble compounds in the leaves, such as proteins and pigments.

The GOGAT and GS isoenzymes have been proposed to play an important role in primary nitrogen assimilation through ammonium incorporation into carbon skeletons (Lea, 1993; Gupta et al., 2012). In plants ammonia is assimilated into an organic form as glutamine and glutamate, representing the nitrogen donors in the biosynthesis of amino acids, nucleic acids, and other nitrogen compounds such as chlorophylls (Lea 1993; Gupta et al., 2012). Water-soluble metabolites contained in microalgae extract can also enter and disperse along the whole plant through translocation, by opening and closing of stomatal pores of the leaves (Ronga et al., 2019). The involvement of N metabolism in the enhanced growth was also observed by treating lettuce seedlings with a *S. quadricauda* extract (1 mg Corg L^{-1}) at the root level, where GOGAT activity increased around 11 times than the control only 1 day after the root treatment (Puglisi et al., 2020a, b). The biostimulant ability of protein hydrolysates and humic-like substances from agro-industrial residues increases the GOGAT and GS activities in two studies, carried out on maize (Schiavon et al., 2008; Ertani et al., 2013). In spinach treated with a commercial algae-based

extract, the increase of total soluble proteins was strictly associated with an increase at the transcription level of regulatory enzymes involved in nitrogen metabolism (Fan et al., 2013). Microalgal extracts applied as foliar spray increased N content in root and shoot tissues by improving nutrient uptake and by regulating the physiological plant mechanisms (Shaaban, 2001a, b; Ronga et al., 2019). In the present work, enzyme activities related to N metabolism in the treated plants were always significantly higher than those in the control (Fig. 4). These results suggest that greater nitrogen absorption as well as a putative increase uptake of the nutrients at the root level, already after two weekly applications, may be involved in enhancement of (i) total proteins at the shoot level (Fig. 3), (ii) photosynthetic pigments (Table 2), and (iii) dry weights (Fig. 2).

As regards carbon metabolism, root drenching application seems to mainly act at the level of the Krebs cycle, showing a simultaneous increase of both CS and MDH enzymes. A similar biochemical response was observed in maize, in which a protein hydrolysate promoted both the activities of CS and MDH, via coordinated regulation of C and N metabolism (Schiavon et al., 2008; Nardi et al., 2016). These results underline that CS represents the most important key enzyme of the Krebs cycle, catalyzing the reaction which controls the regulation of cellular respiration (Alisdair et al., 2004; Taiz et al. 2018). The present results suggest that between the two application methods, a quite different mechanism of action may occur. It is likely that the increase of CS activity in plants treated by foliar spray application could be strictly related to the formation of α-ketoglutarate as a precursor in the GS-GOGAT pathway, supporting the synthesis of N compounds. A coordinated expression level of CS and GS was observed in N-starved tobacco plants after nitrate resupply (Hodges, 2002). These biochemical results on primary metabolism, taken together, suggest that both application methods of *C. vulgaris* extract greatly influenced lettuce growth. However, root application acts both

on nitrogen metabolism and the respiratory metabolism of leaf cells, exerting its effect mostly at the carbon metabolism level, already after 1 day from the first application (Fig. 5). As regards the foliar treatment, it seems to mainly influence nitrogen metabolism (Fig. 4). These findings may be justified by the different mode of action of the *C. vulgaris* extract due to its application, considering that it was shown that also other microalgal extracts applied as a foliar spray mostly affect nitrogen plant metabolism (Shaaban, 2001a, b; Ronga et al., 2019).

The treatments with algae-based extracts activate secondary metabolism by enhancing the biosynthetic pathway of plant defense compounds such as flavonoids and phenylpropanoid (Battacharyya et al., 2015). PAL represents a key role in secondary metabolism of plants (Rani et al., 2012), because it is the key enzyme in the first step of the phenylpropanoid pathway. *Scenedesmus quadricauda* extract positively affects PAL activity when applied at the root level of lettuce seedlings starting from 4 days after the first treatment (Puglisi et al. 2020a, b). Extracts of *C. vulgaris* and *S. quadricauda* in sugar beet (*Beta vulgaris* L. ssp. *vulgaris*), at the molecular level, upregulated some genes linked to secondary metabolism (Barone et al., 2018). Between the two methods of extract application, results suggest that the foliar spray immediately acts on PAL activity (1 day after the first application), whereas root application shows a slight delay in its effect of 4 days (Fig. 6), thus showing that the two methods are also characterized by a different timing effect.

From a metabolic point of view, the foliar spray application of *C. vulgaris* extract positively affects enzymatic activities related to nitrogen (GS and GOGAT), carbon (CS), and secondary (PAL) metabolisms (Fig. 7). A hypothesis of mechanism may envisage that the foliar spray application could increase the CS activity to provide the α-ketoglutarate as a precursor for the GS-GOGAT pathway, suggesting that the metabolic pathway of nitrogen may play a key role in the mechanism of action. Conversely, the root drenching application of

the *C. vulgaris* extract increased GS and GOGAT, as well as strongly influencing other enzymes involved in the Krebs cycle such as CS and MDH, thus suggesting that a coordinated regulation mechanism of the metabolic pathways of carbon and nitrogen may play an important role in the balance of the N/C ratio in the cells. This latter may represent the key points in the mechanism of action of the extract applied by root drenching. Both application methods of the extract also induced secondary metabolism by increasing PAL activity. In the case of the root drenching application, intermediate compounds which can enter in the phenylpropanoid pathway may also be supplied by the Krebs cycle through oxalacetate accumulated by MDH activity and its further transformation into malonyl CoA.

2.5 Conclusion

The use of microalgae as a plant biostimulant is capturing the interest of farmers as well as agrochemical industries to improve yield and quality as well as the sustainability of crop production. This work represents the first study regarding a comparison survey of application methods between foliar spray and root drenching of *C. vulgaris* extract, as well as their effect on morpho-biometric and biochemical responses of lettuce seedlings at different sampling times.
Although both application methods positively affect primary (C and N) and secondary metabolism by activating the monitored key enzymes, the biochemical response suggests that the foliar treatment acts mostly by influencing nitrogen metabolism, whereas root drenching seems to mainly affect Krebs cycle enzymes. Among application methods, foliar spray seems to determine the best results, thus representing an advantage in field conditions, as the foliar spray may result in an easier handling application method. As regards the application strategies, foliar spray application of microalgae-based products is considered a promising and innovative agricultural technique as it is safe for the environment and increases agricultural

sustainability. The direct root application of the extract may influence soil biological and biochemical properties, due to potential addition to the soil of substrates which microorganisms could also metabolize. The results obtained in this study are very promising since both application methods of *C. vulgaris* extract acted as a biostimulant on lettuce seedlings by increasing plant growth and by deeply influencing plant physiology through the coordinated induction of N and C metabolisms. Finally, the *C. vulgaris* extract also induced plant secondary metabolism, suggesting that it might be useful to counteract some stress conditions, although this aspect deserves further investigation.

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3 Chapter II

Comparative Phycoremediation Performance of Three Microalgae Species in Two Different Magnitude of Pollutants in Wastewater from Farmhouse – Experimental activity

Emanuele La Bella[1], Paride Salvatore Occhipinti[1], Ivana Puglisi[1], Ferdinando Fragalà[1], Rossella Saccone[1], Nunziatina Russo[1], Cinzia Lucia Randazzo[1,2], Cinzia Caggia[1,2], Andrea Baglieri[1]

[1] Department of Agriculture, Food and Environment, University of Catania, Italy.
[2] CRIAB – Centro di Ricerca Interdipartimentale per L'implementazione dei Processi di Monitoraggio Fisico, Chimico e Biologico nei Sistemi di Biorisanamento e di Acquacoltura, Department of Medical Science, Surgicals and Advanced Technologies, Hygiene and Public Health "G.F. Ingrassia", University of Catania, Italy.

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Abstract

The cultivation of microalgae using urban wastewater as a nutrient substrate represents a promising bio-refinery concept that can serve multiple purposes; indeed, it allows for the generation of biomass, which can be used for various applications while meanwhile removing nutrients from wastewater. In this study, the potential of urban wastewater collected at two different time periods in a farmhouse as a

nutrient substrate for microalgal growth was assessed. Wastewater samples were treated on a laboratory scale, inoculating reactors with two common species, *Chlorella vulgaris* (CV) and *Scenedesmus quadricauda* (SQ), and with an autochthonous strain of *Klebsormidium* sp. K39 (Kleb.), directly isolated from effluents of the same system. The main aim of the study was to compare the microalgae's performances in terms of wastewater remediation and biomass productivity. In the first case study, which involved an effluent with a lower pollutant level, microalgal cultivation showed removal efficiencies in the range of 57–63% for total nitrogen, 65–92% for total phosphorous, 94–95% for COD, and 100% for *E. coli*. In the second case study, involving an effluent with a higher pollutant level, the remediation performances of the three microalgae strains ranged from 93 to 96% for total nitrogen, from 62 to 74% for total phosphorous, from 96 to 97% for COD, and 100% for *E. coli*. At the end of the experimental trials, treated waters showed values of pollutants suitable for irrigation use, in accordance with environmental and national legislation, which established specific thresholds for irrigation purposes.

3.1 Introduction

In the last few years, the rapidly expanding population, coupled with global climate changes, has represented a considerable pressure on Earth's resources. Indeed, climate change negatively impacts agricultural productivity and affects the water cycle, leading to altered precipitation patterns and increasing water scarcity in some regions, as well as the increase in population putting a strain on freshwater resources (Schewe et al., 2014).

A further important issue is related to the release of municipal wastewaters and, in turn, the environmental challenges they pose to receiving water bodies (Arora and Saxena, 2005; De-Bashan and Bashan, 2010). The high concentration of pollutants, such as excess nitrogen and phosphorus, may cause an important alteration in the health

of the water system (Chai et al., 2021; Olguín, 2003). Furthermore, conventional treatment methods, such as activated sludge systems or chemical coagulation, are still very expensive and often unable to completely eliminate micro compounds or inorganic nutrients (Rizzo et al., 2019; La Bella et al., 2022).

The use of reclaimed water (RW), a suitable strategy in agriculture for irrigation purposes, may represent a risk for plants, soils, and humans (WHO, 2006; Ofori et al., 2021) for the accumulation and propagation of biological (animal and human pathogens, phytopathogens), xenobiotic contaminants (drugs and metals), and antibiotic-resistant genes (Łuczkiewicz et al., 2010; Bouki et al., 2013; Novo et al., 2013). The World Health Organisation guidelines established safety criteria for irrigation purposes, for which RW must comply with standard criteria. In the EU, the use of RW is under Regulation (EU) 2020/741 on minimum requirements for water reuse, which establishes a threshold of 10 CFU 100 mL^{-1} (<1 Log 100 mL^{-1}) of *Escherichia coli* for RW classifying as class "A", useful for irrigation of food crops (Ventura et al., 2019).

In this context, the exploitation of microalgae is emerging as an interesting alternative green source with a low carbon dioxide (CO_2) footprint (González-Fernández et al., 2012; Puglisi et al., 2020). Microalgae are also attracting the interest of worldwide researchers, mainly due to their multipurpose applications as raw materials for the development of new agricultural products (La Bella et al., 2021; La Bella et al., 2022; Puglisi et al., 2022). Moreover, microalgae are taken into account as important sustainable sources of valuable chemicals, pharmaceuticals, and other products (Caporgno and Mathys, 2018; Vaz et al., 2016).

The microalgae-based wastewater treatment process is a sustainable, eco-friendly process with no secondary pollution (Rawat et al., 2011), able to recover wastewater from various organic and inorganic contaminants, ranging from aromatic hydrocarbons, food

residues, solvents, plasticisers, antioxidants, washing and cleaning-related compounds, to high nutrient loads such as nitrogen and phosphorous (Cai et al., 2013). Furthermore, previous studies have shown that microalgae-based wastewater treatment has a rate of coliform removal of up to 99% (Colak and Kaya, 1988; Abdel-Raouf et al., 2012). Microalgae may be adapted to a wide range of types of wastewater, providing a tertiary biotreatment coupled with the production of valuable biomass, a potential feedstock for the development of added-value products for the agricultural sector (Abdel-Raouf et al., 2012).

Among microalgae species suitable for wastewater treatment, the genera *Chlorella* and *Scenedesmus* are the most largely used (Sánchez-Zurano et al., 2021). However, a limitation in applying such a strategy is related to the difficulties of maintaining monoalgal cultures with constant biomass composition (García et al., 2018). The remediation abilities of these two genera are largely reported (La Bella et al., 2022; Law et al., 2022). For instance, Wang et al. (Wang et al., 2010) demonstrated that *Chlorella* sp., employed for urban wastewater treatment, was able to remove high contents of nitrogen, ranging from 62.5 to 82.4%; phosphorus, from 83.2 to 90.6%; and heavy metals. In the same way, Wong et al. (Wong et al., 2015) investigated the lipid production and nutrient removal capabilities of *S. quadricauda* using different types of wastewater from a sewage treatment plant. The results showed interesting performances for both evaluated properties, indicating that the microalga is a viable candidate for wastewater treatment and lipid production. It is relevant to point out that the major pollutants in urban wastewater are nutrients and heavy metals; therefore, a relevant trait for the selection of microalgae strains to be used for this purpose is to detect these abilities (Baglieri et al., 2016).

Moreover, microalgae cultivation can provide an opportunity to produce valuable biomass, which can be utilized to obtain bioproducts for multipurpose applications. It is worth noting that research in this field is ongoing, and further studies are needed to optimize the

processes, explore different microalgae species, and assess the scalability and economic feasibility of using microalgae for wastewater treatment and resource recovery. To achieve a 'win-win' solution by linking wastewater remediation and microalgae biomass accumulation, different types of wastewater could be used as a culture medium for the cultivation of different microalgae species. Based on the above perspectives, this study is aimed at evaluating the phycoremediation performance and biomass accumulation of an indigenous strain of filamentous microalga, previously identified as *Klebsormidium* sp. K39, in urban wastewater treatment, compared to *Chlorella vulgaris* (CV) and *S. quadricauda* (SQ). These performances were evaluated for two different magnitudes of pollutants in wastewater from a farmhouse.

3.2 Materials and methods

3.2.1 Raw wastewaters

Wastewater samples were collected from a constructed wetland active on a farm holiday in Sicily (Italy) in two different periods, as the different host affluence levels (due to the COVID emergency) caused significant differences in their composition. The collected raw wastewaters were preliminary analysed (see detailed methods below in Section 2.2) and used as growth substrates for microalgae.

In Figure 1, a scheme of the phytodepuration system acting in the farm holiday is reported. The wastewater samples used for the experimental trials were collected directly from the Imhoff tank.

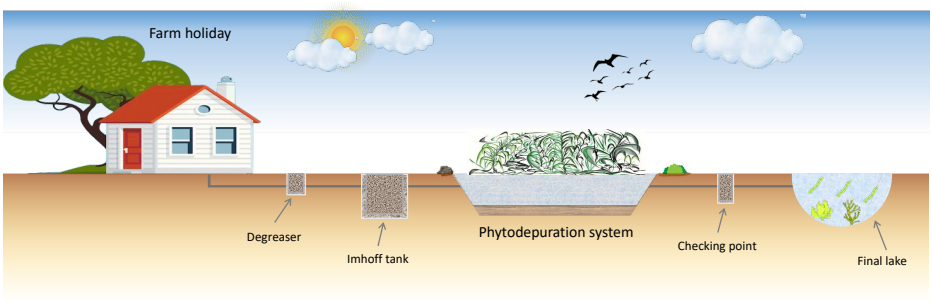

Figure 1. Phytodepuration system scheme at the farmhouse.

The characteristics of the raw wastewaters used in this study are reported in Table 1 (analyses are described in Section 2.2).

Table 1. Composition of raw wastewaters: Total Kjeldahl Nitrogen (TKN), Total Phosphorous (TP), Chemical Oxygen Demand (COD), and *Escherichia coli*.

	Wastewater 1 (MW1)	Wastewater 2 (MW2)
pH	7.24	7.25
EC (mS cm^{-1})	3.95	5.35
TN (mg L^{-1})	10.0	50.7
TP (mg L^{-1})	3.2	10.7
COD (mg L^{-1})	550	753
Zn (mg L^{-1})	nd*	nd
Cu (mg L^{-1})	nd	nd
Cd (mg L^{-1})	nd	nd
Pb (mg L^{-1})	nd	nd
Ni (mg L^{-1})	nd	nd
Hg (mg L^{-1})	nd	nd
E. coli (log CFU 100 mL^{-1})	nd	nd

* nd: not detected.

3.2.2 Chemical Analyses and Nutrient Removal Rate Determination

The wastewater samples were first centrifuged at 4000 g for 5 min, and the supernatants were collected (Ren et al., 2017). Measurements of EC and pH values were performed using an XS Cond 7 and an XS pH 80+ DHS, respectively. In order to evaluate the preliminary composition of wastewaters and the nutrient removal ability of

microalgae, chemical characterization by monitoring several parameters, including TKN, TP, heavy metals, COD, and BOD_5, was performed following the standard methods recommended by the American Public Health Association (APHA, 2005).

TKN was performed by the Kjeldahl method in 50 mL of sample. In a test tube, 2 catalyst tablets were added, each containing 3.5 g of K_2SO_4 and 3.5 mg of Se, and 10 mL of concentrated sulphuric acid. The tubes were placed in the digestor and treated for 60 min at 200 ◦C and 120 min at 370 ◦C. After digestion, samples were treated with an acid solution and boiled in concentrated sulfuric acid. The samples were then distilled according to a pre-defined method of the instrument (Method n◦ 26, VELP UDK 130 A). The distillation of the samples was performed by adding an excess of 35% NaOH to the acid digestion mixture to convert NH_4^+ to NH_3, followed by boiling and condensation of the ammonia (NH_3) gas in a receiving solution (4% H_3BO_3). Finally, to quantify the amount of ammonia in the receiving solution, the water samples were titrated. For the titration, to each sample were added 10 drops of Tashiro's indicator (0.75 g L^{-1} methyl red sodium salt + 0.375 g L^{-1} methylene blue in ethanol 50% (v/v), denatured) and 0.2 N HCl until the endpoint of the titration.

Analysis to determine TP contents was based on the persulfate oxidation under acidic conditions of the samples (APHA, 2005), converting the various forms of phosphate and phosphorus to the orthophosphate form. The phosphorus contents were determined by putting 50 mL of sample, or a diluted amount of 50 mL, into an Erlenmeyer flask, adding 1 drop of phenolphthalein indicator, and 5 M sulphuric acid or 2 M sodium hydroxide until the samples developed a red colour. The next steps were the addition of 1 mL of 10 M sulphuric acid and 0.4 g of potassium persulphate, followed by the transfer of the samples into an incubator at 95–100 ◦C for 2 h. After cooling, the samples were added to 1 drop of phenolphthalein and neutralized to a faint pink colour with 2 M sodium hydroxide, made up to 100 mL with

distilled water. Then, at each sample, 10 mL of a mixed reagent was added, composed of 100 mL of 30 g L^{-1} ammonium molybdate solution, 250 mL of diluted sulphuric acid (1:6.4, H_2SO_4:H_2O), 100 mL of 54 g L^{-1} ascorbic acid solution, and 50 mL of 1.36 g L^{-1} potassium antimony tartrate solution. We allowed at least 10 min for colour development and measured the absorbance at 880 nm using a reagent blank to zero the spectrophotometer. The reagent blank was made using 50 mL of distilled water carried through the digestion and subsequent steps. Finally, the samples' absorbances were checked against the calibration curve phosphate standard, and the concentrations were determined.

The determination of heavy metals was performed by Standard Methods for Examination of Water and Wastewater (APHA, 2005). The metal analyses (Zn, Cu, Cd, Pb, Ni, and Hg) were carried out by means of atomic absorption spectrophotometry (Perkin Elmer 3110, Waltham, MA, USA). Each wastewater sample was filtered through a 0.45-micron nylon filter and acidified to a pH of 4–5 with HCl. Afterwards, 35 mL of Methyl isobutyl ketone (MIBK) and 7 mL of 1% (w/v) ammonium pyrrolidine dithiocarbamate (APDC) were added to 750 mL of the filtered solution, and each sample was equilibrated for 30 min on a mechanical shaker, and the organic layer was separated in a separatory funnel. The concentration of the heavy metals (Zn, Cu, Cd, Pb, Ni, and Hg) was determined by reading the concentrations of the elements of interest directly versus appropriate standards and a reagent blank. Wastewater was analysed for heavy metals only at the beginning of removal experiments because, in both cases (MW1 and MW2), the contents were below the detectable limits.

COD analysis was performed using specific test kits (Nanocolor CSB 40 and Nanocolor CSB 1500), and BOD_5 was monitored using the Velp Respirometric Sensor BOD_5 (Monza – Brianza, Italy). For BOD_5 analysis, all samples were saturated with oxygen using an air pump, and after 5 days of incubation in the dark, the final dissolved

oxygen level was taken directly from the sensor, and the difference between the final and initial levels was recorded.

Each analysis was replicated in triplicate.

To evaluate the nutrient removal ability by microalgae, Total Kjeldahl Nitrogen (TKN), Total Phosphorus (TP), Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD_5), pH, and Electrical Conductivity (EC) were determined according to the standard methods recommended by the American Public Health Association (APHA, 2005). For these parameters, removal quantity (RQ, mg L^{-1}) and removal efficiency (RE, %) were calculated using the following equations (Li et al., 2017):

$$RQ = x_0 - x_i$$

$$RE\ (\%) = [(x_0 - x_i) / x_0] \times 100$$

where x_0 and x_i are defined as the mean values of nutrient concentrations at initial time t_0 and final time t_i, respectively.

3.2.3 Microalgae Strains and Cultivation Conditions

The microalgae tested in the present study were *Chlorella vulgaris* ACUF863 and *Scenedesmus quadricauda* ACUF581, which were kindly provided by the Algal Collection Federico II of Naples (Italy). In addition, a strain of *Klebsormidium* sp. K39, belonging to the Di3A microbial culture collection and previously isolated from the same phytoremediation pond (Occhipinti et al., 2023), was used. All strains were cultured in sterilized standard Bold Basal Medium (BBM) or BBM agar medium.

Microalgae cultivation was carried out in axenic conditions in 2 L Erlenmeyer flasks maintained at 25 ± 1 ◦C in a climate chamber under a light intensity of 100 µmol photons m^{-2} s^{-1} with a light source (PHILIPS SON-T AGRO 400, Eindhoven, The Netherlands), and a photoperiod of 16 h on/off, according to the best microalgae growth conditions. The cultures were bubbled with air with immersion water

pumps (Baglieri et al., 2016).

The microalgae species used in the described experiments were inoculated at their logarithmic growth phase.

3.2.4 Evaluation of Bacterial Removal Efficiency

In order to evaluate the *Escherichia coli* removal efficiency of the tested microalgal treatments, microbiological analyses were performed following the membrane filtration method (APHA, 2017). In detail, 100 mL of sample were treated on membrane filters (0.45 µm pores, Cellulose, Merck, Darmstadt, Germany), and the filters were then poured into RAPID' *E. coli* 2 Agar plates (Bio-Rad, Milan, Italy). Plates were incubated at 37 ◦C for 24 h. The analyses were performed in triplicate, and results were expressed as mean log10 colony-forming units (CFU) per unit of volume.

3.2.5 Experimental Set-up

The experimental set-up consisted of eight lab-scale open photobioreactors (Table 2), each with a 4 L capacity, illuminated for a 12 h photoperiod by an LED lamp (100 µmol photons m^{-2} s^{-1}), in order to simulate the nearest natural environmental conditions. Each reactor was filled with 3 L of wastewater [Wastewater 1 (MW 1) and Wastewater 2 (MW 2)] collected from the Imhoff tank of the phytoremediation system at the farmhouse, as above described (Figure 1).

Each microalga, grown in BBM, was collected by centrifugation at 4000 rpm for 10 min when it reached the logarithmic growth phase. Pellets were washed with deionized water and centrifuged a second time at the same conditions, then were suspended in a small quantity of wastewater, and, finally, inoculated in the reactors (Liu et al., 2016).

The photobioreactors were inoculated with C. vulgaris, *S. quadricauda*, and the autochthonous *Klebsormidium* sp. K39 strains at an initial cell concentration, as determined by cell count in the

Burker counting chamber (Blaubrand), of 100 mg L^{-1}, equal to 1.6, 2.2, and 1.8 10^9 cells L^{-1}, respectively. For each microalga, the cell dry weight and the size of the inoculums were found to be 0.42, 0.44, and 0.45 g (fresh weight), respectively. The wastewater samples that were not inoculated were routinely used as controls. The microalgae were thus fed in the reactors exclusively with the wastewaters as they are, without nutrient addition or dilution, considering that the effluents can supply all inorganic nutrients required for microalgae growth (Ruiz-Martinez et al., 2016).

Table 2. Design criteria and conditions adopted in each photobioreactor used in the experimental trials.

Photobioreac-tors	Substrate	Microalgae Species	Microalgae Biomass (g L^{-1})	Inoculum Size (n. cells 10^9 L^{-1})
1	MW1	0	-	-
2	MW1	*C. vulgaris*	0.42	1.6
3	MW1	*S. quadricauda*	0.44	2.2
4	MW1	*Klebsormidium* sp. K39	0.45	1.8
5	MW2	0	-	-
6	MW2	*C. vulgaris*	0.42	1.6
7	MW2	*S. quadricauda*	0.44	2.2
8	MW2	*Klebsormidium* sp. K39	0.45	1.8

Each microalga, grown in BBM, was collected by centrifugation at 4000 rpm for 10 min when it reached the logarithmic growth phase. Pellets were washed with deionized water and centrifuged a second time at the same conditions, then were suspended in a small quantity of wastewater, and, finally, inoculated in the reactors (Liu et al., 2016). The photobioreactors were inoculated with *C. vulgaris*, *S. quadricauda*, and the autochthonous *Klebsormidium* sp. K39 strains at an initial cell concentration, as determined by cell count in the Burker counting chamber (Blaubrand), of 100 mg·L^{-1}, equal to 1.6, 2.2, and 1.8 × 10^9 cells·L^{-1}, respectively. For each microalga, the cell dry

weight and the size of the inoculums were found to be 0.42, 0.44, and 0.45 g (fresh weight), respectively. The wastewater samples that were not inoculated were routinely used as controls. The microalgae were thus fed in the reactors exclusively with the wastewaters as they are, without nutrient addition or dilution, considering that the effluents can supply all inorganic nutrients required for microalgae growth (Ruiz-Martinez et al., 2016).
Samples of 50 mL were then collected after 2, 5, 10, 30, 45, and 60 days from each photobioreactor in order to evaluate the remediation ability of the tested microalgae, determining the concentrations of Total Kjeldahl Nitrogen (TKN), Total Phosphorus (TP), Chemical Oxygen Demand (COD), Biological Oxygen Demad (BOD_5), pH, and Electrical Conductivity (EC) (as previously described). In order to monitor microbiological parameters, samples were collected at 0, 2, 5, 7, 9, 15, 30, 45, and 60 days after inoculum and immediately processed for *E. coli* detection and microalgae counting (as previously described). All experiments were carried out in triplicate.

3.2.6 Determination of Microalgal Growth

The microalgal growth was determined as cell number by Burker's counting chamber (Blaubrand), as fresh weight, measuring the weight (mg) of fresh biomass per litre and as dry weight, measuring the weight (mg) of dry biomass per litre, obtained oven-dried at 60 °C until a constant weight was reached.
The daily productivity (g L^{-1}·d) was calculated according to the following formula (Pham et al., 2013):

$$\text{Daily productivity} = (CDW_i - CDW_0) / (t_i - t_0)$$

where CDW_i and CDW_0 are the final and initial concentrations of cell dry weight and t_i and t_0 are the final and initial time.
Moreover, at the end of the experimental test, the samples containing

the microalgae were centrifuged at 2500 rpm for 10 min, and the pellet was oven-dried at 60 °C until constant weight and weighed to measure the total biomass (Baglieri et al., 2013).

3.2.7 Statistical Analysis

The collected data were subjected to a two-way analysis of variance (ANOVA) based on a factorial combination (specie × time). Since the laboratory assays were performed in triplicate, F and p values were calculated to evaluate whether the effects of single factors such as as specie, time, and the interaction specie × time were significant. In post-hoc analyses, the means were compared using Fischer's protected least significant difference (LSD) test ($p \leq 0.05$). The calculations were carried out on Excel version 2019 (Microsoft Corporation, Redmond, WA, USA) and Minitab (version 16.1.1, Minitab Inc., State College, PA, USA).

3.3 Results

3.3.1 Dynamics of Microalgae Population

The microalgae strains were cultivated in wastewater for 60 days, and the growth performances, in terms of cell density, are reported in Figure 2. The lag phase, or time necessary for their adaptation to wastewater conditions, was found to be quite short in both case studies (48 h), and in this period the main parameters monitored were not significantly reduced.

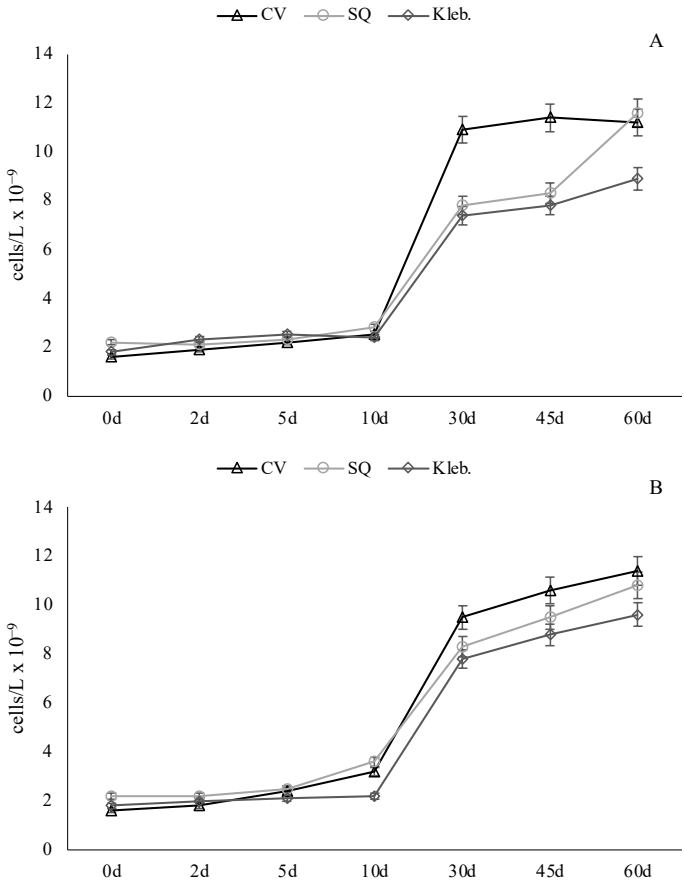

Figure 2. Microalgal growth performance in (A) Wastewater 1 (MW 1) and (B) Wastewater 2 (MW 2). CV: *Chlorella vulgaris*, SQ: *Scenedesmus quadricauda*, Kleb: *Klebsormidium* sp. K39.

However, the effect of a single factor (species) was found to be not significant for any of the parameters monitored in both trials (Tables 3 and 4).

Table 3. Effects of single factors in ANOVA relative to the daily productivity, the fresh weight of biomass collected, and the dry weight of biomass collected in

MW1.

Factor	Daily Productivity		Fresh Weight of Biomass Collected		Dry Weight of Biomass Collected	
	F	*p* Value	F	*p* Value	F	*p* Value
Species	0.95	0.437	1.23	0.356	0.43	0.667

Table 4. Effects of single factors in ANOVA relative to the daily productivity, the fresh weight of biomass collected, and the dry weight of biomass collected in MW2.

Factor	Daily Productivity		Fresh Weight of Biomass Collected		Dry Weight of Biomass Collected	
	F	*p* Value	F	*p* Value	F	*p* Value
Species	0.21	0.813	0.18	0.84	1.58	0.281

In the first case study (MW 1), microalgae quickly adapted to the conditions, as shown by the growth curves (Figure 2A). In details, the *C. vulgaris* strain reached the stationary phase earlier (30 days) compared to the other species, whereas at the end of the trial (60 days), a similar number of cells to those obtained using *S. quadricauda* were counted. As regards *Klebsormidium* sp. K39, a cell number always lower than other species was recorded, although daily productivity and microalgae biomasses collected were similar to those of *C. vulgaris* and *S. quadricauda* (Tables 3 and 5). Furthermore, in Table 5, in which the daily productivity and the microalgae biomasses collected at the end of the trials are reported, it is relevant to point out that no differences in terms of cell density growth or daily productivity were observed.

Table 5. Microalgae daily productivity and biomasses collected at the end of the trial (60 days).

Microalgae Species	Daily Productivity ($g\ L^{-1} \cdot d^{-1}$)		Fresh Weight of Biomass Collected ($g\ L^{-1}$)		Dry Weight of Biomass Collected ($g\ L^{-1}$)	
	MW 1	MW 2	MW 1	MW 2	MW 1	MW 2
C. vulgaris	0.017 ± 0.003	0.016 ± 0.001	5.5 ± 0.4	5.4 ± 0.4	1.10 ± 0.2	1.08 ± 0.06
S. quadricauda	0.015 ± 0.002	0.016 ± 0.002	5.3 ± 0.3	5.3 ± 0.3	1.00 ± 0.1	1.07 ± 0.04

Klebsormidium sp. K39	0.018 ± 0.03	0.015 ± 0.003	5.8 ± 0.3	5.2 ± 0.5	1.08 ± 0.09	0.98 ± 0.11

In the second case study (MW 2), although water samples exhibited a higher nutrient concentration, the three microalgae showed a similar behaviour of adapting to the culturing conditions, as shown by the growth curves reported in Figure 2B. However, the differences in cell numbers among species were less evident, and no significant differences in microalgae growth were detected (Table 4). The daily productivity of the strains was 0.017, 0.015, and 0.018 g $L^{-1} \cdot d^{-1}$ for C. *vulgaris*, *S. quadricauda,* and *Klebsormidium* sp. K39, respectively (Table 5).

3.3.2 Nutrient Removal

Removal pollutant indices were calculated to evaluate the performance of microalgae treatments. As regards the pH values of the wastewaters, they continued to increase from the lag phase through the microalgae growth phase, as shown in Figures 3A (MW1) and 4A (MW2), while EC values showed a decreasing tendency (Figures 3B and 4B), according to nutrient consumption.

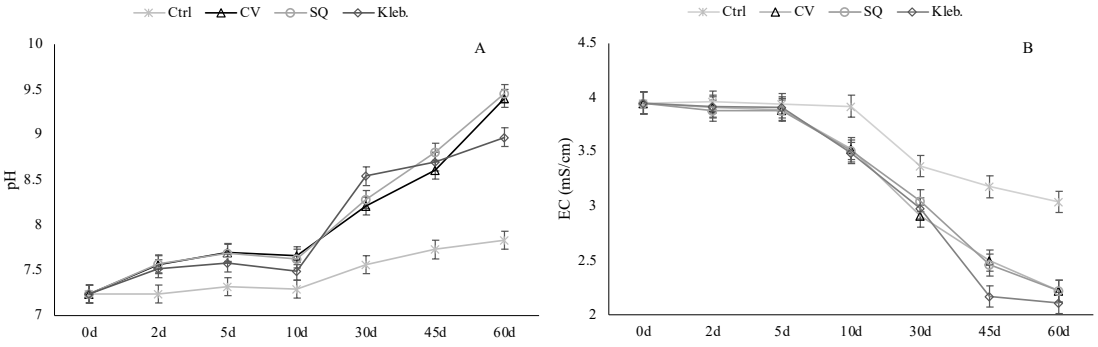

Figure 3. pH (A) and EC (mS·cm^{-1}) (B) values measured at each sampling (MW1).

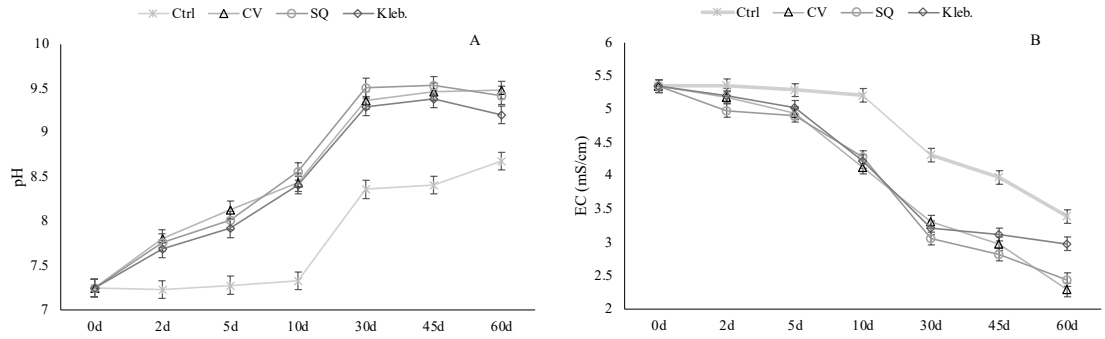

Figure 4. pH (A) and EC (mS·cm^{-1}) (B) values measured at each sampling (MW2).

In the first case study, the effects of single factors, species, and time were always significant on all the parameters monitored, as was the interaction between them on TKN, TP, and COD parameters, except for the BOD_5 parameter (Table 6).

Table 6. Effects of single factors and their interaction in ANOVA—MW1.

Factor(s)	TKN		TP		COD		BOD5	
	F	***p* Value**	**F**	***p* Value**	**F**	***p* Value**	**F**	***p* Value**
Species	2388.08	<0.0001	620.74	<0.0001	338.13	<0.0001	41.85	<0.0001
Time	2618.61	<0.0001	1337.53	<0.0001	16,601.52	<0.0001	1962.83	<0.0001
Species × time	214.24	<0.0001	174.96	<0.0001	17.87	<0.0001	2.53	0.08

The variations in total nitrogen, total phosphorous, chemical oxygen demand, and biological oxygen demand contents during the two experiments are depicted in Figure 5.

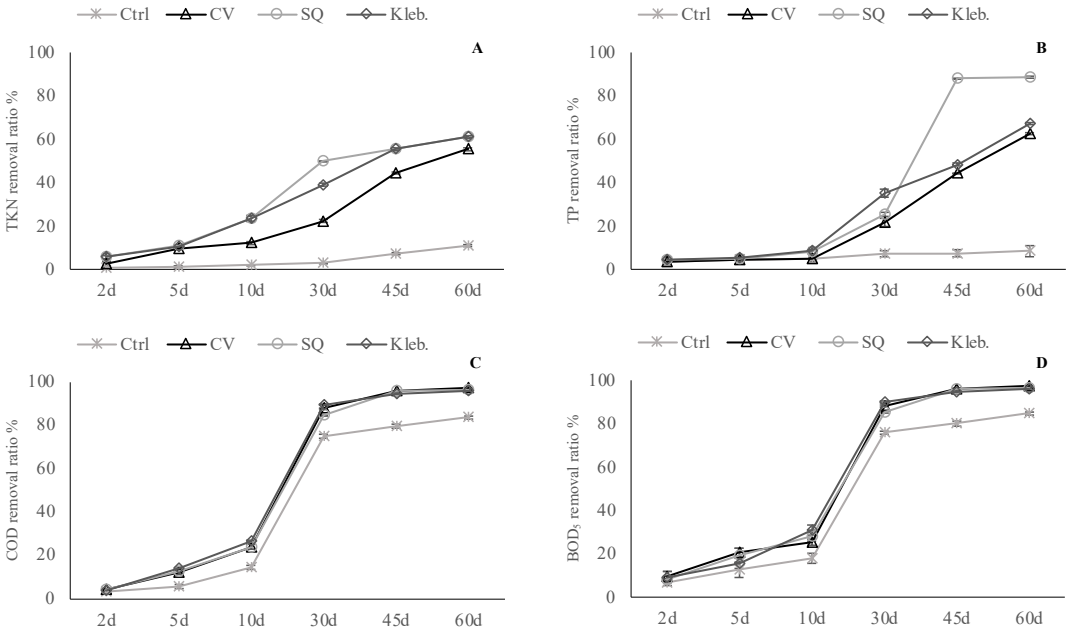

Figure 5. Removal percentage of monitored parameters at each sampling—MW 1 ((A)—TKN; (B)—TP; (C)—COD; (D)—BOD_5).

Post-hoc analyses to establish the ranking of effectiveness at each sampling are shown in Supplementary Materials Table S1. Based on these data, at each sampling, the microalgae significantly reduced all the parameters monitored with respect to the control in MW1. The pollutant concentration in all the tested wastewaters showed a different decrease during the first 2 days. The removal of pollutants gradually levelled off until the end of the experimental trial. At the end of the treatment, the maximum removal efficiency of *C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39 was 55.5, 61.0, and 61.2% for total nitrogen, 62.7, 88.7, and 67.2% for total phosphorous, and 97.3, 96.6, and 96.2% for COD, respectively. The maximum total nitrogen, total phosphorous, and COD removal efficiency from

wastewater control were 11.1%, 8.5%, and 83.8%, respectively.

As regards the second case study, the performance evaluation of microalgae in contaminants degradation showed that the effects of species, time, and species × time were always significant versus all pollutant parameters monitored (Table 7).

Table 7. Effects of single factors and their interaction in ANOVA – MW2.

Factor(s)	TKN		TP		COD		BOD5	
	F	*p* Value	F	*p* Value	F	*p* Value	F	*p* Value
Species	9247.55	<0.0001	968.86	<0.0001	1492.78	<0.0001	164.25	<0.0001
Time	5798.76	<0.0001	2806.37	<0.0001	22,354.06	<0.0001	2353.97	<0.0001
Species × time	416.51	<0.0001	229.33	<0.0001	109.62	<0.0001	13.12	<0.0001

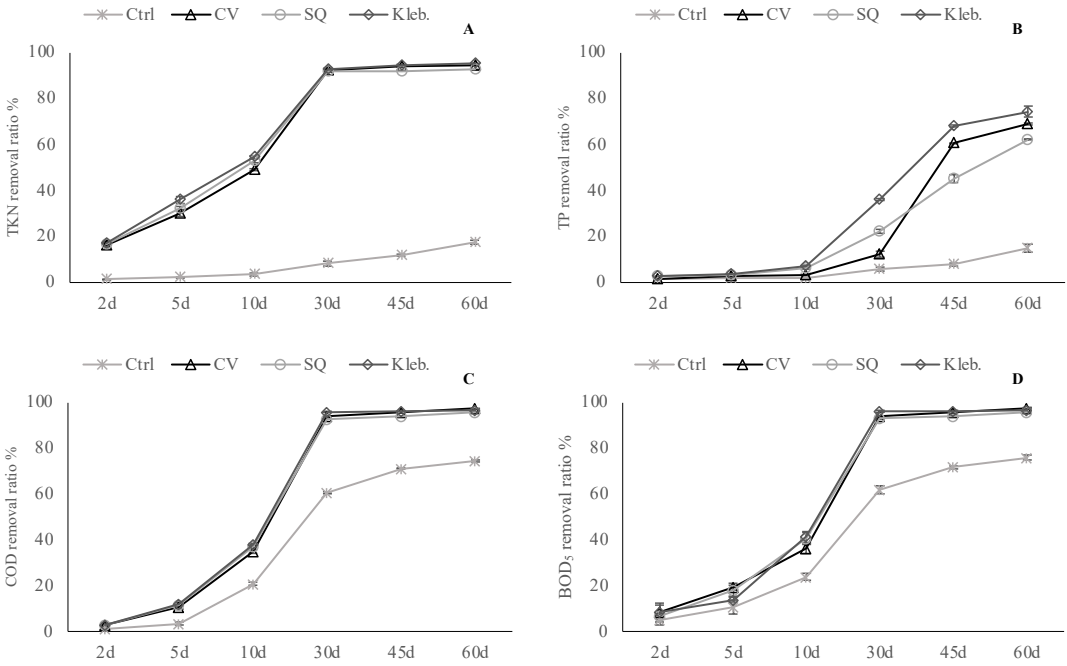

Figure 6. Removal percentage of monitored parameters at each sampling—MW 2 ((A)—TKN; (B)—TP; (C)—COD; (D)—BOD_5).

Post-hoc analyses to establish the ranking of effectiveness at each sampling are shown in Supplementary Materials Table S2. Post-hoc analysis of the data revealed a similar ranking of efficacy among the three tested microalgae, which gradually levelled off until the end of the experimental trial for all parameters monitored (Figure 6). In detail, at this sampling, each microalga significantly reduced the TKN variable with values between 92.7 and 95.5%. As well, concerning the removal of TP, COD, and BOD_5, *C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39 significantly reduced from 62.0 up to 74.3%, from 95.6 up to 97.3%, and from 95.4 up to 97.4% compared to the starting values.

As already seen in the above-mentioned trial, a decrease of the same parameters in the control (not-inoculate wastewater) was observed, and the maximum total nitrogen, total phosphorous, COD, and BOD_5 degradation were 16.9, 14.7, 74.5, and 75.0%, respectively.

3.3.3 E. coli Removal Efficiency

The cell density of *E. coli* detected in MW1 (panel A) and MW2 (panel B) water samples, un-inoculated (control) and inoculated with different microalgal cultures (*C. vulgaris* ACUF863, *S. quadricauda* ACUF581, *Klebsormidium* sp. K39) after 0, 2, 5, 7, 9, 15, 30, 45, and 60 days from the inoculum is reported in Figure 7. Overall, a significant decrease in cell density was observed in all tested samples except the controls. In particular, regarding MW1 samples (Figure 7, panel A), no significant difference was detected in the removal efficiency of the tested microalgae. In detail, 5 days after the inoculum, *S. quadricauda* ACUF581 and *C. vulgaris* ACUF863 induced a decrease of 3.14 and 3.28 unit Log in *E. coli* cell densities, whereas *Klebsormidium* sp. K39 induced a decrease of 2.74 unit Log. After 7 days, higher reductions were registered in microalgal treatments as 1.43 unit Log by *S. quadricauda* ACUF581 and *C. vulgaris* ACUF863 and 1.75 unit Log by *Klebsormidium* sp. K39, while *E. coli* in the control

sample was at 6.1 Log CFU mL^{-1}. After 9 days, *E. coli* showed a cell density of 6.2 Log CFU mL^{-1} while in treated samples higher decreases, as 0.45, 0.50, and 0.55, were observed for *C. vulgaris* ACUF863, *S. quadricauda* ACUF581, and *Klebsormidium* sp. K39, respectively.

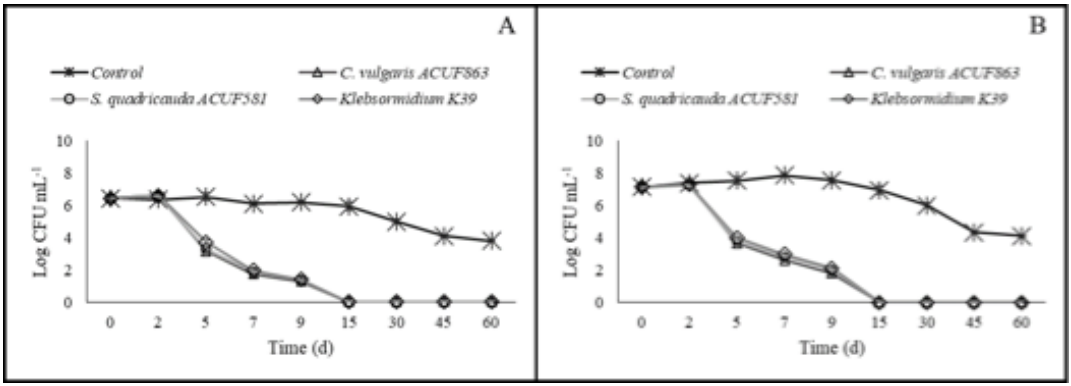

Figure 7. *E. coli* load detected (as Log cells mL^{-1}) detected in MW1 (A) and MW2 (B) samples, un-inoculated (control) and inoculated with different microalgal cultures (*C. vulgaris* ACUF863, *S. quadricauda* ACUF581, or *Klebsormidium* sp. K39) after 0, 2, 5, 7, 9, 15, 30, 45, and 60 days from the inoculum.

In the same samples, no *E. coli* was detected after 15, 30, 45, and 60 days from the microalgal inoculum. A different trend was observed in controls, where *E. coli* was constantly increasing, reaching, at the end of the trial (60 days), a cell density of 3.80 Log CFU mL^{-1}. The bacterial removal results on MW2 samples were significant (Figure 7, panel B). In details, after 5 days from inoculum, in samples treated with *S. quadricauda* ACUF581 and *C. vulgaris* ACUF863, the target bacteria were reduced by 3.34 and 3.49 unit Log, respectively, whereas in samples treated with *Klebsormidium* sp. K39, the target bacteria were reduced by 3.15 unit Log. The reduction values were significantly different compared to the control sample, where the *E. coli* density was found to be 7.53 Log CFU mL^{-1}, while no significant

differences were found among the treatments. After 7 days, more than 0.97, 1.03, and 1.06 unit Log CFU mL^{-1} of reduction were observed for *S. quadricauda* ACUF581, *Klebsormidium* sp. K39, and *C. vulgaris* ACUF863, respectively, when the target bacteria cell density in control samples showed a load of 7.85 Log CFU mL^{-1}. After 9 days, the target bacteria showed a cell density of 7.54 Log CFU mL^{-1}, while the treated samples registered a higher reduction, as 0.80, 0.84, and 0.85 for *C. vulgaris* ACUF863, *S. quadricauda* ACUF581, and *Klebsormidium* sp. K39, respectively. After days 15, 30, 45, and 60 days, *E. coli* was never detected in any treated samples, while its density was found at a mean value of 4.1 Log CFU mL^{-1} in untreated samples at the end of the trial (60 days).

3.4 Discussion

Discharge of wastewater into water bodies represents a serious issue because the high concentrations of contaminants may pose a serious threat to ecosystem health. In this frame, one of the main reasons for removing nutrients from wastewater is to control eutrophication, which is due to the uncontrolled growth of algae or higher hydrophytes triggered by the addition of a nutrient surplus in the ecosystem (Hammouda et al., 1995; Chai et al., 2021). In the present study, a sustainable and eco-friendly wastewater treatment was tested in order to support a circular system in which the microalgae play a key role, representing both the agent of the remediation and the final product of the process, which leads to a useful biomass suitable for several further purposes. The importance of low-cost biomass production is crucial because the economic and environmental drawbacks could be partly overcome using urban wastewater as a microalgae growth substrate (Delrue et al., 2016; La Bella et al., 2022). Because of their ability to perform photoautotrophic, mixotrophic, or heterotrophic metabolism, microalgae represent a promising biological system for a variety of wastewaters. To achieve this aim, employing species able to remediate

wastewater is crucial and guarantees a successful sustainable process, and the best candidate is represented by autochthonous microalgae, which are able to naturally grow in a specific wastewater. Furthermore, microalgal systems are designed mainly to achieve high biomass productivity with minimum energy inputs because essential nutrients and a carbon source, required for an efficient cultivation process, are largely available in the effluent (Nasr, 2019; Nasr, 2022).

The identification process of several isolates recently affiliated with the genus *Klebsormidum* revealed that *Klebsormidum sp.* K39 lacks a proper grouping at the species level due to unclear species boundaries (Škaloud and Rindi, 2013). For this genus, the morphological traits as well as some features considered taxonomically relevant (showing variations depending on the age and the physiological conditions) result in a taxonomically and systematically complex taxon in which phylogenetic relationships are still poorly understood (Škaloud and Rindi, 2013; Rindi et al., 2011). Despite *Klebsormidum sp.* K39 being subjected to molecular analyses for phylogenetic study, further studies are required to cluster this strain into a species, as Novis (Novis, 2006) had already shown, with the description of the *Klebsormidium acidophilum* species. It is relevant to highlight that the K*lebsormidum sp.* K39 strain used in the present study has been recently tested to evaluate its dynamic within an autochthonous microalgal pool in terms of *E. coli* removal efficiency (Occhipinti et al., 2023).

Zooming in on microalgal yields obtained during the phycoremediation process, they were quite different from data reported in the literature due to the different composition of treated effluents (Li et al., 2017; La Bella et al., 2022). In particular, Li et al. (2017), cultivating five microalgae species, among them *C. vulgaris* and *S. quadricauda*, in post hydrothermal liquefaction wastewater, obtained a daily productivity of 0.031 and 0.0071 g $L^{-1} \cdot d^{-1}$, respectively. Regarding *Klebsormidium* sp., available data indicate a biomass production that

may vary from about 0.010 g $L^{-1} \cdot d^{-1}$ in horticultural wastewater to about 0.035 g $L^{-1} \cdot d^{-1}$ in synthetic wastewater (Liu et al., 2016). Although, the yields are quite different than optimal conditions, at the end of the present experimental tests, all the microalgae demonstrated a good growth aptitude in urban wastewaters with different pollutant contents, and this could be mainly related to their physiochemical and biochemical characteristics. Indeed, many studies report the remediation ability and biomass production of *C. vulgaris* and *S. quadricauda* using wastewater from various sources; they have proven abilities of removing nitrogen, phosphorus, and COD and shown their potentiality as a tertiary biotreatment step in the remediation process (La Bella et al., 2022). For instance, Baglieri et al. (2016) investigated the feasibility of cultivating *C. vulgaris* and *S. quadricauda* in agricultural wastewater for inorganic nutrient removal, and the two species showed similar behavior, determining comparable remediation performance in terms of nitrogen (both about 99%) and phosporous (88 and 94%, respectively).

On the contrary, limited studies on the cultivation of *Klebsormidium* sp. K39 in wastewater are still reported. Among *Klebsormidium species*, *Klebsormidium flaccidum* showed good feasibility for nutrient removal from municipal wastewater, being able to provide a complete removal of nitrogen and phosphorous (Umetani et al., 2023). Similarly, Liu and Vyverman (Liu and Vyverman, 2015) evaluated differences in the uptake of nutrients of *Klebsormidium* sp. from wastewater under varying nitrogen and phosphorous contents. The authors observed that the microalgae achieved an approximately 99% phosphorous removal rate and a consistent nitrogen removal rate (about 99%) under almost any tested conditions. However, with a N/P ratio of 20, *Klebsormidium* sp. exhibited a lower nitrogen removal efficiency (76.4%).

Overall, the daily productivity and the growth results confirm the suitability of urban wastewater as a substrate for cultivation of

Klebsormidium sp. K39 and the absence of negative effects. Similarly, the three species showed quite comparable increases in terms of fresh and dry biomass produced. A good adaptability of *Klebsormidium* sp. K39 was also observed in a study under consideration (Occhipinti et al., 2023). In particular, *Klebsormidium* sp. K39, during a lab-scale wastewater treatment at lab scale using a microalgae pool, was the dominant microalgae at the end of the treatment.

Results clearly showed that the initial concentrations of both tested wastewaters did not affect the final biomass accumulation or the daily productivity of the three microalgae species. This may be mainly due to the characteristics of the tested urban wastewater, a kind of effluent usually rich in nutrient compounds and characterized by low concentrations of toxic substances that may inhibit microalgae growth.

In detail, in the first case study using MW 1, *S. quadricauda* showed the highest phosphorous removal rate (91.9%), followed by *Klebsormidium* sp. K39 (69.6%) and *C. vulgaris* (64.7%) of total phosphorous. In terms of nitrogen removal, no significant differences were detected between *S. quadricauda* and *Klebsormidium* sp. K39, which showed the highest removal efficiency (62.8 and 63.1%, respectively), while for *C. vulgaris,* a lower degradation rate was observed at each sampling time. In the control, the decrease of total nitrogen and total phosphorous due to naturally occurring abiotic degradation, was very low. Regarding the removal of COD and BOD_5, slight differences were observed among the tested strains, and both of these parameters always significantly decreased at any time in all treated samples.

In the second case study, using MW 2, *C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39 induced a progressive reduction of measured parameters with increasing treatment time in total nitrogen, total phosphorous, COD, and BOD_5 to values below the reuse for irrigation in agriculture, according to law limits (Italian Ministerial Decree n. 185/2003) for irrigation use. A comparable bioremediation performance, in terms of total nitrogen, COD, and BOD_5, was

recorded regardless of the microalgae species. Instead, the highest phosphorous removal rate was achieved by *S. quadricauda*.

The highest amount of nutrient removal matched the biomass production; in fact, it is well known that the nutrient reduction is mainly related to the metabolic activity of microalgae cells (Li et al., 2017). In both case studies, the *E. coli* removal rates achieved with *C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39 were in line with the values previously reported. Although pathogen removal mechanisms of microalgae have been related to different phenomena such as competition for nutrients, pH increases, and higher dissolved oxygen levels, for *E. coli* removal, adherence to the microalgal surface (Markou et al., 2018) is reported as the most likely mechanism (Ansa et al., 2011; Cho et al., 2022). In a study conducted in photobioreactors, *Chlorella sorokiniana* performed a *E. coli* removal rate of 99.8% in anaerobically treated black water in photobioreactors (Slompo et al., 2020). Overall, as reported in a recent review, the *E. coli* removal rate is on average higher than 98% (Amaro et al., 2023).

The results of the present study indicate that the two different levels of contaminants did not negatively affect the nutrient removal ratio or cell growth, in accordance with findings reported in several studies (Liu et al., 2016; Li et al., 2017; García et al., 2018). In these studies, the authors, starting from effluents with various nutrient concentrations, observed that the microalgae screened, including *C. vulgaris* and *S. quadricauda*, were able to reproduce similar performances in terms of both cell growth and nutrient uptake capacity. In Table 8, a summary of nutrient removal rates reported in various recent studies is provided, supporting and confirming the remediation capacity of the microalgae species tested in the current study.

Table 8. Removal rates by *C. vulgaris*, *S. quadricauda* and *Klebsormidium* sp. K39 in wastewaters.

Microalga Species	Wastewater Type	Starting Values (mg L^{-1})	Treatment Efficiency (%)	Reference
C. vulgaris	Municipal wastewater 1	N: 10 P: 3.2	N: 57 P: 65	Present study
C. vulgaris	Municipal wastewater 2	N: 50.7 P: 10.7	N: 95 P: 69	Present study
C. vulgaris	Agricultural wastewater	NH_4^+: 1.4 NO_3^-: 210.0 P: 4.0	NH_4^+: 99 NO_3^-: 83 P: 88	(Baglieri et al., 2016)
C. vulgaris	Synthetic effluent	NO_3^-: 20.2 PO_4^{3-}: 4.7	NO3−~50 PO43− > 98	(Kube et al., 2019)
C. vulgaris	Municipal wastewater (25%)	NO_3^-: 8.2 PO_4^{3-}: 3.2	NO3−: 88 PO43−: 91	(Singh et al., 2022)
C. vulgaris	Municipal wastewater (50%)	NO_3^-: 16.4 PO_4^{3-}: 6.3	NO3−: 79 PO43−: 88	(Singh et al., 2022)
C. vulgaris	Municipal wastewater (75%)	NO_3^-: 24.6 PO_4^{3-}: 9.5	NO3−: 63 PO43−: 85	(Singh et al., 2022)
C. vulgaris	Municipal wastewater (100%)	NO_3^-: 32.8 PO_4^{3-}: 12.6	NO3−: 54 PO43−: 83	(Singh et al., 2022)
S. quadricauda	Municipal wastewater 1	N: 10.0 P: 3.2	N: 62 P: 92	Present study
S. quadricauda	Municipal wastewater 2	N: 50.7 P: 10.7	N: 93 P: 62	Present study
S. quadricauda	Agricultural wastewater	NH_4^+: 1.4 NO_3^-: 210 P: 4.0	NH_4^+: 99 NO_3^-: 83 P: 88	(Baglieri et al., 2016)
S. quadricauda	Sewage treatment works	N~30.0 P~3.0	N > 95 P > 90	(Ren et al., 2017)
Klebsormidium sp. K39	Municipal wastewater 1	N: 10 P: 3.2	N: 63 P: 69	Present study
Klebsormidium sp. K39	Municipal wastewater 2	N: 50.7 P: 10.7	N: 96 P: 74	Present study

3.5 Conclusions

The use of microalgae as wastewater remediation agents is becoming an interesting alternative to conventional treatments, offering two undeniable benefits, i.e., the wastewater remediation and the production of valuable biomass for multipurpose applications. Overall,

our findings confirm that microalgae-based treatment offers potential for sustainable, eco-friendly, and resource-efficient solutions for wastewater remediation that may also be used for irrigation in agriculture, contributing to a more environmentally friendly approach to water management.

Furthermore, it is noteworthy that this study represents the first investigation into the use of *Klebsormidium* sp. K39, according to the promising performances of other species of this genus for wastewater remediation treatment. Our findings demonstrate that this species exhibits high adaptability to various wastewater conditions and displays efficient nutrient removal capabilities. These results are promising because they suggest that indigenous species like *Klebsormidium* sp. K39 exhibit the potential to deliver similar decontamination performances as the extensively studied microalgae species. However, further studies, as well as a full-scale demonstration, are necessary to verify the practicality, efficiency, and cost-effectiveness of microalgae-based treatment.

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4 Chapter III

Influence of microalgae biomasses retrieved from phycoremediation of wastewaters on yield of lettuce, soil health, and nitrogen environmental fate – Experimental activity

Emanuele La Bella[1], Ivana Puglisi[1], Ferdinando Fragalà[1], Rossella Saccone[1], Erika Salvagno[1], Sergio Terrazzino[1], Andrea Baglieri[1]

[1]Department of Agriculture, Food and Environment, University of Catania, Italy.

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Abstract

Microalgae have emerged as a promising sustainable alternative to enhance crop productivity. The experiments were carried out to assess the effects of *Chlorella vulgaris*, *Scenedesmus quadricauda*, and *Klebsormidium* sp. K39 in lettuce seedlings, focusing on some aspects of the complex soil-plant system. The experimental trials involved the application of microalgae cells at two different concentrations (50 and 500 mg/kg of soil), alone or in combination with standard mineral fertilization. The yield, main morpho-biometric parameters, and protein content of lettuce seedlings, as well as the activities of key enzymes involved in the nitrogen pathway (nitrate reductase, glutamine synthase, and glutamate synthetase) at both root and shoot levels, were monitored and the results were compared to not-inoculated control plants. The nitrate leached due to over irrigation was also evaluated. Furthermore, even the effects of microalgae biostimulants on soil biochemical activity were analysed by monitoring fluorescein diacetate

hydrolysis, dehydrogenase, acid and alkaline phosphomonoesterase, and urease activities. Results showed that all treatments significantly improved lettuce growth, especially when combined with mineral fertilization, providing comparable levels to the control plants treated only with microalgae cells. Furthermore, microalgae treatments positively influenced soil biological activities, as evidenced by increased of the potential biochemical index of soil fertility (Mw). Overall, microalgae soil treatments may be considered as a viable strategy to assist growers in reducing the use of mineral fertilizers, with a view to improve plant growth as well as soil biological activity.

4.1 Introduction

The importance of developing new sustainable techniques able to enhance crop yields as well as prevent their significant loss, mainly due to biotic and abiotic conditions (Dmytryk and Chojnacka, 2018; Jägermeyer, 2020), is becoming a fundamental worldwide issue. In order to improve the quality and yield of the crops and maintain natural agro-ecosystems for future generations, without adding more synthetic inputs, it's fundamental to increase the nutrients uptake and use efficiencies, and enhance, in the meanwhile, the natural mechanisms of plants to face pests and diseases without using chemicals (Costa et al., 2019).

In this contest, any improvement in agricultural practices aimed to increase nutrient uptake could be of great interest to researchers and growers (Lucini et al., 2015). Among interesting new strategies, biostimulants play a key role, representing agents able to enhance plant yields, significantly reducing the cropping systems' dependency on chemical fertilizers and pesticides (Bulgari et al., 2019; Claros Cuadrado et al., 2019). The function of these classes of products is mainly due to the diversity of sources of the raw materials and the complexity of the resulting products, which in most cases may contain many poorly characterized molecules (Brown and Saa, 2015). Furthermore,

biostimulants are considered not only environmentally friendly and cost-effective solutions to sustain agriculture, but also compete with synthetic products in terms of efficiency in enhancing plant growth (Mrid et al., 2021).

An interesting class of biostimulants for their relevance at economic and commercial level, as well as for their great versatility, is represented by microalgae-based products (Mata et al., 2010; Ronga et al., 2019, La Bella et al., 2022). Microalgae are ubiquitarians unicellular photosynthetic organisms able to grow both in marine and freshwater environments (Priyadarshani and Rath, 2012) or even in wastewater, allowing in this way a reduction of the costs production (La Bella et al., 2022). These microorganisms can be easily used to produce a wide range of highly valuable metabolites such as proteins, lipids, carbohydrates, carotenoids, vitamins, and hormone-like substances utilizable in crop production (Priyadarshani and Rath, 2012).

Microalgae biostimulants may positively affect plant growth by enhancing water uptake, root and shoot growth, tolerance to abiotic and biotic stresses, protein content in plant tissues, and the activity of the enzymes involved in the main metabolic pathways, such as nitrogen assimilation, photosynthesis, and carbon cycle (Bulgari et al., 2015; Parrado et al., 2008; Ertani et al., 2013; Puglisi et al., 2020). Furthermore, microalgae biomass might represent an interesting alternative to replace or integrate mineral fertilizers, leading to improve soil quality and increase crop productivity. Introducing these biomasses into the soil, the chemical property of treated soil enhances and the biological activity of microflora boosts, thereby influencing the overall biochemical state of soil fertility (Sharma et al., 2021).

Recently, the relevance of several microalgae, among them *Chlorella vulgaris* and *Scenedesmus quadricauda*, as bioactive agents in the soil, and their ability to improve plant growth, made them interesting products for a sustainable approach to the cultivation process (Puglisi et al., 2022). Several studies have been carried out on the

biostimulant effects of living microalgae. Barone et al. (2019) observed that living cells of *C. vulgaris* and *S. quadricauda* might exert a biostimulant effect on tomato seedlings, growing in a co-cultivation microalgae-plant system in a hydroponic Hoagland solution. Similarly, Zhang et al. (2017) studied the simultaneous cultivation of *Chlorella infusionum* and tomato plants, by using a hydroponic system, with the inputs only for crop production, and showed interesting results both for crop and microalgae, producing low-cost microalgal biomass and providing benefits for plant growth. These effects may be associated with the large number of secondary metabolites produced by microalgae (Puglisi et al., 2020).

It was also shown that extracts from the microalgae *C. vulgaris* and *S. quadricauda* may exert a biostimulant effect on lettuce growth, both through root drench and foliar application, increasing the growth parameters and improving the activity of several enzymes involved both in primary and secondary plant metabolism (Puglisi et al., 2022; La Bella et al., 2021).

Furthermore, another important prerogative to improve the sustainability of the crop production process is related to developing new substances able to reduce the fertilizers doses, often exceeding and causing several environmental problems, such as accumulation in soil and, subsequently, lixiviation of nutrient excess into groundwater (Sharma et al., 2022). The main nutrients that are leached are the nitrates, and depending on the dosage of fertilizers, soil type, and plant cultivation, nitrate leaching, ranging from 70 to 250 kg/ha, may occur (Fragalà et al., 2023). These phenomena may have a serious impact on environment, human and animal health, and lead to eutrophication and environmental pollution.

Lettuce (*Lactuca sativa* L.) is a well-known food plant worldwide grown due to its use and is generally cultivated as an annual crop, requiring relatively low temperatures to prevent it from early flowering. It can suffer from numerous nutrient deficiencies, as well as being

plagued by several pests, fungal, and bacterial diseases (Kim et al., 2016). Due to the importance of lettuce as a food crop, different studies have been carried out on this specie, testing several approaches to reach new sustainable green solutions to improve its production process (Mógor et al., 2018; Silambarasan et al., 2021; Puglisi et al., 2020, 2022; La Bella et al., 2021).

The present study aimed to explore the potential reuse of microalgae biomass (*C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39), which were previously grown on urban wastewater, in order to make them useful for irrigation purposes. Microalgae cells were tested for their effects on plant growth, mainly focusing on the nitrogen metabolism of lettuce seedlings. Furthermore, the study aimed to assess the impact of the addition of *C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39 on the biochemical fertility of the soil, by exploring the principal enzymatic activities of the soil related to the microorganism metabolism. Finally, the effect of microalgae biomasses on the rate of nitrate lixiviation through the soil was also evaluated.

4.2 Materials and methods

4.2.1 Chemicals

Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (Missouri, USA) and Thermo Fischer Scientific Inc. (Oxoid, Limited, Hampshire, UK) and were of analytical grade or higher.

4.2.2 Microalgae culture

The microalgae used in this study were *Chlorella vulgaris* ACUF863, *Scenedesmus quadricauda* ACUF581, and *Klebsormidium* sp. K39. *C. vulgaris* and *S. quadricauda* were originally provided by the Algal Collection Federico II of Naples (Italy), while *Klebsormidium* sp. K39 was obtained in the algal collection of the Department of Agriculture, Food and Environment (Di3A) (University of Catania,

Italy) (La Bella et al., 2023).

All the species were previously cultivated for 46 days, until the reaching of logarithmic growth phase, in a growth chamber in a standard Bold Basal Medium, including the following components: KH_2PO_4 (17.5 g/L), $CaCl_2 \cdot 2H_2O$ (25 g/L), $MgSO_4 \cdot 7H_2O$ (75 g/L), $NaNO_3$ (250 g/L), K_2HPO_4 (75 g/L), NaCl (25 g/L), $Na_2EDTA \cdot 2H_2O$ (10 g/L), KOH (6.2 g/L), $FeSO_4 \cdot 7H_2O$ (4.98 g/L), H_2SO_4 (1 mL/L), and the trace metal solution contains H_3BO_3 (2.86 g/L), $MnCl_2 \cdot 4H_2O$ (1.81 g/L), $ZnSO_4 \cdot 7H_2O$ (0.222 g/L), $Na_2MoO_4 \cdot 2H_2O$ (0.39 g/L), $CuSO_4 \cdot 5H_2O$ (0.079 g/L), $Co(NO_3)_2 \cdot 6H_2O$ (0.0494 g/L) (Wang et al., 2014). The cultures were bubbled with air and were maintained with a photoperiod of 16 h on/off, and a light intensity of 100 µmol photons/$m^2 \cdot s$ with a light source (PHILIPS SON-T AGRO 400) (Baglieri et al., 2016). The microalgal biomasses were centrifuged and the pellets were washed several times with distilled water until to reach a conductivity level <200 µS/cm (Puglisi et al., 2018).

After the cultivation in purity, the microalgae were employed for urban wastewater treatment as described in detail by La Bella et al. (2023). The characteristics of wastewater are summarized in Table 1. Briefly, the pure harvested microalgal biomasses were used as inoculum to evaluate their remediation performances and their ability to grow on this substrate. After 60 days of cultivation in wastewater, the biomasses were collected and separated from the culture medium by centrifugation, as described in La Bella et al. (2023).

Table 1. Composition of wastewater: Total Nitrogen (TN), Total Phosphorous (TP), Chemical Oxygen Demand (COD), heavy metals, and Escherichia coli.

Parameters	Values
pH	7.25
EC (mS cm^{-1})	5.35
TN (mg L^{-1})	50.7
TP (mg L^{-1})	10.7
COD (mg L^{-1})	753
Zn (mg L^{-1})	nd
Cu (mg L^{-1})	nd

Cd (mg L^{-1})	nd
Pb (mg L^{-1})	nd
Ni (mg L^{-1})	nd
Hg (mg L^{-1})	nd
E. coli (log CFU 100 mL^{-1})	nd

*nd: not detected.

4.2.3 Experimental site and plant material

The experiment was carried in a greenhouse located in Sicily. The climate is semi-arid Mediterranean, with dry, warm summers and mild winters. The lettuce seedlings (*Lactuca sativa* L., cv Romana) were provided by a local nursery in Catania and were transplanted at the stage of four true leaves. The cultivation was conducted in plastic pots (15x15x10 cm), filled with local soil, which was previously analysed (Table 2). Soil texture was assessed using the pipette method, which involved determining the particle size classes categorized as clay, silt, and sand (Violante, 2000). The soil was air dried, sieved at 2 mm, and analysed for various parameters including water holding capacity (WHC), moisture content, pH, electrical conductivity (EC), organic carbon, phosphorus, total nitrogen, potassium, and Cation Exchange Capacity (C.E.C). The procedures described by Puglisi et al. (2018) were followed to conduct these analyses. The results of the soil characterization are reported in Table 2.

Before transplantation, microalgal cells were mixed directly as fresh microalgal biomass in the soil in a single dose to obtain two different concentrations, 50 and 500 mg/kg of soil (w/w), respectively, and each of them were used alone or mixed with standard mineral fertilization (MF). MF consisted of solid ternary fertilizer NPK, made of NH_4NO_3, KH_2PO_4, and KNO_3, which was purchased from a local agricultural supplier. Mineral fertilization corresponded to the amounts commonly used in regular practice for lettuce cultivation: 116.60 kg/ha NH_4NO_3, 163.32 kg/ha KH_2PO_4, and 138.60 kg/ha KNO_3 (Muscolo et al., 2022).

Treatments were summarized in Table 3.

Table 2. Physical-chemical properties of the soil used in the experimental trials.

Parameters	Values
Clay (%)	13.5
Silt (%)	18.3
Sandy (%)	68.2
WHC (%)	0.2
Humidity (%)	5.97
pH	7.92
Electrical conductivity (mS/cm)	2.95
Organic carbon (%)	1.57
Total Nitrogen (g/kg)	1.15
P (mg/kg)	10.0
K (mg/kg)	42.0
C.E.C. (cmols(+)/kg)	7.59

Table 3. Experimental scheme used for lettuce cultivation.

Thesis	Treatment to the soil
Ctrl – MF	Control not fertilized
Ctrl + MF	Control with standard mineral fertilization (MF)
Cv 50 mg/kg	50 mg/kg of *C. vulgaris* cells
Cv 500 mg/kg	500 mg/kg of *C. vulgaris* cells
Cv 50 mg/kg + MF	50 mg/kg of *C. vulgaris* cells + MF
Cv 500 mg/kg + MF	500 mg/kg of *C. vulgaris* cells + MF
Sq 50 mg/kg	50 mg/kg of *S. quadricauda* cells
Sq 500 mg/kg	500 mg/kg of *S. quadricauda* cells
Sq 50 mg/kg + MF	50 mg/kg of *S. quadricauda* cells + MF
Sq 500 mg/kg + MF	500 mg/kg of *S. quadricauda* cells + MF
Kleb. 50 mg/kg	50 mg/kg of *Klebsormidium* sp. K39 cells
Kleb. 500 mg/kg	500 mg/kg of *Klebsormidium* sp. K39 cells
Kleb. 50 mg/kg + MF	50 mg/kg of *Klebsormidium* sp. K39 cells + MF
Kleb. 500 mg/kg + MF	500 mg/kg of *Klebsormidium* sp. K39 cells + MF

The seedlings were grown in greenhouse conditions for 45 days. The crop was daily irrigated to avoid water stressful conditions. Furthermore, to simulate rain events and evaluate the phenomenon of leaching of nitrates in groundwater, two supplemental irrigation treatments were carried out, applying an amount of water that was 1/3 greater than the water holding capacity (WHC) of the soil, 8 and 28 days after transplantation, respectively. The water lixiviated from the

pots was collected and stored at –80°C until further analyses.

The experimental design was completely randomized and for each treatment, 5 replicates were performed in 5 independent pots.

At the end of the experimental period, all the plants were sampled, frozen in liquid nitrogen, and stored at –80°C for further analytical determinations.

4.2.4 Physiological parameters of lettuce seedlings

Lettuce plants were divided into roots and leaves and separately measured, recording for each sample fresh weight (FW), length, and number of leaves.

Dry weight (DW) was determined for each plant, placing a set of subsamples of roots and leaves in a driving oven at 105°C until to reach a constant weight, and allowed to cool for 2 h inside a closed bell jar. All parameters were recorded on each plant.

4.2.5 Protein extraction from roots and leaves

Total protein extraction from roots and leaves was performed following Kaiser and Lewis (1984). Briefly, frozen lettuce samples were finely ground using liquid nitrogen, and an extraction buffer, containing 0.1 M phosphate buffer pH 7.5, 1 mM EDTA, 2 mM dithiothreitol, and 1.5 w/V insoluble polyvinylpyrrolidone, was added in 1:12 w/V ratio, both roots and leaves. The crude extract was filtered and centrifuged at 13000 rpm for 30 min at 4°C, and the supernatant was collected and precipitated with $(NH_4)_2SO_4$ at 55% of saturation. The total protein content was determined by the Bradford (1976) method, using bovine serum albumin (BSA) as a standard curve (from 2 to 10 µg/mL protein), and expressed as mg protein/g DW. Analyses were performed for each sample.

4.2.6 Plant enzymatic activities

Nitrate reductase (NRA) was determined as described by Kaiser and Lewis (1984). For the reaction, 100 µL of fresh leaf protein extract

(as above described) or 200 µL of fresh root protein extract (as above described) was mixed with 0.1 M potassium phosphate buffer pH 7.5, 1 mg/ml NADH, and 0.1 M KNO_3, and the final volume was made up to 2 mL with distilled water. The samples were maintained at 28°C for 15 min, and then the reaction was stopped by adding 1 mL of 1% (w/V) sulphanilamide in 1.5 M HCl and 1 mL of 0.02% (w/V) n-1-napthyl-ethylenediamine dihydrochloride solution. All samples were centrifuged at 500 rpm for 5 minutes to remove interfering matter. NRA activity was measured spectrophotometrically (Jasco V-730 UV–vis spectrophotometer), by recording the absorbance at 540 nm and was expressed as units of nitrite/mg protein, using a standard curve of sodium nitrite.

All other enzymatic activities were performed on aliquots of precipitated total protein extracts obtained as above described. The extracts were previously centrifuged at 13000 for 30 min at 4°C, the supernatant was discarded, and the pellet was dissolved in the smallest volume possible of the appropriate buffer.

The glutamate synthase (GOGAT) activity of lettuce extracts was determined via the procedure reported by Avila et al. (1987). Each assay mixture, with a final volume of 1.1 mL, contained 25 mM Hepes-NaOH (pH 7.5), 2 mM L-glutamine, 1 mM α-ketoglutaric acid, 0.1 mM NADH, 1 mM Na_2EDTA, and 100 µL of enzyme extract. Samples were read at 340 nm, monitoring NADH oxidation for 4 minutes, and the activity was expressed as nmol NAD^+/min·mg protein, using a molecular extinction coefficient of 6220 L/mol·cm.

The glutamine synthetase (GS) activity was quantified through the method proposed by Cánovas et al. (1991) and was evaluated as transferase activity. For the reaction, in a final volume of 750 µL, 100 µL of enzyme extract solution was added to the assay mixture containing 90 mM imidazole-HCl (pH 7.0), 60 mM hydroxylamine (neutralized), 20 mM $KAsO_4$, 3 mM $MnCl_2$, 0.4 mM ADP, and 120 mM glutamine. The reaction tubes were incubated for 15 min at 37°C, and

next 250 µL of a mixture 1:1:1 of 10% (w/V) $FeCl_3 \cdot {}_6H_2O$ in 0.2 M HCl, 24% (w/V) trichloroacetic acid, and 50% (w/V) HCl was added to stop the reaction. The γ-glutamyl hydroxamate produced was quantified at 540 nm, and GS activity was calculated from a standard curve prepared by plotting the change in absorbance against different concentrations of γ-glutamyl hydroxamate and was expressed as µmol γ-glutamyl hydroxamate/min·mg protein.

All enzymatic activities were measured using three replicates for each separate extraction, and the protein concentration of each enzyme aliquot was calculated by Bradford method (1976).

4.2.7 Soil enzymatic activities

Soil samples were analysed after the sampling at the end of the experimental period, after the harvesting of the lettuce seedlings.

Total hydrolytic activity in the soil was performed by monitoring fluorescein diacetate activity (FDA), according to Green et al. (2006). Briefly, 1 g of soil, soon after the sampling, was dissolved in 60 mM sodium phosphate buffer, pH 7.6, the reaction was started by adding 4.9 mM fluorescein diacetate as substrate. and then the samples were incubated for 3 h at 37 °C. The hydrolysis reaction was stopped by adding 2 mL of acetone, and then samples were centrifuged at 8820 × g for 5 min. The supernatant was recovered, filtered, and the absorbance was measured by spectrophotometry (JascoV-730 UV-vis spectrophotometer) at 490 nm. The fluorescein concentration hydrolysed by the soil enzymes was calculated using a fluorescein standard calibration curve.

Determination of soil dehydrogenase activity (DHA) (EC 1.1) was carried out as described by von Mersi and Schinner (1991). To perform the assay, 1 g of soil was mixed with 1 M Tris(hydroxymethyl) aminomethane (TRIS buffer) and a solution of 9.88 mM 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-5phenyl tetrazolium chloride (INT), daily prepared. The soil samples were incubated at 40°C for 2 hours.

Subsequently, a mixture of ethanol and dimethylformamide in a 1:1 ratio was used to extract the reduced iodonitrotetrazolium formazan (INTF). The quantification of INTF was carried out spectrophotometrically, by measuring the absorbance at 464 nm. To determine the concentration of INTF in the samples, a calibration curve was prepared using known concentrations of INTF as standard.

Acid (EC 3.1.3.2) and alkaline (EC 3.1.3.1) phosphomonoesterase activities, indicated as ACP and ALP, respectively, were assayed according to Tabatabai and Bremner (1969) and Eivazi and Tabatabai (1977), with a few modifications. Briefly, the amount of p-nitrophenol released was determined by incubating 1 g of soil sample at 37°C for 1 h with the buffer and the substrate. The buffer consisted of 28 mM TRIS, 28 mM maleic acid, 19 mM citric acid, and 28 mM boric acid pH 6.5 and pH 11 for ACP and ALP, respectively. The substrate was composed of 115 mM p-nitrophenyl phosphate and was daily prepared. At the end of the incubation, the reaction was stopped by adding 0.5 M calcium chloride and 0.5 mM sodium hydroxide, and the sample was then filtered. The content of p-nitrophenol (PNP) released in the soil samples was spectrophotometrically determined at 400 nm and was calculated using a standard calibration curve, prepared by plotting the change in absorbance against different concentrations of p-nitrophenol.

Urease activity (URE) (EC 3.5.1.5) was carried out as reported by Kandeler and Gerber (1988) with a few modifications. 5 g of soil samples were placed in an Erlenmeyer flask and mixed with 720 mM buffered urea solution. The mixture was then incubated for 2 h at 37°C. Afterward, the samples were treated with 2 M potassium chloride, and filtered, and the filtrates were analysed for ammonia by the following colorimetric procedure. Under alkaline conditions (pH 10), a green-coloured complex was formed as a result of the reactions between NH_3 and sodium salicylate in the presence of sodium dichloroisocyanurate. Urease activity was measured spectrophotometrically

recording the absorbance at 690 nm and was expressed as N released in the reaction, using a standard curve of NH_4Cl.

ACP, ALP, DHA, and URE activities, as well as organic carbon content (C), were used to calculate the potential biochemical index of soil fertility (Mw), according to Kalembasa and Symanowicz (2012), using the following formula:

$$Mw = (ACP + ALP + DHA + URE \times 10^{-1}) \times \%C$$

4.2.8 Determination of the N-NO_3 in leached water

The concentration of nitrate in leached water was measured as described in Fragalà et al. (2023), by extraction with 1 M KCl for 1 h. The extracted solution was then determined spectrophotometrically, recording the absorbance at 540 nm. To quantify the nitrate concentration a standard curve of NO_2^- was used.

4.2.9 Statistical procedures

The collected data were subjected to a one-way analysis of variance (ANOVA). Means were compared using Fischer's protected least significant difference (LSD) test ($p \leq 0.05$). The calculations were carried out on Excel version 2019 (Microsoft Corporation, Redmond, WA, USA) and Minitab (version 16.1.1, Minitab Inc., State College, PA, USA).

4.3 Results

4.3.1 Physiological parameters of lettuce seedlings

The impact of microalgae cells on lettuce physiological parameters, such as root length, shoot height, root and shoot fresh and dry weight, and number of leaves, is reported in Table 4.

Statistical analysis of data revealed significant differences ($p \leq 0.05$) at both the shoot and the root levels between the treatments and the fertilized control (Table 4). However, the unfertilized control consistently exhibited lower morphobiometric results compared to all

other treatments. Microalgae treatments showed a positive trend in the treated plants' shoot fresh weight, especially Cv 50 mg/kg and Cv 500 mg/kg + MF, which exhibited 91% and 76% increases over the Ctrl + MF, respectively. Similarly, all tested microalgae significantly improved root growth compared to the Ctrl + MF. In detail, *S. quadricauda* at the concentration of 50 mg/kg provided the best performance in terms of root fresh and dry weight. Noteworthy, it's interesting to highlight the tendency of microalgae to positively affect the monitored root parameters in every treatment with respect to the Ctrl + MF.

Table 4. Morpho-biometric parameters of lettuce seedlings.

Sample	Root length (cm)	Root fresh weight (g)	Root dry weight (g)	Shoot height (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	n° Leaves
Ctrl – MF	8.38 ± 0.41^{h}	11.39 ± 0.51^{g}	1.47 ± 0.07^{j}	7.33 ± 0.67^{NS}	37.88 ± 3.51^{f}	4.11 ± 0.36^{NS}	8.00 ± 1.00^{NS}
Ctrl + MF	11.97 ± 0.58^{g}	16.04 ± 0.72^{f}	2.14 ± 0.07^{ij}	10.00 ± 0.00	51.57 ± 2.52^{e}	4.93 ± 0.66	14.00 ± 1.00
Cv 50 mg/kg	11.33 ± 0.93^{g}	25.78 ± 1.63^{cd}	3.92 ± 0.23^{ef}	10.67 ± 1.20	98.34 ± 2.42^{a}	7.32 ± 1.37	14.00 ± 1.00
Cv 500 mg/kg	15.83 ± 0.84^{cd}	28.96 ± 0.93^{bc}	5.30 ± 0.23^{b}	11.33 ± 0.67	84.62 ± 6.27^{b}	6.61 ± 0.98	14.00 ± 1.53
Cv 50 mg/kg + MF	12.60 ± 0.21^{fg}	22.90 ± 0.85^{de}	3.35 ± 0.12^{fg}	10.33 ± 0.88	62.23 ± 4.41^{de}	5.01 ± 0.36	14.67 ± 0.33
Cv 500 mg/kg + MF	17.70 ± 0.96^{bc}	28.77 ± 0.94^{bc}	5.05 ± 0.07^{bc}	13.67 ± 0.88	90.70 ± 1.03^{ab}	5.87 ± 0.58	16.00 ± 0.58
Sq 50 mg/kg	14.67 ± 1.20^{def}	34.08 ± 1.66^{a}	8.56 ± 0.37^{a}	13.33 ± 2.40	68.53 ± 0.27^{cd}	6.50 ± 1.58	16.67 ± 2.33
Sq 500 mg/kg	15.87 ± 0.47^{cd}	29.07 ± 0.63^{bc}	4.19 ± 0.17^{de}	10.33 ± 0.88	64.08 ± 7.72^{de}	4.41 ± 0.53	14.33 ± 0.33
Sq 50 mg/kg + MF	11.60 ± 0.38^{g}	23.66 ± 0.77^{de}	4.42 ± 0.34^{cde}	11.33 ± 1.86	61.89 ± 8.77^{de}	4.86 ± 0.69	14.67 ± 2.19
Sq 500 mg/kg + MF	19.33 ± 0.88^{b}	28.32 ± 1.42^{bc}	3.96 ± 0.35^{def}	10.00 ± 0.58	63.51 ± 6.46^{de}	4.92 ± 0.50	15.00 ± 2.31
Kleb. 50 mg/kg	21.90 ± 1.10^{a}	25.90 ± 1.15^{cd}	2.46 ± 0.07^{hi}	11.67 ± 1.67	80.23 ± 0.15^{bc}	2.98 ± 0.46	14.00 ± 1.53
Kleb. 500 mg/kg	15.43 ± 1.20^{cde}	30.27 ± 1.39^{b}	5.26 ± 0.25^{b}	9.33 ± 0.33	65.43 ± 0.77^{d}	4.93 ± 0.48	15.33 ± 0.33
Kleb. 50 mg/kg + MF	13.10 ± 0.95^{efg}	21.23 ± 1.34^{e}	4.65 ± 0.29^{bcd}	10.67 ± 0.67	59.45 ± 4.09^{de}	4.56 ± 0.31	15.00 ± 1.00
Kleb. 500 mg/kg + MF	13.13 ± 0.59^{efg}	23.57 ± 2.43^{de}	3.01 ± 0.31^{gh}	13.00 ± 0.58	66.61 ± 3.82^{d}	4.93 ± 0.28	15.67 ± 0.67

All data are expressed as mean ± standard error of the mean (SEM). Different letters within each column indicate significant differences according to Fisher's protected LSD test (p = 0.05); NS: not significant; ± indicates the standard error mean.

4.3.2 Protein contents

The protein contents both in the shoot and root of the treated seedlings showed a significant increase when compared to the fertilized controls. In this sense, mainly the protein content increased proportionally with the increase of microalgae cell concentration applied to the soil (Figure 1). As already seen for the morphobiometric parameters, the protein contents in both the root and shoot are significantly lower compared to all other treatments.

As regard the root protein contents, Sq 500 mg/kg + MF showed the highest increase (+50%) when compared to the mean content of the Ctrl + MF plants. Similarly, *C. vulgaris* combined with mineral fertilization increased protein root content from 16 (Cv 50 mg/kg + MF) to 33% (Cv 500 mg/kg + MF) with respect to the Ctrl + MF (Figure 1A). Although with lower performances, even *Klebsormidium* sp. K39, in all the treatments, significantly increased the average protein content if compared to those of Ctrl + MF.

At the shoot level, the microalgae also increased the protein content in the treated plants (Figure 1B). In detail, a major increase was noticed in the samples Cv 500 mg/kg + MF, Sq 500 mg/kg + MF, and Kleb. 500 mg/kg + MF (about 12% compared to the fertilized control).

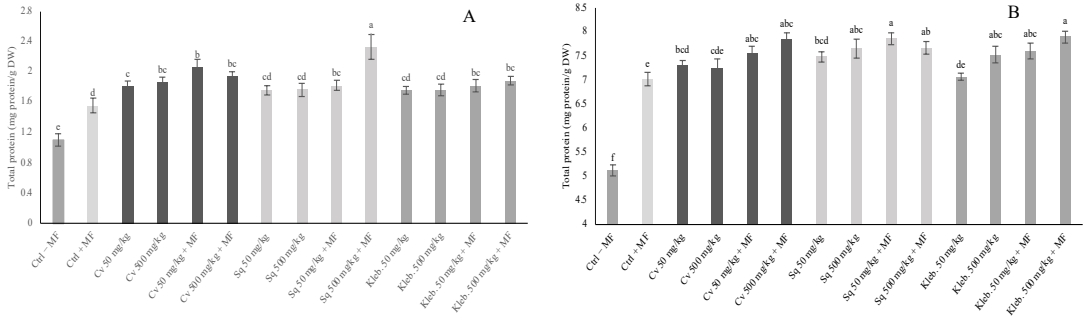

Figure 1. Total protein content in roots (A) and leaves (B) of lettuce seedlings subjected to microalgae treatments. Different letters indicate significance

according to Fisher's protected LSD test (p = 0.05). Error bars indicate the standard error of the mean.

4.3.3 Enzyme activities in lettuce seedlings

The effects of microalgae cells on enzymes related to nitrogen assimilation in lettuce seedlings are reported in Figures 2 and 3. All microalgae associated with mineral fertilization significantly increased the activity of nitrate reductase, glutamine synthetase, and glutamate synthase, both in roots and shoots, when compared to the Ctrl + MF. Furthermore, at the shoot level, all microalgae determined significant increases in glutamate synthase even when applied alone. Regarding the unfertilized control, all measured activities exhibited significantly lower values than those observed in other treatments. This finding can be attributed to the absence of inputs during the experimental trial.

Figure 2A shows the NRA activity measured in lettuce roots. The treatments with *C. vulgaris*, at both concentrations (50 and 500 mg/kg), associated with mineral fertilization produced the highest increases (50 and 66%, respectively) in the activity compared to fertilized control plants. A similar increase was observed in samples treated with *S. quadricauda* (50 and 500 mg/kg) and mineral fertilization (52 and 47%, respectively). Overall, there was a consistent positive trend in NRA activity, and plants treated with microalgae in combination with fertilization showed the highest increases.

Likewise, in Figure 2B, the trend of GS activity in roots is shown, which is like that observed for NRA. The activity was consistently higher in plants treated with microalgae and fertilization compared to Ctrl + MF plants, except for plants treated with only microalgae, where the activity levels did not differ from the Ctrl + MF. The main increases were observed in plants treated with Cv 50 and 500 mg/kg + MF, Sq 50 and 500 mg/kg + MF, and Kleb. 50 + 500 mg/kg, exhibiting increases ranking from 18 to 33% over the Ctrl + MF.

GOGAT activity is shown in Figure 3C. As observed with

previous activities, treatments with *C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39 in combination with mineral fertilization increased all the values of GOGAT activity compared to Ctrl + MF plants. The main increases were recorded in plants treated with Cv 50 and 500 mg/kg + MF (24 and 20%, respectively) and Sq 50 and 500 mg/kg + MF (21 and 23%, respectively).

Regarding the activity levels recorded at the shoot level (Figure 3), the addition of microalgae cells into the soil tendentially increased all enzymatic activities. Just as observed for root data, the formulations with microalgae and fertilization provided the main improvements. In detail, the highest increases in NRA activity were achieved in plants treated with *C. vulgaris* and *S. quadricauda* at a concentration of 500 mg/kg + MF, showing increases ranging from 23 to 28% with respect to untreated plants, respectively.

As shown in Figures 3B and 3C, even GS and GOGAT activities were positively influenced by microalgae, leading to major increases in samples treated with microalgae and fertilization. Specifically, the highest increases in GS activity were observed in plants treated with Cv 500 mg/kg + MF and Kleb. 50 mg/kg + MF, increasing by approximately 15%. Similarly, as regard GOGAT, the main improvement of the activity, a 26% increase compared to the Ctrl + MF, was reached in plants treated with Kleb. 50 mg/kg + MF.

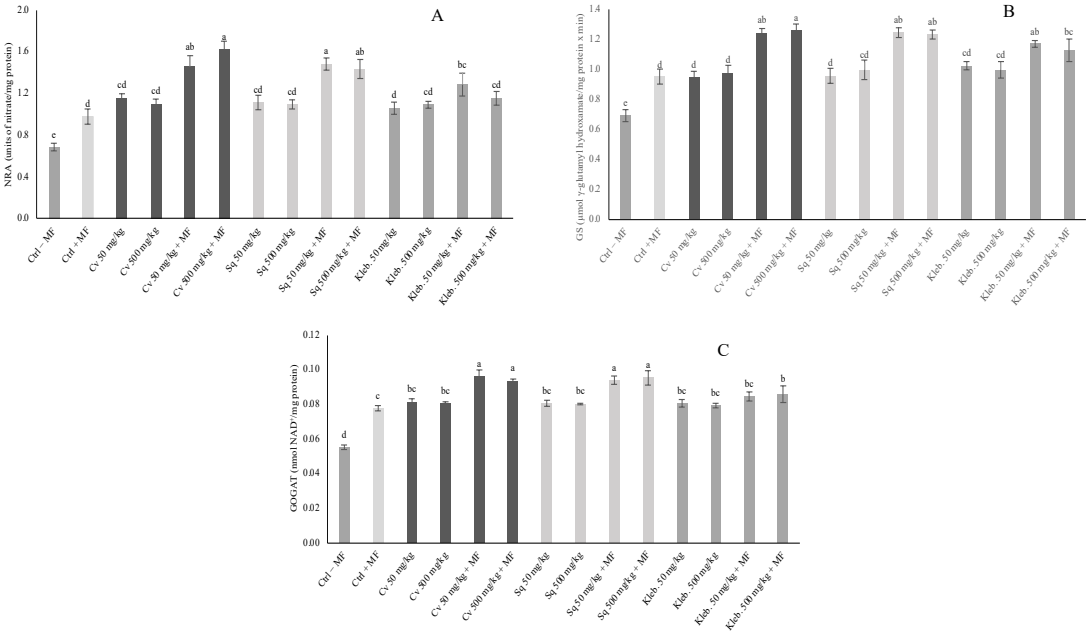

Figure 2. Nitrate reductase (NRA) activity (A), glutamine synthetase (GS) activity (B), and glutamate synthase activity (C) in roots of lettuce seedlings subjected to microalgae treatments. Different letters indicate significance according to Fisher's protected LSD test (p = 0.05). Error bars indicate the standard error of the mean.

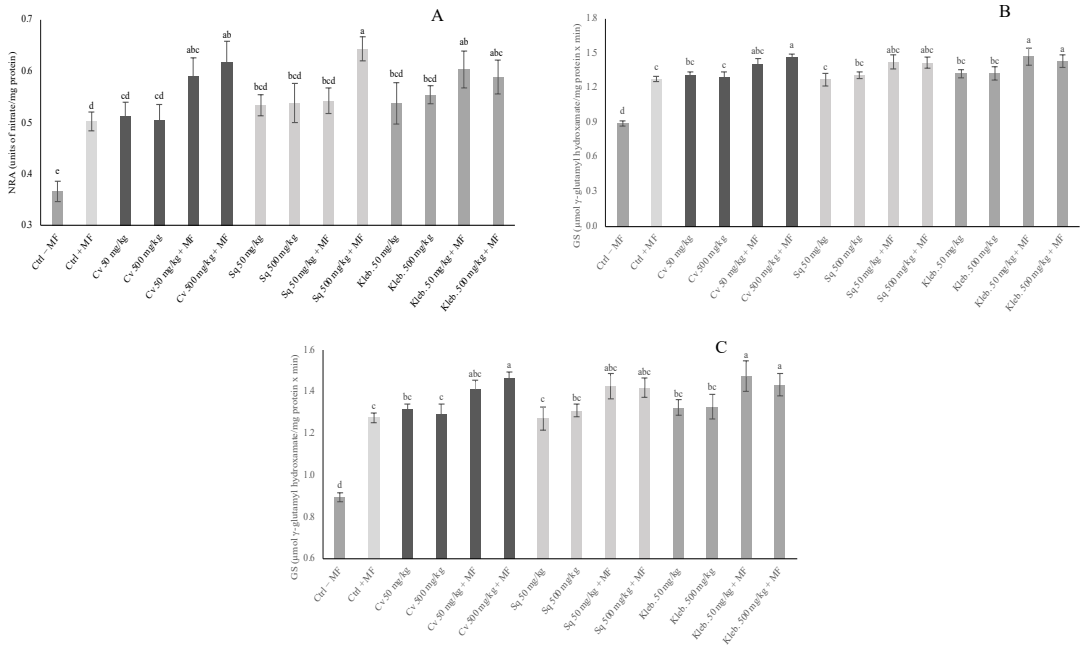

Figure 3. Nitrate reductase (NRA) activity (A), glutamine synthetase (GS) activity (B), and glutamate synthase activity (C) in shoots of lettuce seedlings subjected to microalgae treatments. Different letters indicate significance according to Fisher's protected LSD test (p = 0.05). Error bars indicate the standard error of the mean.

4.3.4 Soil enzymatic activities

The effects of living microalgae cells on the monitored soil enzymatic activities (FDA, DHA, ACP, APL, and URE) are reported in Figures 4 and 5.

The addition of microalgae cells showed a generally positive impact on FDA, ACP, ALP, and URE soil enzymes; however, no significant differences were observed between fertilized control and treatments on DHA (Figure 5A). Regarding the unfertilized control soil, all enzymatic activities showed significantly lower levels than those observed in other treatments. This result can be related to the

absence of inputs during the experimental period.

Fluorescein diacetate hydrolysis was increased by microalgae treatments, and significant differences among treatments were observed (Figure 4). Soil FDA activity was strongly influenced by treatments with *S. quadricauda* at 50 and 500 mg/kg, which resulted in the highest increases in activity levels of about 150 and 173%, respectively, compared to the Ctrl + MF. Nonetheless, all other treatments positively affected the hydrolytic activity. Furthermore, treatments involving only microalgae cells exhibited noteworthy results, showing higher recorded activity levels compared to Ctrl + MF soil. These results suggest the beneficial role of these microorganisms in enhancing the total soil microbial activity, thus contributing to the overall improvement soil quality, even in absence of fertilizer inputs.

Concerning ACP activity (Figure 5B), all treatments with *S. quadricauda* and *Klebsormidium* K39 showed significant increases compared to the Ctrl + MF soil, highlighting that the presence of microalgae cells may lead to an improvement in activity. Among the different treatments, Sq 50 mg/kg + MF reached the highest increase (about 85%) in ACP activity. However, the treatments Cv 50 mg/kg, Cv 500 mg/kg and Cv 50 mg/kg + MF did not show a significant increase in ACP activity compared to the Ctrl + MF soil.

Regarding ALP activity (Figure 5C), all microalgae treatments were found to be effective in increasing the activity compared to the fertilized control soil, showing similar behaviour in terms of their impact on ALP activity. As observed for ACP, Sq 50 mg/kg + MF showed the highest increase (about 69%) in ALP activity.

In contrast to the results for the ACP and ALP activities, the addition of microalgae resulted in a higher URE activity in the treated soils compared to the Ctrl + MF soil for only four treatments. Specifically, Cv 500 mg/kg, Cv 50 mg/kg + MF, Sq 50 mg/kg + MF, and Kleb. 50 mg/kg achieved statistically significant improvements in URE activity compared to the fertilized control soil. Among these

treatments, Cv 50 mg/kg + MF exhibited an approximate increase of 59.9% in URE activity, while the sample Sq 50 mg/kg + MF demonstrated a more substantial increase of approximately 82.5% in URE activity.

Finally, to assess overall soil fertility, the potential biochemical index of soil fertility (Mw) was calculated to include activities of ACP, ALP, URE, and DHA, as well as organic carbon content. Interestingly, Mw values were quite different between treatments, but in each case, Mw values were always higher than the Ctrl + MF (Figure 6). The most efficient treatment proved to be Sq 50 mg/kg + MF (81.5 % increase).

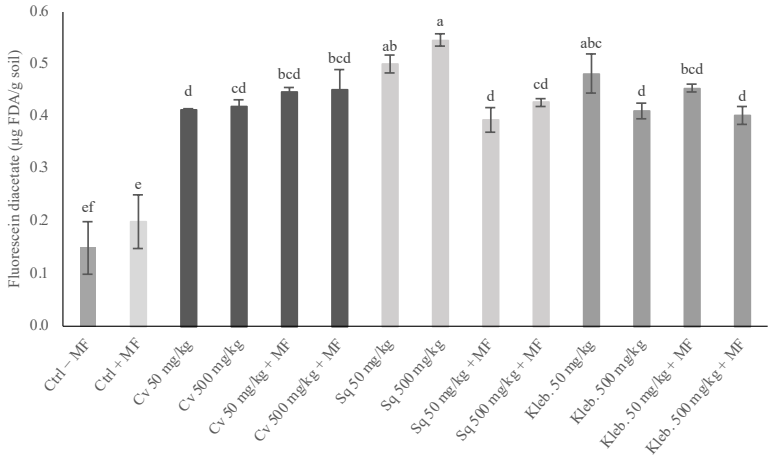

Figure 4. Fluorescein diacetate (µg INTF per g of soil). The values are means of data from 5 pots and three replicates each. Different letters indicate significance according to Fisher's protected LSD test (p = 0.05). Error bars indicate the standard error of the mean.

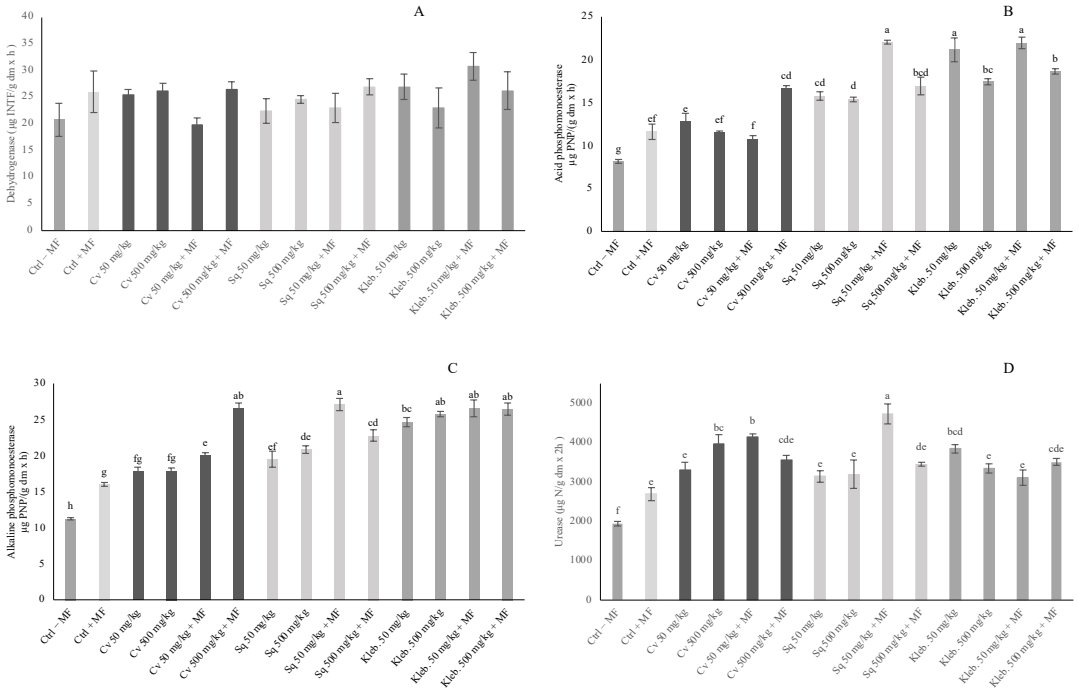

Figure 5. Dehydrogenase activity (µg INTF per g of dry matter in 1 h) (A), acid phosphomonoesterase activity (µg PNP per g of dry matter in 1 h) (B), alkaline phosphomonoesterase activity (µg PNP per g of dry matter in 1 h) (C), urease activity (µg N per g of dry matter in 2 h) (D). The values are means of data from 5 pots and three replicates each. Different letters indicate significance according to Fisher's protected LSD test (p = 0.05), absence of letters means not significant differences. Error bars indicate the standard error of the mean.

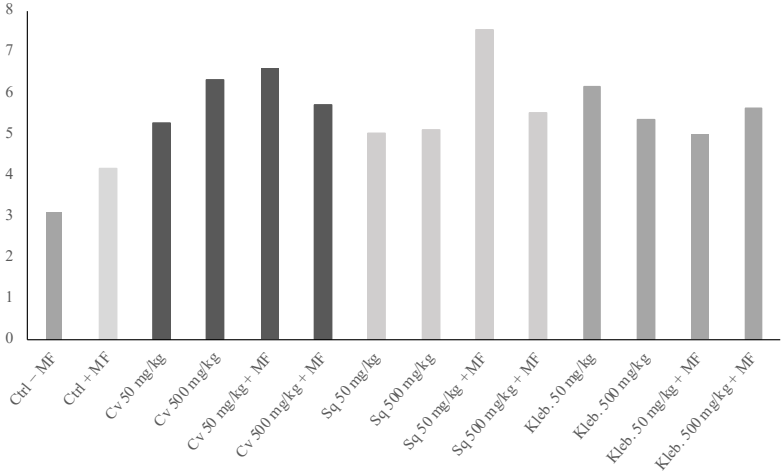

Figure 6. Biochemical index of potential soil fertility (Mw) in soils treated with living cells of *C. vulgaris*, *S. quadricauda*, or *Klebsormidium* sp. K39. The values were calculated using the following formula: Mw = (ACP + ALP + DHA + URE x 10^{-1}) x % C, considering average values of activities.

4.3.5 N-NO_3 content in leached water

Figure 7 displays the N-NO_3 content in the leached water during the experimental trials. As expected, the Ctrl + MF sample exhibited the highest average value overall, approximately 1600 mg/L. However, only specific microalgal treatments showed a significant reduction in N-NO_3 content when combined with mineral fertilization. These treatments include Sq 500 mg/kg + MF, Kleb. 50 mg/kg + MF, and Kleb. 500 mg/kg. On the other hand, when the microalgae were applied alone, only Cv 500 mg/kg appeared to slightly reduce the N-NO_3 content in the leached water, compared to the unfertilized control sample.

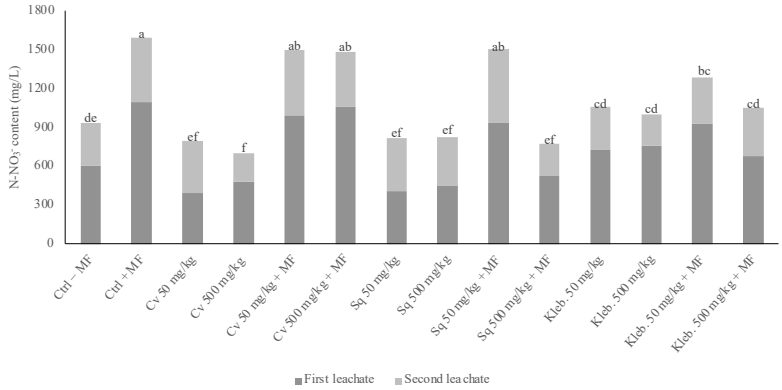

Figure 7. Nitrogen form N-NO_3 in leached water (first and second leachate) after two supplemental irrigation treatments, simulating rain events, during experimental trials. Different letters indicate significance according to Fisher's protected LSD test (p = 0.05). The error bars indicate the standard error of the mean.

4.4 Discussion

The massive use of chemical fertilizers over time has led to negative effects on environmental health. Therefore, in this study, sustainable and environmentally friendly alternative compounds were tested to improve plant growth in lettuce seedlings, one of the most common leaf vegetables in the Mediterranean area, and soil ecosystem health. Moreover, several studies were recently carried out to evaluate the effects of microalgae on a wide range of crops, among these *C. vulgaris* and *S. quadricauda* were largely tested mostly as cellular extracts (La Bella et al., 2022). For instance, Puglisi et al. (2022) showed biostimulant effects on lettuce plants treated with a *C. vulgaris* extract, both at root and shoot levels, with a concentration of 1 mg C_{org}/L. Similarly, Barone et al. (2018) observed that *C. vulgaris* and *S. quadricauda* extracts were able to act as biostimulants in the early stages of sugar beet cultivation, improving root and plant growth. Instead, little is known about possible biostimulant effects of *Klebsormidium* sp. K39.

The effectiveness of microalgae as biostimulants, alone and

combined with conventional mineral fertilization, was assessed in lettuce cultivation: the use of microalgae-based products in agriculture might reduce the use of mineral fertilizers and other chemical products, improving, in the meanwhile, plant growth and sustainability of the process (Apone et al., 2010). However, conventional microalgae biomass production is often too expensive to justify its use for crop production. To exclude this limit, the microalgae used in this study were grown on a large scale in urban wastewater, in order to achieve their phycoremediation, as described in La Bella et al. (2023), proposing a cheap productive methodology in the system of microalgae biomass production (Enzing et al., 2014). As reported by La Bella et al. (2023), the high levels of organic and inorganic compounds in urban wastewater can positively affect microalgae growth. Furthermore, the low concentration of microbiological pollutants and absence of heavy metals (La Bella et al., 2023) exclude the possible contamination risk in microalgae biomasses, leading to a final by-product that can be safely employed in agriculture, in the perspective of a sustainable and circular economy.

Biostimulants, unlike organic or mineral fertilizers, have no mineral-nutrition effects on plant growth since their nutrient concentration is too low (Ertani et al., 2011). This is well-confirmed by the low doses at which they are able to affect plant metabolism (Miller, 2020).

Our results showed that although conventional mineral fertilization always provided good results in terms of plant growth, microalgae-based treatments also showed noteworthy results. In detail, *C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39, at both concentrations (50 and 500 mg/kg), combined with mineral fertilization positively affected plant growth, such as the protein contents and the plant enzymatic activities monitored. Otherwise, microalgae alone were effective in improving plant development but determined only a general slight increase compared to the fertilized control. These results suggest

the possibility of reducing chemical fertilization in lettuce cultivation, mainly due to the ability of microalgal biostimulants to optimise the nutrient uptake efficiency of plants. However, the most significant improvements were recorded at root levels: this is probably due to the modality of application of microalgae cells, leading to a major activity of the enzymes detected, providing, consequently, a major development of roots and a major content of dry matter. Furthermore, among the microalgae tested, the performances of *C. vulgaris* and *S. quadricauda* were slightly better than *Klebsormidium* sp. K39.

Therefore, as shown in Figure 2, treatments with microalgae significantly increased the activities of NRA, GS, and GOGAT at the root level, while they had only a minor influence on the same enzymes in the shoots (Figure 3). The increases in the root apparatus resulting from microalgae applications may also have contributed to an increase in nitrogen uptake, as previously demonstrated by Ertani et al. (2009), who reported that root applications of protein hydrolysates in corn cultivation can enhance nitrogen assimilation through an increase in key enzymes (such as NRA and GS). Furthermore, the positive effects of various biostimulant applications on plant nitrogen content, following an improvement in enzymes of nitrogen cycle, have also been observed in several vegetable crops such as lettuce, radish, and red pepper (Liu and Lee, 2012; Tsouvaltzis et al., 2014).

NRA is an enzyme found in the cytosol of plant cells and is considered a critical point in the nitrate assimilation pathway (Tischner, 2000). It plays a basic role in reducing NO_3^- to NO_2^- and acts as a key component in plant nitrogen metabolism (Nemie-Feyissa et al., 2013). NRA is commonly recognised as the rate-limiting step in this pathway and can impact plant growth and development. Our findings indicate that when soils were treated with mineral fertilization and microalgae at both concentrations, the NRA in the roots significantly increased. However, the improvements observed in the leaves were comparatively lower than those achieved at the root level.

Regarding GS and GOGAT, these enzymes are also considered key players in the process of incorporating ammonium into carbon skeletons and assimilating it into organic forms such as glutamine and glutamate (Gupta et al., 2012). Our results show that treatments involving microalgae in combination with mineral fertilization significantly increased GS and GOGAT activities. These increases were associated with better growth of the seedlings and higher protein content. The positive effects of microalgae on key enzymes of nitrogen cycle in roots are consistent with previous studies, as indicated by Barone et al. (2019) in a co-cultivation system of tomato plants and microalgae *C. vulgaris* and *S. quadricauda*. These authors evaluated simultaneously microalgae growth in the presence of plant roots in the medium for crops as well as the effects of microalgae on tomato plants. Furthermore, our findings are consistent with several other studies, highlighting the involvement of nitrogen metabolism in the promotion of the growth of various crops using common products with biostimulant properties (Bulgari et al., 2015). Overall, microalgae significantly stimulated the enzymatic activities of nitrogen cycle, both at the root and leaf levels, particularly when applied at a higher concentration. It is also worth noting that lettuce seedlings treated solely with microalgae consistently exhibited enzymatic levels that were comparable to or slightly higher than the levels recorded in fertilized control plants, underlining the possibility of reducing mineral fertilization in the lettuce cultivation process.

Various soil enzymes are involved in soil fertility characteristics, biological cycling, soil nutrient conversion processes, and overall soil quality. Furthermore, these enzymes can be used as indicators and sensitive methods to assess the effects of environmental pollutants, agricultural practices, ecological differences, vegetation type, and different soil properties (Zornoza et al., 2006; Nannipieri et al., 2012; Utobo et al., 2015).

Each soil has a specific level of enzyme activities, and the types

and number of enzymes may vary depending on factors such as the quality and amount of harvest residues in the soil, as well as the type and amount of organic and inorganic fertilizers applied (Akça et al., 2015; Koc et al., 2018; Barone et al., 2019). However, it is worth highlighting the importance of soil enzymatic activity and exploring new sustainable solutions to enhance microbial activities in the soil. Our results demonstrated that the monitored soil enzymatic activities were positively affected by microalgal biomasses, specifically FDA, ACP, ALP, and URE. Previous studies demonstrated that several soil enzymatic activities, such as dehydrogenase, urease, and alkaline phosphomonoesterase, were substantially promoted and increased by blue-green algae treatments (Rao and Burns, 1990). Likewise, De Caire et al. (2000) reported that soil treatments with two microalgae species (*Tolypothrix tenuis* and *Microchaete tenera*) as inoculants led to increased activities of extracellular enzymes, such as glucosidase, phosphomonoesterase, arysulfatase, protease, and urease, as well as the accumulation of intracellular dehydrogenase. These findings are in agreement with our results.

FDA (hydrolytic activity) allows estimation of soil microbial activities (Liao et al., 2020). Our results indicate that native soil microbial populations were positively affected by microalgae treatments, as a notable increase in FDA hydrolysis was observed in soil samples treated with microalgae, both alone and in combination with mineral fertilization, compared to Ctrl + MF soil. Barone et al. (2019) obtained similar results and observed an improvement in FDA activity in soil samples treated with living cells of *C. vulgaris* and *S. quadricauda* or their cellular extracts. Furthermore, the inoculation of microalgal cells further increased FDA hydrolysis, suggesting an improvement in the indigenous microbiota by bioinoculants, probably related to increased substrate availability that stimulates the metabolic activity of microbes in the soil.

DHA is an intracellular enzyme, and its action represents total

microbial activity (Saha et al., 2019). However, contrary to what was observed in other soil enzymatic activities, DHA was not significantly affected by the addition of microalgae cells to the soil.

ACP and ALP catalyse the hydrolysis of ester-phosphate bonds, leading to the release of phosphate, which can be taken up by plants or microorganisms (Nannipieri et al., 2012). Overall, ACP and ALP exhibited similar behaviour after the application of microalgal cells. Substantial increases in both enzymatic activities were observed with *C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39 in combination with mineral fertilization, leading to an elevated availability of phosphorous. However, treatments containing solely microalgae did not show any significant differences compared to the Ctrl + MF soil. In general, our results suggest that ALP activity is more stimulated in quantitative terms of PNP released compared to ACP activity, and it is consistent with the pH values of the soils (Table 1), which remained quite constant throughout the experimental period (data not shown). Our findings are also in accordance with Eivazi and Tabatabai (1977) and Dick and Tabatabai (1992), who previously demonstrated that ACP is predominantly active in acid soils, while ALP is active in neutral or alkaline soils.

URE is a potential factor for evaluating soil nitrogen content; indeed, it is a crucial soil enzyme that plays an important role in the hydrolysis of urea to ammonia and carbamic acid, which is further converted to ammonia and carbon dioxide through a chemical hydrolysis process (Sharma et al., 2022). In our study, soil URE was enhanced only by four treatments. These findings are in accordance with a previous study of Barone et al. (2019), where an improvement in URE activity in soil samples treated with living cells of *C. vulgaris* and *S. quadricauda* or their cellular extracts was reported. Furthermore, Kwiatkowski et al. (2020) conducted a three-year study to assess the impact of organic agriculture on soil quality and found that the continuous application of organic manure had a positive influence

on soil urease and dehydrogenase activities.

In general, the positive effects of microalgae treatments on FDA activity, coupled with the relatively high sensitivity of ACP and ALP to treatments, indicate that microalgae soil inoculation has great potential to improve the indigenous microbiota and the release of inorganic phosphorus (orthophosphate) from organic phosphomonoesters (Alef, 1995). Furthermore, increases in URE activity, although observed only in some treatments, indicate the potentiality of microalgal cells as a biostimulant for nitrogen cycling (Siczek and Lipiec, 2016). On the other hand, the less pronounced improvement in DHA might be associated to the low potential of these treatments for the production of adenosine triphosphate through the oxidation of organic matter in the soil (Siczek and Lipiec, 2016).

Concerning the Mw index, a very useful index as it takes into account all the enzymatic activities analysed in the present study to establish the most effective treatment in terms of soil fertility, our findings showed Mw values quite different among the treatments. It appears that *C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39 are able to positively affect soil functioning compared to the Ctrl + MF, alone as well as in combination with mineral fertilization. The increased values of the Mw index indicate that microalgae cells have a positive impact on the biological or biochemical activity of the rhizosphere, as previously reported by Pii et al. (2015), determining as a consequence an improved crop growth. Our results are also in agreement with Barone et al. (2019), who observed higher Mw values in soils treated with microalgae cells and cellular extracts of *C. vulgaris* and *S. quadricauda*. Furthermore, it is noteworthy microalgae cells may positively improve Mw index at a low application dose compared to other substances (Kalembasa and Symanowicz, 2012). Taking all these results together, it is possible to hypothesize that the direct use of microalgae cells in soil treatment, alone or in combination with mineral fertilization, may have a bioactive effect by inducing enzyme

activities using small amounts of biomass.

Regarding the leached water, the maximum leaching of nitrate was taken place from the treatment with only mineral fertilization, while the least was recorded from treatments with only microalgae cells. Statistically, among the treatments, the nitrate contents in the leachates from Cv 50 mg/kg, Cv 500 mg/kg, Sq 500 mg/kg, and Sq 500 mg/kg + MF were significantly lower than the Ctrl + MF treatment by approximately 50%, thus suggesting that the presence of microalgae reduce the loss of nitrate from the soil. Probably, the microalgae biomass in the soil, increasing the soil enzymatic activities and therefore the soil microorganism, led to an increased use of nitrogen by microbiota, and consequently a lower nitrate lixiviation.

Overall, nitrate leaching contents in all the treatments with microalgae cells in the presence of MF were significantly lower than in the fertilized control. Therefore, it might be concluded that the leaching of nitrate to the water bodies may be greatly alleviated by the application of microalgae biomasses to the soil. Our findings are in agreement with Sharma et al. (2021), who observed that the application of microalgal biomass (*Chlorella minutissima*) to soil significantly reduced nitrate leaching compared to chemically fertilized treatment soil. Our experiment may be also supported by the long-term study conducted by Nguyen et al. (2013) on corn and soybean crops to determine the effect of poultry manure and chemical fertilizer on nitrate leaching and found that the leaching loss of nitrate was less in poultry manure compared to urea ammonium nitrate. This hypothesis is further supported by Fragalà et al. (2023), who tested bioproducts from pre-treated municipal biowaste as soil biostimulants. The authors found that these bioproducts may improve plant growth, affect various metabolic pathways, and reduce the environmental impact associated with nitrogen leaching, thus reducing the loss of nitrogen through lixiviation in groundwater. Similarly, microalgae may reduce nitrate lixiviation by increasing the uptake of nitrogen from the plant, as well as

the microbial community living in the soil. Indeed, all microalgae treatments resulted in an improved plant uptake of nitrogen from the soil, determining enhanced seedlings growth. This was supported by the increase in total protein content in the edible portion of plants, as well as the improvements of the main plant enzymes involved in the nitrogen pathway and of the soil microbial activities monitored, hence leading to a great reduction in the amount of leached nitrate in the groundwater. This hypothesis is supported by the evidence that increased nutrient uptake, such as nitrogen from the soil, is one of the main processes studied among the mechanisms of biostimulant products (Chilom et al., 2013; Puglia et al., 2021).

However, this study presents only preliminary results on the effectiveness of microalgae, and further trials are needed to confirm their practical effectiveness in reducing nitrogen loss in groundwater.

Reduction in chemical fertilizers by the use of sustainable and eco-friendly compounds can bring about benefits for microbial communities and environmental health. At this regard, as the present study demonstrates, microalgae are able to improve yields and reduce, as a consequence, the doses of chemical fertilizers, maintaining a high standard of production. Furthermore, microalgae also seem to be able to reduce nitrogen loss of nitrogen through lixiviation, contributing to a global issue as the reduction of environmental pollution is often related to common agriculture practices.

These results might be confirmed under different operating conditions, different crops, and/or different combinations with fertilizers, proposing a solution to improve yields and sustainability of process crop cultivation. These efforts will allow a significant decrease over time in the use of conventional fertilizers, in accordance with global green policies.

4.5 Conclusion

Increased awareness of resource shortages, environmental

protection, food safety, and nutrition has created a need for more sustainable and resource-intensive agricultural production systems. In this context, this study demonstrated that microalgae biomasses, produced using wastewater as a growth substrate, may be considered as sustainable alternatives to enhance the lettuce cultivation process, improving seedlings development and soil quality.

The microalgae biomasses (*C. vulgaris*, *S. quadricauda,* and *Klebsormidium* sp. K39) obtained through the phycoremediation process have shown a great potential as biostimulants in agriculture field, alone or in combination with mineral fertilization, thereby suggesting an auspicable reduction in the use of the latter in common practises. The application of microalgae as a soil biostimulant results in a higher or equivalent yield of lettuce plants compared to the standard supply of recommended doses of mineral fertilizers. Furthermore, at the best of our knowledge, this is the first investigation on the effects of direct application of cells of *Klebsormidium* sp. 39 to the soil. Our results also indicated that microalgae biomasses are able to increase lettuce growth, mainly stimulating nitrogen metabolism in plants while simultaneously reducing nitrogen loss through leaching, and soil microbial wellness, although further studies are needed to evaluate the potential long-term effects of microalgae biomass, obtained after phycoremediation, on various crops.

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5 General conclusion

The PhD thesis aimed to provide further insights into the research field concerning the utilization of microalgae species suitable for agricultural applications. In detail, the review article provided an overview of the versatile applications of microalgae in agriculture, as well as the possible use of various species in the wastewater remediation process. The review article highlighted the feasibility of implementing an integrated and circular approach to the production of microalgae biomass for further agricultural purposes, using discharged raw materials (i.e., wastewater) and thereby reducing the cost of biomass production. Furthermore, the review thoroughly examines the most studied microalgae species, focussing on their potential as biostimulants, biofertilizers, and biopesticides. The bibliographic research conducted to write the present review article led to the selection of a main microalgae species *C. vulgaris* as one of the widely studied microalgae species, which was then further investigated in Chapter 1 of this thesis.

Chapter 1 presents a preliminary study on the potential biostimulant effects of a methanol cellular extract of *C. vulgaris* applied through two different methods in lettuce seedlings: root drenching and foliar spray. This study confirms the biostimulant properties of the extract, enhancing crop yield and positively affecting several key plant enzymes involved in the nitrogen and carbon pathways.

Based on the findings of the Introduction section and Chapter 1, the idea to found new microalgae species suitable for agricultural application emerged. Therefore, in the Chapter 2 further microalgae species were evaluated. In particular, the ability of *C. vulgaris* and *S. quadricauda*, the latter being another commonly studied microalgae species, to remove organic, inorganic, and microbiological pollutants from municipal wastewater with two different magnitudes of contaminants was investigated. Moreover, an autochthonous microalga,

Klebsormidium sp. K39, isolated from a phytoremediation system active in a Sicilian farmhouse was compared to the phycoremediation performance of the two previous mentioned species, *C. vulgaris* and *S. quadricauda*. The results of Chapter 2 demonstrate the effectiveness of all three microalgae in wastewater remediation, achieving high levels of biomass productivity.

In order to support a sustainable and circular bioeconomy model, the potential reuse of microalgae biomass resulting from the above phycoremediation process was further investigated in the study presented in Chapter 3. The final objective was to evaluate potential agricultural applications related to the presence of biostimulant substances in microalgal biomasses. By applying living microalgae cells directly to the soil, differences in terms of lettuce yield and soil biochemical activities were detected and compared with the performance of the treatments with the standard mineral fertilization, commonly used in lettuce cultivation. The results suggest that the use of microalgae inoculants is promising as soil biostimulant, contributing to improve the plant growth and the health of the microbial soil community. Furthermore, the findings indicate that living microalgae cells can mitigate nitrate loses through lixiviation, thus contributing to the amelioration of environmental impact caused by leaching of nutrients in groundwater.

In conclusion, the PhD pathway led to the evidence that the microalgae *C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39 may be successfully used for both wastewater treatment and as biostimulant on lettuce seedlings. Therefore, based on the obtained results, a rational use of microalgae in agriculture could be envisaged. In this regard, it is possible to hypothesize that the direct application of living microalgae cells may offer a more manageable and cost-effective treatment method to achieve similar beneficial effects to those obtained with microalgae-based extracts (Chapter 1). Furthermore, the direct application of microalgal biomasses as soil inoculants, obtained

when feasible through wastewater purification, may present a valuable approach for producers to improve growth performances.

Finally, this thesis confirms the hypothesis that microalgae may represent a viable option in the actual contest, where green-sustainable solutions are needed to address various common issues related to emerging global challenges.

6 Other activity 1

Foliar spray application of *Chlorella vulgaris* extract: Effect on the growth of lettuce seedlings – Research article

Emanuele La Bella[a], Andrea Baglieri[a], Ermes Ivan Rovetto[a], Piergiorgio Stevanato[b], Ivana Puglisi[a]

[a] Department of Agriculture, Food and Environment, University of Catania, Italy.
[b] Department of Agronomy Food Natural Resources Animals and Environment, University of Padova, Italy.

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Abstract

Lettuce seedlings often require the use of fertilizers for their cultivation management to achieve appropriate yield. However, for eco-sustainable chemical-fertilizers-free agronomy, the implementation of totally organic farming often cannot support lettuce productivity, therefore new natural biostimulants able to increase lettuce yield could be considered of great interest. In this preliminary work, the foliar spray application of a *Chlorella vulgaris* extract in lettuce seedlings was investigated in order to achieve better yield performance. Its biostimulant effect was evaluated by monitoring the morphobiometric parameters, chlorophylls, carotenoids, total protein contents, and several enzymatic activities involved in primary and secondary metabolisms of the plant. The experimental trials were carried out by growing

lettuce seedlings on inert substrate (pumice) with a 16 h photoperiod for 21 days. The treatment consisted of three consecutive applications by foliar spraying using a concentration of the *C. vulgaris* extract, corresponding to 1 mg Corg L^{-1}, which were performed one week apart. The results showed that the *C. vulgaris* extract positively influenced the growth of lettuce seedlings, by increasing the fresh and dry weights, chlorophylls, carotenoids, protein content, and ashes at shoot level. From a biochemical point of view, primary and secondary metabolisms of shoots, in particular nitrogen metabolism, were positively influenced. At the root level, the extract increased dry matter, proteins, and ash content.

6.1 Introduction

Among vegetable crops cultivated in the Mediterranean area, lettuce (*Lactuca sativa* L.) often requires for its cultivation the use of biostimulants and chemical fertilizers to reach a high degree of productivity and maximum growth, since it is a crop moderately sensitive to salt (Lucini et al., 2015). Unfortunately, the implementation of totally organic farming in some regions often cannot increase lettuce productivity (Adiloğlu et al., 2018). In this respect, the application of natural biofertilizers and/or biostimulants to the agricultural field is becoming an attractive research topic. Researchers have discussed for a long time about the definition of biostimulants. Du Jardin (2012) defined biostimulants as "substances or a mixture of molecules or microorganism which when applied to plants are able to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, independently of its nutrient content" (Du Jardin, 2012; 2015). Yaknin et al. (2017) proposed to describe a biostimulant as "a formulated product of biological origin improving plant productivity as a consequence of the novel or emergent properties of the complex of constituents, and not as a sole consequence of the presence of known essential plant nutrients, plant growth regulators, or plant protective

compounds." In Europe, the legislative framework on plant biostimulant is very complex, and it is regulated at a state level, since specific legislation or definitions are still not issued (Du Jardin, 2015; Caradonia et al., 2019). In this complex context, du Jardin (2015) also proposed to deem biofertilisers as a subcategory of biostimulants, which are able to increase nutrient use efficiency and allow new prospect for nutrients acquisition by plants.

Among these natural biostimulants, microalgae and their extracts showed to be good candidates, since it was shown that they may increase plant growth as well as improve the germination process, aiming to attain sustainable and environmentally friendly agricultural systems (Faheed and Fattah, 2008; Elhafiz et al., 2015; Zhang et al., 2017; Barone et al., 2018; Ronga et al., 2019; Barone et al., 2019a; 2019b; Puglisi et al., 2020a; 2020b).

Regarding the microalgae effect on lettuce growth, Elhafiz et al. (2015) successfully used *Chlorella vulgaris* and *Chlorella pyrenoidosa* living cells as biofertilizers for lettuce seedlings, providing them in the irrigation water of the culture, which strongly improved the dry weights and the chlorophyll content of cultivated lettuce. The same biofertilizer effect was also proven for other crops such as rice, cucumber, and eggplant (Elhafiz et al., 2015). Moreover, a formulation composed of *C. vulgaris* and plant growth-promoting bacteria (*Bacillus licheniformis*, *Bacillus megatherium*, *Azotobacter* sp., *Azospirillum* sp., and *Herbaspirillum* sp.) showed to positively affect the fresh weight, total antioxidant capacity, and total carotenoids content in lettuce cultivated for spring and summer crop (Kopta et al., 2018). More recently, an extract from *Scenedesmus quadricauda* showed a biostimulant effect on lettuce seedlings, increasing their growth at shoot level, and by influencing the activities of several enzymes involved in the primary and secondary plant metabolisms (Puglisi et al., 2020a).

In recent years, several studies have been carried out on the

biostimulant effect of microalgae and their extracts containing biologically active compounds on a great variety of vegetable crops (Ronga et al., 2019; Chiaiese et al., 2018). Among these studies, the application of a mixture of microalgae (MaB-flocs and *Nannochloropsis* biomass) to the substrate showed to positively affect the growth of tomato seedlings (Coppens et al., 2016). The living cells of microalgae *C. vulgaris* and *S. quadricauda* showed to exert a biostimulant effect on tomato plants by increasing their growth parameters, both when tomato plants were grown in a microalgae co-cultivation system in Hoagland solution and when living cells were directly applied into the soil (Barone et al., 2019a; 2019b).

Moreover, the use of microalgal extracts applied by foliar spraying was proven to increase N-content in treated plants by improving nutrient uptake and by regulation of physiological plant metabolism (Ronga et al., 2019; Shaaban, 2001). Indeed, foliar spray application of microalgae-based products was recently considered as a promising and innovative agricultural technique, as it is safe to the environment, increases agricultural sustainability, and achieves high yield in crop production (Ronga et al., 2019; Shaaban, 2001a; 2001b). The application of 5% and 10% microalgal suspensions of *C. vulgaris* by spraying plants of Swiss chard and in soil, respectively, positively affected the initial growth of Swiss chard, and the content of photosynthetic pigments (Hajnal-Jafari et al., 2020).

The aim of this work is to investigate, as a first approach, the biochemical response of lettuce seedlings treated by foliar spray application of an extract from *C. vulgaris*. The novelty of this study consists in the use of a little amount of the methanolic extract of *C. vulgaris* directly sprayed on the surface of the lettuce seedlings. In order to test the biostimulant effect of the treatment, both at leaf and root level, a set of morpho-physiological parameters, the protein contents, and ash contents of lettuce seedlings were investigated. Moreover, the chlorophyll and carotenoid contents of leaf tissues were also

measured. Finally, the biochemical response at the shoot level was estimated by measuring the activities of glutamate synthase and glutamine synthetase (enzymes involved in nitrogen metabolism), citrate synthase and malate dehydrogenase (enzymes involved in carbon metabolism), phenylalanine ammonia-lyase (the key enzyme involved in secondary metabolism, leading to the synthesis of phenylpropanoids).

6.2 Materials and methods

6.2.1 Microalgae culture and extract preparation

Chlorella vulgaris (CCAP 211/11C) was obtained and maintained in the algal collection of the Department of Agriculture, Food and Environment (Di3A) (University of Catania, Catania, Italy). *C. vulgaris* was cultivated as detailed in Puglisi et al. (2018). Briefly, microalgae were grown in standard BG11 algae culture medium in a growth chamber, bubbled with air using a pump at around 180 bubbles per minute through a plastic tube fitted to an air regulator, illuminated by a 3500-lux, average photon flux (PPF) 100 µmol m^{-2} s^{-1} light source (SON-T AGRO 400, PHILIPS, Eindhoven, the Netherlands), with a 12 h photoperiod (microphotography image is provided in Supplementary Figure S1). The microalgal biomass was collected when it reached the plateau growth phase and was centrifugated at 2500 rpm for 10 min at room temperature. The pellet was washed further with distilled water to reach a conductivity <200 µS cm^{-1} (Stanier et al, 1971; Baglieri et al., 2016). The final *C. vulgaris* biomass was treated with methanol (99.9% *v*/*v*) to lyse the cell walls and release the intracellular contents. Lysed cells were centrifugated 2500 rpm for 10 min at room temperature, and the organic solvent was evaporated, then the extract was collected with distilled water to obtain the microalgal extract stock solution. The complete characterization of the biomass of *C. vulgaris* and its extract was reported in Barone et al. (2018), and the distribution of C intensity of 13C NMR and element composition are summarized in Supplementary Tables S1 and S2.

6.2.2 Experimental conditions

The experiments were carried out using pumice as an inert substrate in transparent containers (40 × 20 × 10 cm) as reported in Puglisi et al. (2020a). The substrate was wetted with 1 L of Hoagland solution: $Ca(NO_3)_2 \cdot 4H_2O$, 1180 mg L^{-1}; KNO_3, 505 mg L^{-1}; KH_2PO_4, 68 mg L^{-1}; $MgSO_4 \cdot 7H_2O$, 493 mg L^{-1}; NH_4NO_3, 80 mg L^{-1}; H_3BO_3, 2.86 mg L^{-1}; $MnCl_2 \cdot 4H_2O$, 1.81 mg L^{-1}; $ZnSO_4 \cdot 7H_2O$, 0.22 mg L^{-1}; $CuSO_4 \cdot 5H_2O$, 0.051 mg L^{-1}; $Na_2MoO_4 \cdot 2H_2O$, 0.12 mg L^{-1}; NaFeEDTA, 22.5 mg L^{-1} (Armon and Hoagland, 1940; Gent, 2017). Lettuce seedlings (*Lactuca sativa* L.) at four true leaves, with a weight of around 4 g and height 8 cm, were provided by a local nursery in Catania. In a completely random design, 10 seedlings were transplanted in each container and were acclimatized by growing them for 6 days in a growth chamber at 25 ± 2 ◦C, with a 16 h photoperiod. Irrigation consisting of 100 mL distilled water was supplied every day, then 3 consecutive treatments one week apart were performed by spraying the seedlings with a solution of Hoagland (500 mL) containing *C. vulgaris* extract at the concentration of 1 mg of organic carbon per liter (Corg L^{-1}), whereas the control plants were sprayed with 500 mL of Hoagland solution. Experimental trials were composed of five replications for treatment and control, and each replicate was made of 10 seedlings. The seedlings were then grown for 21 days (from the first treatment) in a growth chamber at 25 ± 2 ◦C, with a 16 h photoperiod, being irrigated every day with 100 mL distilled water according to the experimental condition described in Puglisi et al. (2020a).

At the end of the experimental period, five plants for each treatment and replica were used for the morphobiometric parameters, whereas the remaining five plants were immediately frozen with liquid nitrogen and stored at −80 ◦C until further analysis.

6.2.3 Morpho-biometric and physiologic parameters in lettuce seedlings

Lettuce seedlings (five plants for treatment and replica) were collected, separated into roots and shoots, and their lengths were measured by using a digital ruler to the nearest 0.5 mm, and the leaf number for each seedling was recorded. On the same seedlings, the fresh weights (FW) of leaves and roots were separately measured, and the dry weights (DW) were obtained by placing tissues in a drying oven at 105 ◦C until the constant weight was reached. Then, each sample was allowed to cool for 2 h inside a closed bell jar, then the dry weights of leaves and roots were separately measured.

Tissue ash contents were determined separately for shoots and roots by incineration of samples in a muffle furnace at 550 ◦C up to constant mass and were expressed as % respect to DW.

Relative growth rate (RGR) was determined, as reported in Gent (2017), from the shoot weights harvested just before the treatment and at the end of the experimental period (21 days after the first treatment) using the following equation:

$$RGR = [\ln(weight_2) - \ln(weight_1)] / (day_2 - day_1)$$

where $weight_2$ represents the fresh weight at the end of the experimental period (21 days), $weight_1$ represents the fresh weight at the beginning of the experimental period, day_2 and day_1 represent the end and the beginning of the experimental period (21 and 0 days), respectively.

The pigment content in leaves (chlorophyll a, chlorophyll b, and carotenoids) were photometrically determined according to Vanni et al. (2006) and Sumanta et al. (2014). Leaf tissues (0.5 g) were homogenized in 10 mL 80% acetone used as extraction solvent, then samples were centrifuged at 10,000 rpm for 15 min at 4 ◦C, and 0.5 mL of supernatant was mixed with 4.5 mL of the extraction solvent. Sample

absorbance was then recorded at three different wavelengths: 470, 646.8, and 663.2 nm (Jasco V-530 UV-vis spectrophotometer) and the relative amount of Chlorophyll-a (Ch-a), Chlorophyll-b (Ch-b), and total carotenoids (C) were calculated as follows:

$$\text{Ch-a} = 12.25\ A_{663.2} - 279\ A_{646.8}$$
$$\text{Ch-b} = 21.5\ A_{646.8} - 5.1\ A_{663.2}$$
$$\text{C} = (1000\ A_{470} - 1.82\ \text{Ch-a} - 85.02\ \text{Ch-b}) / 198$$

Pigments amounts were expressed as mg g^{-1} leaf dry weight (DW).

6.2.4 Total protein extraction from lettuce tissues

Extraction of total proteins and enzymes from leaves and roots of lettuce seedlings was performed as described in Puglisi et al. (2014). Briefly, samples of frozen leaves and roots of lettuce were ground with an extraction buffer made of 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM cysteine, and 5 mM HEPES−KOH pH 7.5, in a 1:1.25 *w/v* ratio. The homogenate was then filtered with three layers of cheesecloth and centrifuged at 13,000 rpm for 30 min at 4 ◦C. The resulting supernatant was recovered, and the total proteins were precipitated with solid $(NH_4)2SO_4$ at 55% of saturation. Total protein content, expressed as mg protein g^{-1} DW, was quantified according to the Bradford (1976) method, using BSA as standard curve.

6.2.5 Enzyme activities in lettuce leaves

Enzymatic activities were performed by using the total protein extract from lettuce leaves. Enzymatic aliquots (1 mL) were centrifuged at 13,000 rpm for 30 min at 4 ◦C, the supernatant was discarded, and the pellet was dissolved in the smallest volume possible with the appropriate buffer for each enzymatic activity. All enzymatic activities were performed as described in Puglisi et al. (2020).

Glutamate synthase (GOGAT) activity was performed in an assay mixture containing 25 mM Hepes-NaOH (pH 7.5), 2 mM L-glutamine, 1 mM α-ketoglutaric acid, 0.1 mM NADH, 1 mM Na2EDTA, and 100 µL of enzyme extract (Avila et al., 1987). GOGAT activity was determined by a spectrophotometer (V-530 UV-vis spectrophotometer, Jasco, Japan), monitoring NADH oxidation at 340 nm by using a molar extinction coefficient of 6220 L mol^{-1} cm^{-1}, and was expressed as nmol NAD+ min^{-1} mg^{-1} protein.

Glutamine synthetase (GS) was measured as transferase activity according to Canovas et al. (1991). The assay mixture (750 µL) contained 90 mM imidazole-HCl (pH 7.0), 60 mM hydroxylamine (neutralized), 20 mM $KAsO_4$, 3 mM $MnCl_2$, 0.4 mM ADP, 120 mM glutamine, and 100 µL of enzyme extract. The enzymatic reaction was incubated at 37 ◦C for 15 min, then 250 µL of a mixture (1:1:1) made of 10% (*w*/*v*) $FeCl_3 \cdot 6H_2O$ in 0.2 M HCl, 24% (*w*/*v*) trichloroacetic acid, and 50% (*w*/*v*) HCl was added. The γ-glutamyl hydroxamate produced during the reaction was spectrophotometrically quantified at 540 nm using a standard curve of γ-glutamyl hydroxamate, and activity was expressed as µmol γ-glutamyl hydroxamate mg^{-1} protein min^{-1}.

Citrate synthase (CS) activity was performed in an assay mixture of 3 mL, containing 50 µL of 0.17 mM oxalacetic acid, 50 µL of 0.2 mM acetyl coenzyme A (acetyl-CoA), and 100 µL of enzyme extract in 0.1 M Tris-HCl, pH 8.0 (Schiavon et al., 2008). CS activity was spectrophotometrically determined by following the reduction of acetyl-CoA to CoA at 232 nm using a molar extinction coefficient of 5400 L mol^{-1} cm^{-1} and was expressed as nmol CoA mg^{-1} protein min^{-1}.

Malate dehydrogenase (MDH) activity was carried out as described in Schiavon et al. (2008). The assay mixture (1 mL) was made of 94.6 mM phosphate buffer pH 6.7, 0.2 mM NADH, 0.5 mM oxalacetic acid, 1.67 mM $MgCl_2$, and 100 µL of enzyme extract. MDH

activity was spectrophotometrically measured by monitoring NADH oxidation at 340 nm using a molar extinction coefficient of 6220 L mol^{-1} cm^{-1} and was expressed as nmol NAD^{+} min^{-1}, mg^{-1} protein.

Phenylalanine ammonia-lyase (PAL) activity was performed as reported in Mori et al. (2001). The assay mixture (final volume of 1 mL) was made of 0.4 mL of 100 mM Tris–HCl buffer (pH 8.8), 0.2 mL of 40 mM phenylalanine, and 200 µL of enzyme extract. The reaction was developed for 30 min at 37 ◦C, then stopped with 200 µL of 25% (*v*/*v*) TCA. Samples were then centrifugated at 10,000 rpm for 15 min at 4 ◦C, and the absorbance of the supernatant was registered at 280 nm. PAL activity was calculated by using a molar extinction coefficient of 16,890 L mol^{-1} cm^{-1} and was expressed as nmol cinnamic acid mg^{-1} protein min^{-1}.

All leaf enzymatic activities were performed as three separated extractions (on tissues sampled from five plants) for each replicate. Protein concentration in each aliquot used for the different enzymatic assays was measured by using the Bradford (1976) method.

6.2.6 Statistical analysis

Data were preliminarily checked for normality using the Shapiro–Wilk test. Data from the repeated experiments about growth performances, chlorophylls, carotenoids, proteins, and enzymatic activities of lettuce seedlings were analyzed using Statistica package software (version 10; Statsoft Inc., Tulsa, OK, USA) by one-way ANOVA ($p < 0.05$), followed by post hoc Tukey's test for multiple comparison procedures.

6.3 Results and discussions

The foliar spray treatment with *C. vulgaris* extract (CV) showed to strongly affect the morphological traits of lettuce seedlings mainly at shoot level as shown in Figure 1. Therefore, these results suggest that foliar spray CV extract treatment seems to be a good strategy to

obtain a greater yield of the edible portion of lettuce without the application of chemical fertilizers. *C. vulgaris* extract could be then considered a biostimulant, increasing lettuce growth according to the biostimulant definition provided by du Jardin (2012) and Yakhin et al. (2017).

Growth and the morphological traits of lettuce seedlings subjected to the foliar treatments were then measured, and the results are shown in Table 1. As confirmed by Figure 1, *C. vulgaris* extract positively affected all the morphological traits of lettuce seedlings at the shoot level (height, number of leaves, FW, and DW). On the contrary, at the root level, no significant differences were detected in length and FW, whereas DW of treated seedlings resulted significantly higher than in control plants (Table 1). These findings are in accordance with Puglisi et al. (2020a), who found that a biostimulant extract prepared from *Scenedesmus quadricauda* applied by root drenching on lettuce seedlings showed better effectiveness above all at shoot level. In particular, CV extract spray application resulted an increase of around 23% of leaf FW and around 20% of leaf DW as compared to those of control (Table 1). Similar values (around 22%) were also reported in lettuce seedlings treated with a *S. quadricauda* extract applied at the root level and grown for 14 days (2020a). Data regarding root DW cannot be compared, as in lettuce treated with *S. quadricauda* extract at root level this parameter was not measured (2020a). Even so, these results suggest that the spray treatment with *C. vulgaris* resulted to be as effective as those performed with *S. quadricauda* on the lettuce seedlings at the root level. Moreover, similar results were also obtained by Barone et al. (2019a) using the *C. vulgaris* extract at the concentration 1 mg Corg L^{-1} on tomato plants (the same concentration applied at leaf level in lettuce), grown in pots of soil for 18 days and treated by a singular soil application, which recorded an increase in their leaf dry weights of around 33% concerning the control. On the contrary, with respect to the unaffected root length, which was

observed in these experiments (Table 1), in the early stages of plant growth in sugar beet, the addition to Hoagland solution of the same amount used in the present study of *C. vulgaris* extract (1 mg Corg L^{-1}) significantly increased total root length of treated plants (Barone et al. 2019c). These different taken together suggest that in the functioning of the CV extract great importance should be referred both to the application method and different variety of plant species.

CV **Control**

Figure 1. Lettuce seedlings sprayed (CV) and not sprayed (Control) with *Chlorella vulgaris extract* after 21 days from the first treatment.

Table 1. Morphological traits of lettuce seedlings subjected to *Chlorella vulgaris* extract treatment (CV) by the foliar application after 21 days from the first treatment. Data are means ± SD. The values are the means of data from five plants for each replica. Values in the same column followed by different letters are significantly different ($p < 0.05$). Ctr: control; FW: fresh weight; DW: dry weight.

	Shoot Height (cm)	Leaves (N°)	Shoot FW (g)	Shoot DW (g)	Root Length (cm)	Root FW (g)	Root DW (g)

Ctr	19.37 ± 0.89 b	14.67 ± 1.15 b	13.66 ± 1.05 b	0.43 ± 0.04 b	11.72 ± 0.56 a	1.95 ± 0.20 a	0.099 ± 0.02 b
CV	23.53 ± 0.75 a	18.00 ± 1.15	16.85 ± 0.95 a	0.60 ± 0.05 a	11.33 ± 0.89 a	1.89 ± 0.16 a	0.154 ± 0.03 a

The FW root/shoot ratios confirmed that CV positively affected the plant weights mostly at the shoot level (Table 2), showing better growth performance. Indeed, it is well-known that, except for injury to the roots, the reduction in the root/shoot ratio is an index of more favorable growing conditions (Bohne and Hasler, 2009). Moreover, the DW root/shoot ratios showed no significant difference among treated and untreated lettuce seedlings (Table 2). These results taken together suggest that the dry matter in the root system and epigeous part grow at the same rate, thus confirming that plants were not affected by stress conditions, simultaneously the lower FW root/shoot ratio in treated plants may be attributed to the general wellness of plants, enhancing the growth of the epigeous part (Bohne and Hasler, 2009). Interestingly, the lowest values of FW/DW ratios, calculated both for shoot and root, were observed in treated plants, suggesting that the treatment positively influenced the biomass accumulation in term of dry matter both at the shoot and root level (Table 2).

Table 2. Growth parameters of lettuce seedlings subjected to *Chlorella vulgaris* extract treatment (CV) by foliar application after 21 days from the first treatment. Data are means ± SD. The values are the means of data from five plants for each replica. Values in the same column followed by different letters are significantly different ($p < 0.05$). Ctr: control; FW: fresh weight; DW: dry weight; RGR: Relative Growth Rate.

	Root/Shoot FW Ratio	Root/Shoot DW Ratio	Shoot FW/DW	Root FW/DW	RGR
Ctr	0.14 ± 0.01 a	0.23 ± 0.01 a	31.77 ± 1.15 a	19.70 ± 1.05 a	0.035 ± 0.004 b
CV	0.11 ± 0.01 b	0.25 ± 0.02 a	28.08 ± 1.04 b	12.27 ± 1.25 b	0.042 ± 0.002 a

Finally, as reported in Table 2, the relative growth rate (RGR) calculated for treated seedlings resulted to be significantly higher than that estimated in control lettuce. Gent (2017) showed that in lettuce

the RGR, representing the relative increase in weight per day, slowly changes when plants grow in a constant environment, whereas environmental and nutritional alterations have effects on their growth. Therefore, being fixed in the experimental conditions, as it is in the present trial, the increase of RGR in treated lettuce was certainly linked to the biostimulant effect of CV extract.

As reported in Table 3, the pigment contents (chlorophylls a and b, and carotenoids) in treated lettuce seedlings showed values always significantly higher than the respective amounts in untreated plants. These data are in agreement with the results reported in other studies on a wide range of crops, including lettuce, in which an increase in chlorophyll contents was observed in plants treated with algae extracts (Puglisi et al., 2020a; Spinelli et al., 2010; Fan et al., 2013). Interestingly, the chlorophyll a and b ratio of treated plants resulted higher than the value calculated for control lettuce (Table 3). Indeed, this ratio being used as an indicator of N partitioning in leaves, it seems to be positively correlated with the ratio of PSII cores, supporting higher light captures by the chlorophyll-protein complex (Kitajima and Hogan, 2003). In accordance with previous results, Hajnal-Jafari et al. (2020) found that treatments with 5% and 10% *C. vulgaris* suspensions applied on soil and Swiss card, respectively, positively affected the content of photosynthetic pigments, showing a correlation analysis between chlorophyll a content and leaf number, and chlorophyll b content and fresh leaf weight.

Table 3. Chlorophyll and carotenoid contents in leaves of lettuce seedlings subjected to *Chlorella vulgaris* extract treatment (CV) after 21 days from the first treatment. Data are means ± SD. The values are the means of data from five replications. Values in the same column followed by different letters are significantly different ($p < 0.05$). Ctr: control; Ch-a: chlorophylls a; Ch-b: chlorophylls b; C: total carotenoids.

	Ch-a (mg g^{-1} DW)	Ch-b (mg g^{-1} DW)	C (mg g^{-1} DW)	Ch-a/Ch-b ratio
Ctr	0.484 ± 0.042 b	0.239 ± 0.024 b	0.153 ± 0.010 b	2.02 ± 0.10 b

CV	0.699 ± 0.035 a	0.282 ± 0.023 a	0.282 ± 0.025 a	2.48 ± 0.11 a

Total protein contents extracted from the shoots and roots of lettuce seedlings are reported in Table 4. The foliar spray treatment strongly increased total protein contents (around 20% and 10% in shoot and root, respectively) compared to the control. An increase in protein contents was also observed in leaves of lettuce treated with *S. quadricauda* extract in Puglisi et al. (2020a), and it is probably related to the raised growth of plants subjected to the treatment. According to previous results of dry weights, ash content in shoots and roots also resulted higher in treated plants concerning the control (Table 4), showing that CV treatment promoted an accumulation of mineral content both at the shoot and root level. These results suggest that the weight increase of the edible part of treated lettuce seedlings (Figure 1 and Table 1) probably was supported by the cumulative increase in pigments (Table 3), proteins, and ashes (Table 4). Meanwhile, the root apparatus supported epigeous growth by increasing the uptake of minerals by soil, since the photosynthates from leaves may be used either for new growth of the shoot itself or may be exported by phloem in root cells, which then increase their biomass (Taiz et al., 2018). This hypothesis is in agreement with Murchie et al. (2009), who reported that an improvement in carbon fixation due to higher interception of solar radiation (chlorophyll content) is strictly related to an increase in yield and biomass in the most important crops. The effect on growth of lettuce seedlings is putatively linked to the action of one or more bioactive compounds present in *C. vulgaris* extract, and exerting their effect above all at shoot levels, determining the manifestation of the biostimulant effect in accordance with the definition of biostimulant proposed by du Jardin (2012; 2015) and Yaknin (2017).

Table 4. Total protein and ashes contents in leaves and roots of lettuce seedlings subjected to *Chlorella vulgaris* extract (CV) treatment after 21 days from the first treatment. Data are means ± SD. The values are the means of data from five replications. Values in the same column followed by different letters are significantly different ($p < 0.05$). Ctr: control.

	Shoot Protein Content (mg g^{-1} DW)	Root Protein Content (mg g^{-1} DW)	Shoot Ashes (%)	Root Ashes (%)
Ctr	92.10 ± 2.2 b	51.93 ± 2.0 b	18.55 ± 1.2 b	6.48 ± 1.0 b
CV	110.53 ± 2.3 a	56.91 ± 2.1 a	21.91 ± 1.5 a	11.28 ± 2.0 a

Finally, to deepen the effect of spray *C. vulgaris* extract on lettuce seedling metabolism, this preliminary study monitored the activities of GOGAT and GS as key enzymes involved in nitrogen primary metabolism, CS and MDH, involved in carbon primary metabolism, and PAL, as the key enzyme of the secondary metabolism (Figure 2). All the enzyme activities calculated in treated samples, except MDH, were always significantly higher than those measured in the controls (Figure 2).

GOGAT and GS isoenzymes play an important role in the primary nitrogen uptake through ammonium assimilation processes into organic form as glutamine and glutamate, representing the nitrogen donors in the biosynthesis of amino acids, nucleic acids, and other nitrogen compounds such as chlorophylls (Lea, 1993; Gupta et al., 2012). Our hypothesis is that greater nutrient absorption, in particular of nitrogen, may occur at the root level, involving an increase in biomass (Table 1), total proteins, and ashes (Table 4), thus contributing to enhancing the growth at the shoot level of the treated seedlings through the increase of nitrogen metabolism (Figure 2A,B). This hypothesis is in accordance with the results reported in several other studies and other crops. Among these studies, the ability of biostimulants to stimulate nitrogen metabolism was shown in lettuce (Puglisi et al., 2020a), maize (Schiavon et al., 2008; Ertani et al., 2009; 2013), and spinach (Fan et al., 2013). Moreover, the application by foliar spray of microalgal extracts was proven to increase N content both in

root and shoot tissues, by improving nutrient uptake and by a regulation of physiological plant mechanisms (Ronga et al., 2019; Shaaban, 2001a; 2001b).

Regarding carbon metabolism, the treatment significantly increased CS activity when compared to that of untreated plants, whereas MDH activity was not significantly affected (Figure 2C,D). These results suggest that the increase of CS activity in the treated lettuce may be strictly related to the formation of α-ketoglutarate as a precursor in the GS-GOGAT pathway supporting N compounds synthesis. This hypothesis may also be confirmed by Hodges (2002), who found that in N-starved tobacco plants after nitrate resupply, a coordinated expression level of CS and GS was measured.

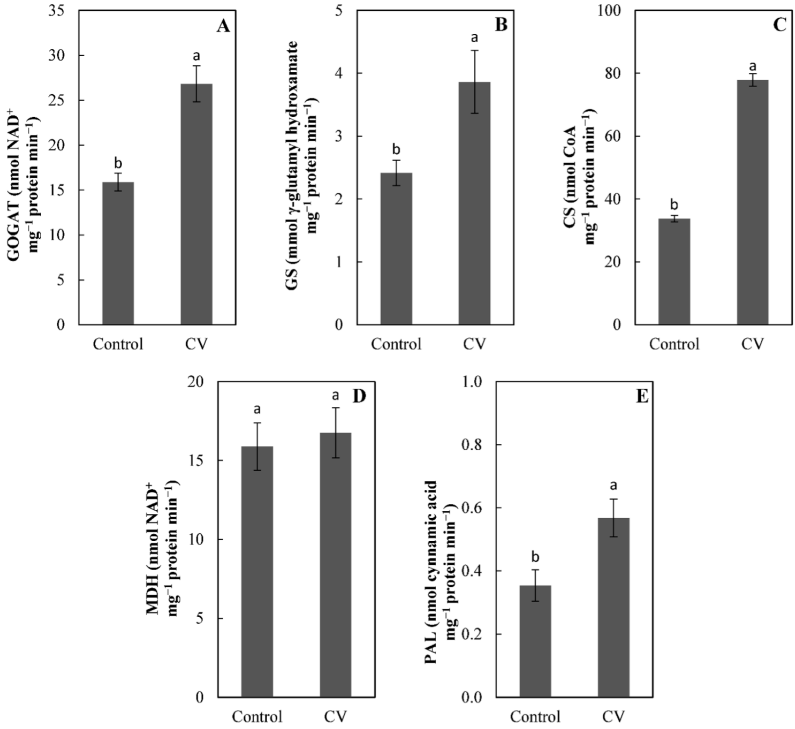

Figure 2. Glutamate synthase (GOGAT) activity (A), glutamine synthetase (GS) activity (B), citrate synthase (CS) activity (C), malate dehydrogenase (MDH) activity (D) and phenylalanine ammonia-lyase (PAL) activity (E) in leaves of lettuce seedlings. Error bars indicate standard deviation. The values are the means of data from five replications. Values followed by different letters are significantly different ($p < 0.05$).

Finally, to evaluate the effect of *C. vulgaris* extract on secondary metabolism, PAL activity was also evaluated (Figure 2E), resulting always significantly higher in treated plants respect to the control. Similarly, *S. quadricauda* extract applied at the root level of lettuce seedlings positively influenced PAL activity (Puglisi et al., 2020a). Indeed, it is well-known that treatments with algae-based extracts

activate either primary metabolism or secondary metabolism by enhancing the biosynthetic pathway of plant defense compounds such as flavonoids and phenylpropanoid (Battacharyya et al., 2015). Given that in seaweeds and their extracts the major reason associated to biostimulation activity on crop plants has often been associated with hormonal effects (Du Jardin, 2015), similarly, the increased growth performance observed in plants treated with microalgae extracts might be due to hormone-like substances, although other possible synergisms among different substances cannot be excluded.

6.4 Conclusions

The foliar spray application of microalgae-based biostimulant in agriculture practice is to be considered a promising and innovative agricultural technique, as it is safe to the environment, eases agricultural sustainability, and achieves high yield in crop production. Indeed, taking all the results together, *C. vulgaris* extract can be considered a biostimulant, being able to increase lettuce yield by enhancing crop growth and inducing plant metabolism. In this regard, these preliminary results represent the first study about a foliar spray application of *C. vulgaris* methanolic extract on lettuce seedlings, reporting a successful biostimulant effect on their growth and metabolism. For future studies it would be very interesting to investigate the comparison of different application strategies of the *C. vulgaris* extract and evaluate the best rate of dosage which allows to obtain the best biostimulant effect on lettuce. Although the application methods of *C. vulgaris* extract would deserve further investigation, the presented results are very promising, since the extract shows to act as a biostimulant on lettuce seedlings by increasing their growth and influencing plant physiology through coordinated induction of N and C metabolisms.

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7 Other activity 2

Transcriptomic profile of lettuce seedlings (*Lactuca sativa*) response to microalgae extracts used as biostimulant agents – Research article

Danilo Fabrizio Santoro[a], Ivana Puglisi[a], Angelo Sicilia[a], Andrea Baglieri[a], Emanuele La Bella[a], Angela Roberto Lo Piero[a]

[a] Department of Agriculture, Food and Environment, University of Catania, Italy.

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Abstract

To reduce the use of chemical fertilizers and maximize agricultural yields, the use of microalgae extracts as biostimulants has recently attracted significant attention due to their favorable impact on both plant growth and their ability to induce tolerance towards environmental stressors. Lettuce (*Lactuca sativa*) is one of the most important fresh vegetables that often requires applications of chemical fertilizers to increase quality and productivity. Accordingly, the purpose of this study was to analyse the transcriptome reprogramming of lettuce (*L. sativa*) seedlings in response to either *Chlorella vulgaris* or *Scenedesmus quadricauda* extracts by applying an RNAseq approach. Differential gene expression analysis revealed that the core gene set that responded to microalgal treatments in a species-independent manner includes 1330 clusters, 1184 of which were down-regulated and 146 up-regulated, clearly suggesting that the repression of gene

expression is the main effect of algal treatments. The deregulation of 7197 transcripts in the *C. vulgaris* treated seedlings compared to control samples (*Ls*Cv vs. *Ls*CK) and 7118 transcripts in the *S. quadricauda* treated seedlings compared to control samples (*Ls*Sq vs. *Ls*CK) were counted. Although the number of deregulated genes turned out to be similar between the algal treatments, the level of deregulation was higher in *Ls*Cv versus *Ls*CK than in *Ls*Sq versus *Ls*CK. In addition, 2439 deregulated transcripts were observed in the *C. vulgaris* treated seedlings compared to *S. quadricauda* treated samples (*Ls*Cv vs. *Ls*Sq comparison) suggesting that a specific transcriptomic profile was induced by the single algal extracts. 'Plant hormone signal transduction' category includes a very elevated number of DEGs, many of them specifically indicating that *C. vulgaris* actives both genes involved in the auxin biosynthesis and transduction pathways, whereas *S. quadricauda* up-regulates genes implicated in the cytokinin biosynthesis pathway. Finally, algal treatments induced the deregulation of genes encoding small hormone-like molecules that are known to act alone or by interacting with major plant hormones. In conclusion, this study offers the groundwork to draw up a list of putative gene targets with the aim of lettuce genetic improvement that will allow a limited or even null use of synthetic fertilizers and pesticides in the management of this crop.

7.1 Introduction

Over the past few years, several studies have been conducted to quantify the impact of climate change on crop productivity (Asseng et al., 2015; Webber et al., 2015; Zhao et al., 2015; Bennici et al., 2019). Considering that it has been reported that the human population might grow to reach 9.6 billion by 2050 (Bruinsma, 2009), emerging breakthroughs are needed to increase crop productivity worldwide and to meet the human requirements in terms of food supplies. From an organic agricultural point of view, the use of chemical fertilizers and

pesticides should be limited (Pascual et al., 2018) as they pose unsolved issues to human health and the environment. Consequently, the necessitates for new eco-sustainable organic compounds have arisen to reduce the dependency on agrochemical compounds which is typical of the conventional agricultural practice. Plant biostimulants are gaining an increasing attention to address environment-friendly crop management due to their positive effects on plant growth (Martínez-Viveros et al., 2010; Bhattacharyya and Jha, 2012), resulting in enhanced nutrient use efficiency, tolerance to abiotic stresses, and improved crop quality and yield (Drobek et al., 2019). Microalgae are photosynthetic, autotrophic, or heterotrophic unicellular microorganisms which are mostly found in freshwater and marine environments. Among the wide range of biostimulant resources (Abbott et al., 2018), microalgae and their extracts have been shown to positively influence plant physiology by affecting both the transcriptomic and metabolomic patterns of the treated plants (Jannin et al., 2013; Battacharyya et al., 2015; Ali et al., 2022) acting either on the plant primary metabolism or secondary metabolism pathways and leading to a generalized increase of plant fitness (Franzoni et al., 2022). Notably, these compounds can be added to the soil in small quantities (Bulgari et al., 2015) provoking an enhancement of water uptake, root and shoot growth, tolerance to abiotic stress conditions, protein content, and the activity of several enzymes related to nitrogen assimilation and photosynthesis processes (Parrado et al., 2008; Baglieri et al., 2014). The effectiveness of biostimulants on plant physiology is not due to single components of extracts but depends on the synergistic action of different bioactive molecules (Rouphael and Colla, 2018) including polysaccharides, phenolics, fatty acids, vitamins, osmolytes and phytohormones (Franzoni et al., 2022). Moreover, the quantity and quality of biologically active metabolites in microalgal extracts largely depend on the species used and on the extraction technique (Puglisi et al., 2018). The characterization of the biostimulant action of *Chlorella*

vulgaris and *Scenedesmus quadricauda* microalgae extracts has been carried out on several crops such as sugar beet (Barone et al., 2018) and tomato (Barone et al., 2019) registering in both cases a sharp enhancement of shoot and root dry and fresh weight (FW) (Barone et al., 2018, 2019).

Lactuca sativa is one of the major horticulture crops grown in the Mediterranean basin, which often requires the use of chemical fertilizers to reach high levels of productivity also being a moderately salt-sensitive crop (Lucini et al., 2015). Recently, the effect of either *C. vulgaris* or *S. quadricauda* extracts on lettuce seedling growth was investigated (Puglisi et al., 2020a, 2022). The results showed that both algal extracts ameliorated seedling growth by promoting an increase in dry matter, in photosynthetic pigment content, and inducing the activities of several enzymes involved in primary and secondary metabolism (Puglisi et al., 2020a, 2022). Similarly, a formulation based on *C. vulgaris* extract combined with plant growth-promoting bacteria was also evaluated revealing a positive effect on the yield and nutritional parameters, on the total antioxidant activity as well as on the carotenoid content in romaine lettuce leaf (Kopta et al*.,* 2018).

The characterization of the global molecular mechanisms by which microalgae extracts exert their effects on plants can be obtained using *-omics* approaches. Transcriptomic analysis based on Next-Generation Sequencing made the development of genomic resources progressively simpler and cheaper. It represents one of the most powerful tools allowing the quantitative determination of all the virtually expressed genes in a specific organ, as well as of the biological processes and metabolic pathways deregulated in response to an external stimulus (Sicilia et al., 2019, 2020; Russo et al., 2021). *De novo* transcriptome analysis has been also applied in lettuce to identify genes specifically induced by UV-B radiation (Zhang et al*.*, 2019) or by inoculation with the necrotrophic fungus *Botrytis cinerea* (de Cremer et al., 2013). More recently, the transcriptomic profiles of young and old

leaves of lettuce grown under different light sources were also unraveled to identify the optimal illumination conditions for green-vegetable production (Nagano et al., 2022).

Taking into account both the acquired knowledge regarding the stimulating effects of microalgae extracts on lettuce seedling's growth (Puglisi et al., 2020, 2022) and the worldwide increasing interest in biofertilizers, the objective of this work was to shed light upon the effects of *C. vulgaris* and *S. quadricauda* extracts on lettuce seedling transcriptomic profile. As far as we know, this is the first report on the global transcriptomic analysis of lettuce leaves treated with algal extracts.

7.2 Materials and methods

7.2.1 Microalgae culture and extract preparation

The microalgae used in this study were *C. vulgaris* (Beijerinck, CCAP 211/11C) and *S. quadricauda* (isolated from an algal company raceway pond, located in Borculo, Gelderland, the Netherlands, in 2011). They were obtained and maintained in the algal collection of the Department of Agriculture, Food and Environment (Di3A) of University of Catania. Microalgal growth was conducted in 250-mL flasks containing 150 mL of sterile standard BG11 algae medium (Stanier et al., 1971) at pH 8.4, incubated on a mechanical shaker (100 rpm) at 25–30 °C, bubbled with air and illuminated by a 3500-lux, average photon flux (PPF) 100 μmol m^{-2} s^{-1} light source (PHILIPS SON-T AGRO 400) with a 12-h photoperiod for 30 days in a growth chamber and aerated by pumps with 20 L h^{-1} 1.5 % CO_2. Microalgal biomasses were harvested by centrifugation at 5000 rpm for 15 min, then the pellet was washed several times with distilled water to reach a conductivity <200 μS cm^{-1} and finally freeze-dried as described by Puglisi et al. (2018, 2019). Microalgae extract stock solutions were prepared as described by Barone et al. (2018). Briefly, microalgae cells were centrifuged at 5000 rpm for 15 min and methanol was added (1:12 w/v

ratio) to the final pellet. The mixture was mechanically shaken overnight to disperse the biomass in the solvent system, lyse the cell wall and obtain the intracellular extracts. Then, the organic solvent was removed through centrifugation at 5000 rpm for 15 min and evaporation via rotary vapour. Finally, the extracts were freeze-dried and collected with distilled water to obtain the extract of microalgae stock solution as reported in Puglisi et al. (2020*a*). The characterization of the biomass of *C. vulgaris* and *S. quadricauda* and their extracts are reported in detail in Barone et al. (2018).

Table 1. Growth parameters of lettuce seedlings subjected to *C. vulgaris* (*Ls*Cv) and *S. quadricauda* (*Ls*Sq) treatments (*Ls*CK: control; FW: fresh weight; DW: dry weight; RGR: relative growth rate). Different letters indicate significance according to Fisher's protected LSD test ($P = 0.05$); *, **, and *: significance of $P \leq 0.05$, 0.01, and 0.001, respectively. ns: not significant.**

Sample	Root/shoot FW ratio	Root/shoot DW ratio	Shoot FW/DW	Root FW/DW	RGR
Control	0.26a	0.39a	16.59a	10.60a	0.0068c
*Ls*Cv	0.20b	0.35a	16.67a	9.98a	0.0255a
*Ls*Sq	0.13c	0.27a	17.61a	9.80a	0.0163b
	**	ns	ns	ns	***

7.2.2 Experimental conditions

The experiment was conducted in transparent boxes (40 × 20 × 10 cm), containing pumice as an inert substrate wetted with 1 L of Hoagland solution (Arnon and Hoagland, 1940) as detailed in (Puglisi et al., 2020*b*). Lettuce seedlings (*Lactuca sativa*) were provided by a local nursery and 10 seedlings at 'four true leaves' stage were transplanted in each box in a completely random design, performing five biological replicates for treatments. The seedlings were grown for 6 days in a growth chamber at 25 ± 2 °C, with a 16-h photoperiod and they were irrigated every day with 100 mL distilled water. After this period of acclimatization (6 days), the treatment was performed by

irrigating the inert substrate with Hoagland solution (500 mL) containing either *C. vulgaris* (*Ls*Cv sample) or *S. quadricauda* (*Ls*Sq sample) extracts at the concentration of 1 mg of organic carbon per litre (Corg L^{-1}), whereas the untreated plants (*Ls*CK) received only 500 mL of Hoagland solution (Puglisi et al., 2020*b*). Leaf tissue was collected both in treated (*C. vulgaris* and *S. quadricauda*) and untreated plants after 4 days from the treatment and immediately frozen in liquid nitrogen and stored at −80 °C until further use (sampling T4[I]).

7.2.3 Morpho-biometric parameters in lettuce seedlings

Lettuce seedlings were collected, separated into roots and shoots, and the FW of leaves and roots was separately determined (0.01 g accuracy). The dry weight (DW) was obtained by placing a set of subsample tissue in a drying oven at 105 °C until constant weight, and, after allowing to cool for 2 h inside a closed bell jar, the DW was recorded. For each sample, the Relative Growth Rate (RGR) index was also determined. It represents the relative increase in weight per day, calculated according to the following equation (Gent, 2017): RGR = [ln(weight2) − ln(weight1)]/(t2 − t1), where weight2 and weight1 represent the DW at the sampling time [sampling T4(I)] and the FW at the beginning of the experimental period, respectively; t2 and t1 represent the end and the initial time of the experimental period (11 and 0 days, respectively). Statistical analysis was performed by evaluating the effects of single factor on lettuce seedlings by using Minitab (version 16.1.1, Minitab Inc., State College, PA) by one-way ANOVA ($P < 0.05$). The arithmetic mean of each parameter was calculated by averaging the values of ratios and RGR determined for the single replicates of each treatment. Post-hoc analysis was performed by Fisher's least significant difference test ($P = 0.05$). The biochemical characterization of seedling samples used in the following transcriptome analysis is reported in Puglisi et al. (2020*b*) and Puglisi et al. (2022) [sampling T4(I)], and includes protein and pigment content,

as well as several enzyme activities involved in primary (carbon and nitrogen) and secondary metabolism.

7.2.4 Sample collection and RNA extraction

RNA isolation was carried out by using the Spectrum Plant Total RNA Extraction kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions (Santoro et al., 2022). RNA purity and concentration were assayed using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA integrity was assessed using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA).

7.2.5 Library preparation for transcriptome sequencing

A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) following the manufacturer's recommendations. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First-strand cDNA was synthesized using random hexamer primers and M-MuLV Reverse Transcriptase (RNase H). Second-strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, NEBNext Adaptors with hairpin loop structure were ligated to prepare for hybridization. To select cDNA fragments of preferentially 150–200 bp in length, the library fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, MA). Then 3 μL of USER Enzyme (NEB) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before polymerase chain reaction (PCR).

Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

7.2.6 Clustering and next-generation RNA sequencing

Cluster generation and sequencing were performed by Novogene (UK) Company Limited (Cambridge, UK). The clustering of the samples was performed on a cBot Cluster Generation System using a PE Cluster kit cBot-HS (Illumina). After cluster generation, the library preparations were sequenced on the Illumina HiSeq2000 platform to generate paired-end reads whose size was paired-end 2 × 150bp reads. Raw reads in fastq format were first processed through in-house perl scripts. In this step, clean data were obtained by removing reads containing adapters, reads containing poly-N and low-quality reads. At the same time, Q20, Q30, GC content and sequence duplication levels of the clean data were calculated. All the downstream analyses were based on high-quality clean data (see Table 2).

Table 2. Summary statistics of the RNA quality and sequencing results.

Average RIN	6.6
Clean reads	216 million
No. of transcripts	94179
No. of unigenes	39253
Average of read mapped rate	84.03%
Transcripts N50 (bp)	1897
Unigenes N50 (bp)	1854
Q30 (%)	95.24
GC content (%)	43.31

7.2.7 De novo assembly and gene functional annotation

De novo transcriptome assembly was made up by Trinity software (2.6.6 version) with min_Kmer_Cov = 3 and min_glue = 4 (Grabherr et al. 2013). Hierarchical Clustering was carried out by Corset (4.6 version) to remove redundancy (parameter – m 10) so that the

longest transcript of each cluster has been selected as unigene (Davidson and Oshlack, 2014). The assembly assessment and gene prediction were performed by Benchmarking Universal Single-Copy Orthologous (BUSCO software, 3.0.2 version; Simão et al. 2015), whereas the unigene functional annotations were obtained by exploiting seven different databases: National Centre for Biotechnology Information (NCBI), non-redundant protein sequences (Nr, Diamond software, 0.8.22 version, e-value threshold 1e-5; Buchfink et al. 2014), NCBI non-redundant nucleotide sequences (Nt, NCBI blast software, 2.9.0 version, e-value threshold 1e-5), Protein family (Pfam, hmmscan software, HMMER 3.1 version, e-value threshold 0.01; Finn et al. 2011), Cluster of Orthologous Groups of Proteins (KOG/ COG, Diamond software, 0.8.22 version, e-value threshold 1e-5; Buchfink et al., 2014), Swiss Prot (Diamond software, 0.8.22 version, e-value threshold 1e-5), Kyoto Encyclopaedia of Genes and Genome (KEGG, Diamond and KAAS software, 0.8.22 version, e-value threshold 1e-5; Moriya et al. 2007; Buchfink et al. 2014) and GO (blast2GO software, b2g4pipe_ v2.5 version, e-value threshold 1e-6; Götz et al., 2008). The *L. sativa* transcriptome was submitted to NCBI (https://www.ncbi.nlm.nih.gov/geo/) accession number (GSE227491).

7.2.8 Quantification of gene expression and differential expression analysis

Gene expression level was estimated by RSEM software (1.2.28 version) by mapping back each clean read onto assembled transcriptome and the read counts for each gene were then obtained from the mapping results. Furthermore, the read counts of each gene have been used as input data for DESeq2 (1.26 version, padj ≤ 0.05), to obtain differentially expressed genes (DEGs; Love et al. 2014). The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. The genes with an adjusted *P*-value ≤ 0.05 were assigned as differentially expressed.

7.2.9 Real-time validation of selected DEG candidates using qRT-PCR

Leaf total RNA (2.5 µg) was reverse transcribed using Super-Script Vilo cDNA synthesis kit by Thermo Fischer Scientific, according to the manufacturer's instructions. Real-time qRT-PCR was carried out for nine DEGs with PowerUp SYBR Green Master mix by Thermo Fischer Scientific. All the genes have been normalized with the endogenous reference gene encoding the ribosomal RNA small subunit methyltransferase (LOC111912865) and the fold change was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The sequences of primers used for real-time PCR are provided in Supporting Information—Table S1.

7.2.10 KEGG, GO, Mapman and iTAK enrichment analysis

For enrichment analysis, all the DEGs were submitted to KOBAS software (version 3.0, corrected *P*-value ≤ 0.05) to identify the significantly enriched pathways in the KEGG database (Mao et al., 2005). The GO functional enrichment analysis of the DEGs was implemented by using either blast2go (b2g4pipe, version v2.5, e-value threshold 1e−6) or GOSeq (version 1.32.0, corrected *P*-value ≤ 0.05) softwares. Moreover, a pathway analysis was conducted using MapMan3.6.0RC1 (https://mapman.gabipd.org/). All the unigenes were annotated and mapped using Mercator4 V2.0, an online tool of MapMan (https://www.plabipd.de/portal/mercator4) which accurately assigns hierarchal ontology providing a visual representation of genes in different plant processes. The significant DEGs (*P*adj ≤ 0.05), with the corresponding log2fold change values, were used as dataset to align with the Mercator map. Furthermore, we focused on those clusters showing a threshold of ±1.5 $\log_2$fold change. For each cluster, sequence alignment has been performed and the score of these alignments (*L. sativa*, 100% identity and e-value = 0) provided clear indications of the cluster identity. iTAK (hmmerscan software) tool was

used to identify the transcription factor (TF) families among DEGs (Pérez-Rodríguez et al. 2009; Jin et al. 2014). Furthermore, to identify the core gene set responding to microalgal treatments, the significant DEGs (*P*adj ≤ 0.05) belonging to both *Ls*Cv versus *Ls*CK and *Ls*Sq versus *Ls*CK comparisons (threshold of ±1.50 $\log_2$fold change) were retrieved and merged in a list of genes responding to both algal treatments and deregulated in the same direction (up- or down-regulated). All these genes were subjected to GO and Mapman enrichment analysis as described above.

7.3 Results

7.3.1 Effect of microalgae extracts upon lettuce seedling morphobiometric parameters

As shown in Table 1, the application of microalgae extracts positively influenced the seedling morphological traits. In detail, the application of *C. vulgaris* and *S. quadricauda* extracts reduced the root/shoot FW ratios, indicating that better-growing conditions have been reached (Bohne et al., 2009). *C. vulgaris* and *S. quadricauda* extracts did not affect both the shoot and root FW/DW ratios, thus suggesting that the treatments determined a biomass accumulation in terms of dry matter at a comparable extent to the control conditions (Table 1). As reported in Table 1, the RGR, whose value increases as function of an ameliorated nutritional status of the plant (Gent, 2017), resulted to be higher in treated samples than that calculated for control seedlings. In particular, the RGR in *Ls*Cv samples was higher than that measured in the *Ls*Sq thesis, thus suggesting that *C. vulgaris* extract could exert a more pronounced biostimulant effect on lettuce seedlings.

7.3.2 Transcript assembly and annotation

In this study, a comprehensive identification of the transcriptional response of *L. sativa* seedlings to *C. vulgaris* and *S.*

quadricauda extracts was conducted by applying a RNASeq approach. The quality of RNA was assessed before the preparation of the libraries by the RNA integrity number (RIN) measurement. The mean RIN value was 6.6, indicating that a low level of RNA degradation occurred, thus all samples were adequate for further processing and sequencing (Table 2). After library sequencing, we filtered the raw reads to remove the adapter-based or poor-quality reads, obtaining a total of 216 million clean reads (Table 2), representing the 98.02% of the total reads. Downstream analysis was further performed on about 36 million reads (10.82 Gb per sample), showing Q30 and GC content equal to 95.24% and 43.31%, respectively (Table 2). The clean read *de novo* assembly yielded 94,179 transcripts and 39,253 unigenes with N50 length of 1897 bp and 1854 bp, respectively (Table 2), consistent with previously reported N50 values (Sicilia et al. 2019, 2020; Zhang et al., 2019) and indicating that a good coverage of the transcriptome has been achieved. To assess assembly consistency, filtered unique reads were mapped to the reconstructed transcriptome and the average read mapping rate using bowtie2 alignment software was equal to 84.03 % (Table 2). The completeness of the assembled transcriptome was evaluated by comparing it to the set of Embryophyta genes using the BUSCO quality assessment tool coupled with the OrthoDB (9.0 version) database of orthologs (Simão et al., 2015). The quality of the *L. sativa* leaf transcriptome was comparable to those of the majority of transcriptome assemblies listed in Simão et al. (2015). Among the searched 1440 BUSCO groups, 76.25 % (1098 BUSCOs) was complete (1046 single-copy orthologs and 52 duplicated), 10.9 % (157 BUSCOs) was represented by fragments and 12.9 % (185 BUSCOs) was missing. In addition, both transcript and unigene length distributions were reported [see Supporting Information—Fig. S1].

Functional annotation of the lettuce unigenes was conducted by performing BLAST searches against public databases, such as the National Center for Biotechnology Information (NCBI), Protein Family

(Pfam), Protein Ortholog Group Clusters (KOG/COG), SwissProt, Ortholog Database (KO), Gene Ontology (GO) (Table 3). A total of 33 819 unigenes were annotated in at least one database, and the frequency of unigenes annotated in at least one searched database was 86.15 %. Among them, 29 515 (75.19 %) and 30 498 (77.69 %) unigenes showed identity with the sequences in the Nr and Nt databases, respectively. The distributions of unigene homologous to the sequences in the KO, SwissProt, Pfam, GO, and KEGG databases were 25.24 %, 56.84 %, 52.81 %, 52.81 % and 18.77 %, respectively (Table 3).

7.3.3 Identification of differentially expressed genes

The characterization of leaf *L. sativa* transcriptome was carried out by the identification of those unigenes whose expression level changed upon microalgal extract treatments. Based on the experimental design, a total of 16,754 DEGs were observed from all the comparisons. Among them, 3254 up-regulated genes and 3943 down-regulated genes were detected in the *Ls*Cv versus *Ls*CK (samples treated with *C. vulgaris* vs. untreated samples), whereas in the case of *Ls*Sq versus *Ls*CK (samples treated with *S. quadricauda* vs. untreated samples) a total of 2773 up-regulated genes and 4345 down-regulated genes were identified (Table 4). Table 4 also reports the number of deregulated genes in the *Ls*Cv versus *Ls*Sq comparison (samples treated with *C. vulgaris* vs samples treated with *S. quadricauda*). A total of 2439 DEGs were in this last comparison, 1374 of them resulted up-regulated and 1065 down-regulated, thus indicating that a distinct response was induced upon lettuce seedlings in a species-specific manner by the two algal extracts under investigation. However, transcripts belonging to both the *Ls*Cv versus *Ls*CK and *Ls*Sq versus *Ls*CK comparisons and showing the same direction of deregulation (up- or down- regulated) were retrieved and included in a list representing the core gene set that responded to treatments in a microalgal species-

independent manner [see Supporting Information—Table S2]. The list includes 1330 clusters, 1184 of which were down-regulated and 146 up-regulated, suggesting that the effects of algal extracts mainly involve the repression of a high number of lettuce genes.

Table 3. The number and percentage of successful annoted genes.

Database	Number of unigenes	Percentage (%)
Annotated in NR	29515	75.19
Annotated in NT	30498	77.69
Annotated in KO	9908	25.24
Annotated in SwissProt	22314	56.84
Annotated in PFAM	20733	52.81
Annotated in GO	20731	52.81
Annotated in KOG	7369	18.77
Annotated in at least one database	33819	86.15

7.3.4 Validation of RNAseq experiments by real-time PCR

The validation of gene expression levels for nine selected DEG candidates was carried out by quantitative real-time PCR (coefficient of determination $R2 = 0.91$), indicating the reliability of RNA Seq in the quantification of gene expression [see Supporting Information—Fig. S2]. In addition, the selected genes could also constitute useful markers of microalgal extract response in lettuce.

7.3.5 GO and Mapman enrichment analysis of the core gene set deregulated in algal species-independent manner

The GO functional enrichment analysis of those clusters belonging to both the *Ls*Cv versus *Ls*CK and *Ls*Sq versus *Ls*CK comparisons and showing the same direction of deregulation (146 up-regulated and 1184 down-regulated) is shown in Fig. 1. 'Protein kinase domain' (GO:0051603) (6 up- and 16 down-regulated genes), 'Protein tyrosine and serine' (GO:0016310) (6 up- and 10 down-regulated genes), 'Leucine rich repeat' (GO:0006913) (8 up- and 7 down-regulated genes) and 'ABC transporter' (GO:0006810) (1 up- and 10 down-regulated genes) are the most enriched GO terms found in the

Biological Process (BP) category. 'Oxidation-reduction process' (GO:0016702) (6 up- and 60 down-regulated genes), 'ribosome biogenesis' (GO:0042254) (0 up- and 41 down-regulated genes), 'regulation of transcription, DNA- templated' (GO:0006355) (8 up- and 29 down-regulated genes) and 'transmembrane transport' (GO:0055085) (8 up- and 28 down-regulated genes) are the most enriched GO terms in the Molecular Function (MF) category. Among the DEGs belonging to the Cellular Component (CC) category, the most represented GO terms are 'protein binding' (GO:0005515) (15 up- and 60 down-regulated genes), 'ATP binding' (GO:0005524) (11 up- and 24 down-regulated genes) and 'DNA binding' (GO:0003677) (11 up- and 19 down-regulated genes). All the significant DEGs were also analysed with the Mapman 3.6.0RC1 software and 'protein homeostasis' (33 DEGs, 4 up- and 29 down-regulated), 'lipid metabolism' (18 DEGs, 3 up- and 15 down-regulated), 'phytohormone' (9 DEGs, 5 up- and 4 down-regulated) and 'amino acid metabolism' (6 DEGs, 0 up- and 6 down- regulated) are the categories mainly deregulated by the algal treatments [see Supporting Information—Table S3].

Table 4. DEG number of different comparisons under microalgae treatments.

	Up-regulated	**Down-regulated**	**Total DEGs**
*Ls*Cv vs *Ls*CK	3254	3943	7197
*Ls*Sq vs *Ls*CK	2773	4345	7118
*Ls*Cv vs *Ls*Sq	1374	1065	2439
Total DEGs	7401	9353	16754

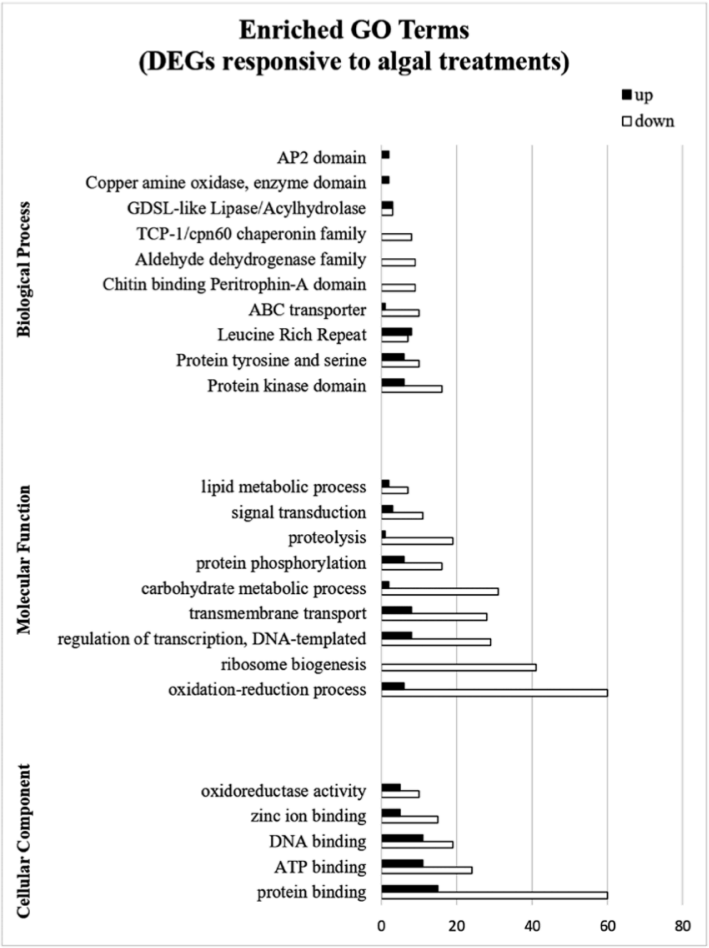

Figure 1. GO enrichment analysis for the DEGs in common between the *Ls*Cv vs *Ls*CK and *Ls*Sq vs *Ls*CK comparisons in *L. sativa*. The Y-axis indicates the subcategories, and the X-axis indicates the numbers related to the total number of GO terms. BP, biological process; MF, molecular functions; CC, cellular components.

7.3.6 Functional classification of DEGs

Gene Ontology terms, Clusters of Orthologous Groups of proteins (KOG) classification and KEGG pathway functional enrichment were carried out to identify biological processes or pathways specifically involved in lettuce seedling response to microalgal extract treatments. Considering the *Ls*Cv vs *Ls*CK data set (Fig. 2A), 'Cellular nitrogen compound metabolic process' (GO:0034641) (229 up- and 227 down-regulated genes), 'biosynthetic process' (GO:0009058) (177 up- and 177 down-regulated genes) and 'transport' (GO:0006810) (143 up- and 106 down-regulated genes) are the most enriched GO terms found in the BP category. 'Ion binding' (GO:0043167) (267 up- and 194 down-regulated genes), 'oxidoreductase activity' (GO:0016491) (134 up- and 64 down-regulated genes) and 'DNA binding' (GO:0003677) (71 up- and 97 down- regulated genes) are the most enriched GO terms in the MF category. Among the DEGs belonging to the CC category, the most represented GO terms are 'intracellular' (GO:0005622) (202 up- and 215 down-regulated genes), 'protein-containing complex' (GO:0032991) (155 up- and 174 down-regulated genes) and 'organelle' (GO:0043226) (141 up- and 168 down-regulated genes).

Among the DEGs belonging to *Ls*Sq versus *Ls*CK dataset, 'Transport' (GO:0006810) (155 up- and 81 down-regulated genes), 'cellular nitrogen compound metabolic process' (GO:0034641) (126 up- and 88 down-regulated genes), 'biosynthetic process' (GO:0009058) (115 up- and 78 down- regulated genes) and 'small molecule metabolic process' (GO:0044281) (116 up- and 80 down-regulated genes) are the most represented GO terms identified in the BP category. 'Ion binding' (GO:0043167) (241 up- and 142 down-regulated genes), 'oxidoreductase activity' (GO:0016491) (96 up- and 65 down-regulated genes), and 'transmembrane transport activity' (GO:0022857) (96 up- and 48 down- regulated genes) are over-represented in the MF category (Fig. 2B). In the CC category, 'intracellular' (GO:0005622) (225 up- and 137 down-regulated genes), 'protein-

containing complex' (GO:0032991) (184 up- and 110 down-regulated genes) and organelle (GO:0043226) (172 up- and 106 down- regulated genes) were highly represented. A similar trend also characterized the comparison *Ls*Cv versus *Ls*Sq since the same categories are represented (Fig. 2C).

To predict and classify possible functions, all the 39,253 unigenes were aligned to the KOG database and were assigned to the KOG categories [see Supporting Information—Fig. S3]. Among the KOG categories, the cluster for 'posttranslational modification, protein turnover and chaperones' (12.30 %) represented the largest group, followed by 'general function prediction only' (11.40 %) and 'translation, ribosomal structure, and biogenesis' (10.46 %) [see Supporting Information—Fig. S3]. To identify biological pathways activated in response to microalgae extracts, DEGs were also mapped onto the KEGG database. Figure 3 shows the main metabolic pathways sorted by the decreasing gene number involved in each pathway in relation to all the comparisons under investigation (*Ls*Cv vs. *Ls*CK, *Ls*Sq vs. *Ls*CK and *Ls*Cv vs. *Ls*Sq). Interestingly, the results indicate that the maximum number of DEGs were observed in the 'biosynthesis of amino acids', 'cell cycle', 'plant hormone signal transduction' and 'starch and sucrose metabolism', indicating that a deep metabolic reprogramming occurred in presence of the microalgal extracts (Fig. 3). The remodulation of the metabolic machinery is also supported by the involvement of other important pathways, such as 'carbon metabolism' and 'phenylpropanoid biosynthesis', which play a pivotal role both in primary and secondary metabolisms thus confirming our previous results (Puglisi et al., 2020b). It is also worth to note that among the most enriched metabolic pathways, 'protein processing in endoplasmic reticulum', 'ribosome' and 'RNA transport' which are involved in mRNA translation to a polypeptide chain were deeply regulated by microalgae extracts (Fig. 3).

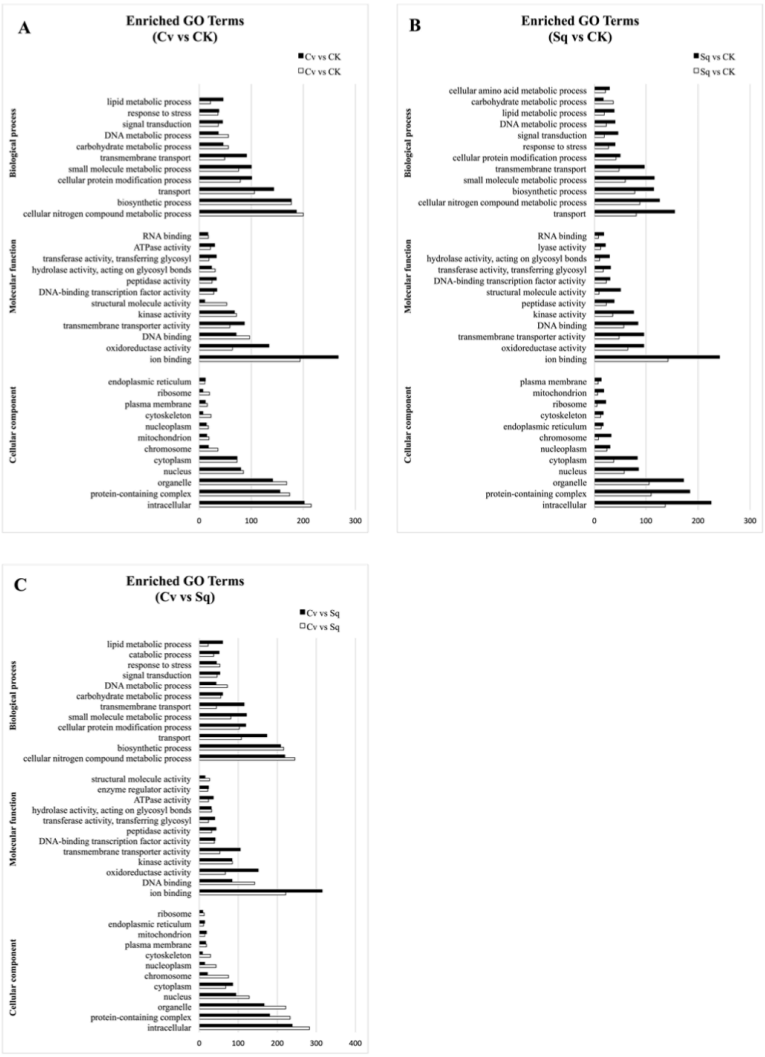

Figure 2. GO enrichment analysis for the DEGs in *L. sativa*. (A) *Ls*Cv vs *Ls*CK. (B) *Ls*Sq vs *Ls*CK (C) LsCv vs LsSq. The Y-axis indicates the subcategories, and the X-axis indicates the numbers related to the total number of GO terms. BP, biological processes; CC, cellular components; MF, molecular functions.

7.3.7 Comprehensive analysis of the main pathways induced by microalgal extracts

To obtain a complete picture of the metabolic changes occurring in lettuce seedlings treated by microalgae extracts, all the significant DEGs were mapped to the Mapman 3.6.0RC1 software. As shown in Fig. 4, several genes resulted deregulated (activated or inhibited) by either algal treatments (Fig. 4A and B). However, a shaper response was obtained in the *Ls*Cv versus *Ls*CK comparison (Fig. 4A) than in the other comparisons (Fig. 4B and C) thus indicating that the global response of lettuce to *C. vulgaris* was more pronounced than the response to *S. quadricauda*. Accordingly, to decipher the lettuce leaf response to algal treatments, we filtered the significant Mapman enriched DEGs by applying a ±1.5 log_2fold change filter and counted the DEGs belonging to each category. As shown in Supporting Information—TableS4, 'proteinhomeostasis'(253DEGs), 'phytohormone' (148 DEGs), 'lipid metabolism' (140 DEGs) and 'amino acid metabolism' (95 DEGs) are the categories mainly deregulated by the algal treatments. In the 'protein homeostasis' category, the up-regulation of several genes responsible for protein turnover was observed, including those encoding for chaperone, ubiquitin ligase, serine carboxypeptidase and proteases thus indicating that a strong rearrangement of the protein metabolism is strictly induced in response to microalgae extract treatment (data not shown). A second group of categories includes those clusters counting between 52 and 25 DEGs. Among these categories, 'cell division' (46 DEGs) and 'cell wall organization' (25 DEGs) are probably related with higher seedling growth induced by the algal treatment (Puglisi et al., 2022). In addition, the 'redox homeostasis' category (32 DEGs) lists a group of genes encoding glutathione peroxidases and glutathione transferases confirming their role in protecting plant cells both in normal and stressful conditions (data not shown; Lo Piero et al., 2010; Puglisi et al., 2013). Finally, a third group comprises categories including from 18 to 3 DEGs such as 'protein

translocation' (18 DEGs) and 'photosynthesis' (10 DEGs) [see Supporting Information—Table S4].

7.3.8 Dissection of the 'phytohormone' and 'transcription factor' categories

Considering their main role in transcriptome reprogramming, we further dissected both the 'phytohormones' (Tables 5 and 6) and 'transcription factor' categories (Fig. 5). Table 5 includes the DEGs of the 'phytohormone' category that have been found specifically deregulated *by C. vulgaris* (*Ls*Cv vs. *Ls*Sq comparison) and reports the log fold change of each deregulated cluster. The gene 2 encoding the isopentenyltransferase (IPT), responsible for the rate-limiting step of cytokinin biosynthesis, was drastically down-regulated in the *Ls*Cv versus *Ls*Sq (−8.50 log2fold change), and concordantly, the cytokinin independent 1 histidine kinase, an activator of the cytokinin signalling pathway, was down-regulated. In addition, cytokinin phosphoribohydrolase (LOG) encoding gene catalysing the direct activation pattern was also down-regulated. Zeatin-type-cytokinin synthase (CYP735A), involved in later steps of cytokinin biosynthesis, was found up-regulated in *C. vulgaris* treated samples in comparison with those seedlings treated with *S. quadricauda*. However, the gene encoding the zeatin O-glucosyltransferase (ZOG) which glycosylates cytokinins leading to the cytokinin forms with reduced biological activity, was also up-regulated indicating that in *C. vulgaris* treated samples these hormones and the induction of the related signal cascade are repressed with respect of the *S. quadricauda* treated seedlings (Table 5). Clusters encoding the ligands negatively influencing stomatal density (EPF/ EPFL, epidermal patterning factor; Rychel et al., 2010) were found down-regulated in the *C. vulgaris* treated samples. Another group of clusters related to cell proliferation was also found down-regulated in the *Ls*Cv versus *Ls*Sq comparison (Table 5): (i) the TDIF (Tracheary element Differentiation Inhibitory Factor) peptide and the

TDR/PXY (TDIF receptor/Phloem intercalated with Xylem) membrane protein kinase, promoting the proliferation of procambial cells and suppressing their xylem differentiation (Hirakawa et al., 2010), (ii) the EMS1 (EXCESS MICROSPOROCYTES1) LRR-RLK and its small protein-ligand TPD1 (TAPETUM DETERMINANT1), that play a fundamental role in somatic and reproductive cell differentiation during early anther development in *Arabidopsis* (Li et al., 2017), and (iii) the phytosulfokine receptor which regulates a signalling cascade involved in plant cell differentiation, organogenesis, somatic embryogenesis, cellular proliferation and plant growth.

Interestingly, two clusters encoding indole-3-pyruvate monooxygenase involved in auxin biosynthesis during embryogenesis and seedling development (Zhao, 2010) were up-regulated in *Ls*Cs vs *Ls*Sq comparison suggesting that auxin is strongly implicated in the response of lettuce towards *C. vulgaris* treatment. Moreover, the down-regulation of regulatory protein kinase (PINOID) of auxin transport functioning as a positive regulator of polar auxin transport (Benjamins et al., 2001) indicates that the fine-tuning of polar auxin transport during organ formation in response to local auxin concentrations is affected in *C. vulgaris* treated samples.

Finally, in Table 6 the DEGs in common between the *Ls*Cv versus *Ls*CK and *Ls*Sq versus *Ls*CK comparisons related to the 'phytohormone' category are reported. All of them are subjected to the same de-regulation type (up- or down-regulated) in both comparisons, but in all cases, the extent of gene de-regulation is higher in *Ls*Cv versus *Ls*CK than in *Ls*Sq versus *Ls*CK (Table 6) as encountered by MapMan analysis (Fig. 4). Among the down-regulated genes we enumerate the transcriptional repressor (IAA/AUX) that represses the expression of primary/early auxin response genes (Tiwari et al., 2004), confirming the crucial role of auxin signal transduction during algal treatment. Moreover, the CASPARIAN STRIP INTEGRITY FACTOR (CIF) that triggers the spatially precise deposition of designated cell wall

components, enabling plants to establish transcellular barrier networks correctly (Table 6). RALF-peptide receptor (*Catharanthus roseus* receptor-like kinase 1-like, CrRLK1L)—THESEUS, previously shown in *Arabidopsis* to trigger growth inhibition and defense responses upon perturbation of the cell wall (Gonneau et al., 2018) was also down-regulated in both comparisons (Table 6). Among the up-regulated genes, we found an essential regulator of plant stress responses, RALF-peptide receptor (CrRLK1L)—FERONIA, and the B-type ARR response regulator of cytokinin initiating both the transcriptional response to cytokinin and a negative feedback loop that desensitizes the plant to cytokinin (Zubo et al., 2020). (PYR/PYL/RCAR) receptors, responsible for the regulation of the ABA signalling pathway, PIN-LIKES (PILS) proteins contributing to intracellular auxin homeostasis (Zhao et al., 2021) and Plant Natriuretic Peptides (PNPs) which have an important and systemic role in plant growth and homeostasis (Morse et al., 2004) were among the most induced genes under algal treatments. Interestingly, the PILS protein-encoding gene, identified to be putative auxin carrier at the endoplasmic reticulum (ER) and control intracellular auxin accumulation (Zhao et al., 2021), was highly up-regulated in algal-treated samples.

Figure 5 categorizes the DEGs encoding TFs in the three comparisons (*Ls*Cv vs. *Ls*CK, *Ls*Sq vs. *Ls*CK and *Ls*Cv vs. *Ls*Sq). Overall, for each considered family, the highest number of deregulated TFs was encountered within the *Ls*Cv versus *Ls*CK samples, and AP2/ERF, WRKY, MYB and NAC TF are numerically the most represented families.

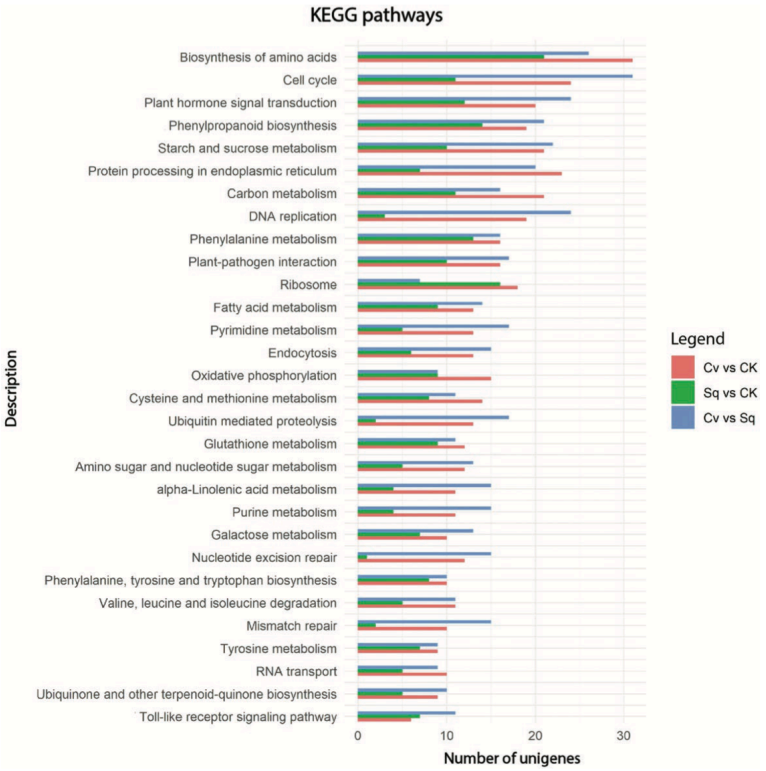

Figure 3. The main KEGG biological pathways for the DEGs in lettuce leaf transcriptome. The Y-axis indicates the KEGG categories, and the X-axis indicates the numbers of unigenes.

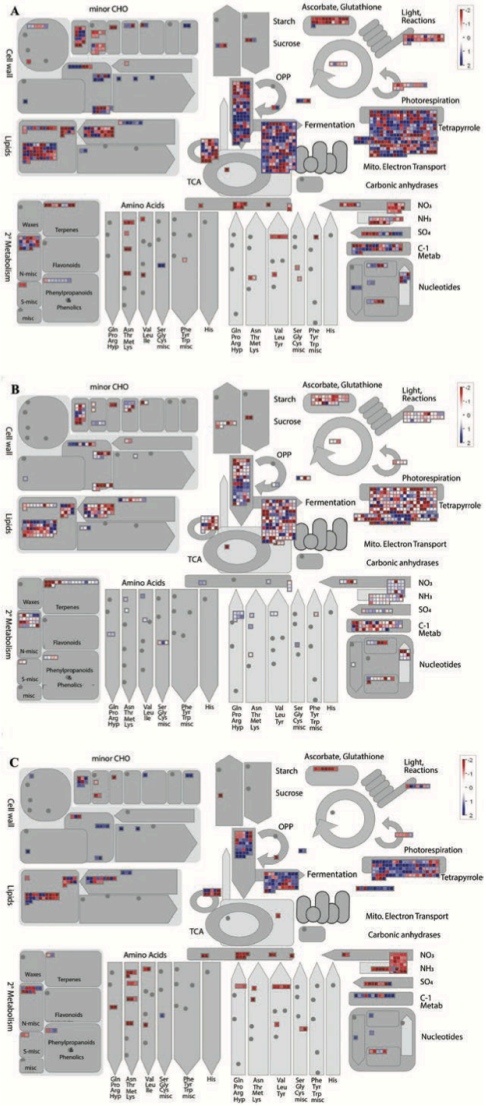

Figure 4. MapMan analysis of differentially expressed genes in *L. sativa*. (A) *Ls*Cv vs *Ls*CK. (B) *Ls*Sq vs *Ls*CK. (C) *Ls*Cv vs *Ls*Sq. Blue spots represent up-regulated genes and red spots represent down-regulated genes.

Table 5. DEGs listed in the 'Phytohormone' category specifically deregulated in *Ls*Cv versus *Ls*Sq.

Cluster ID	Database description	log_2fold change LsCv vs LsSq
9839.0	*IP-type-cytokinin synthase* (*IPT3*)	–8.50
15809.3160	*Cytokinin signalling pathway activator* (*CKI1*)	–2.12
15809.14844	*Cytokinin phosphoribohydrolase* (*LOG3*)	–1.45
16226.0	*Cytokinin hydroxylase*	+2.86
3690.0	*Zeatin O-glucosyltransferase* (*ZOG*)	+3.20
15809.11964	*EPF/EPFL epidermal patterning factor*	–2.26
11176.0	*TDL-peptide receptor* (*EMS1/MSP1*)	–2.03
6384.0	*Regulatory protein kinase* (*PINOID2*) *of auxin transport*	–1.76
15809.1007	*TDIF-peptide receptor* (*PXY*)	–1.69
10341.0	*Pythosulfokine peptide receptor* (*PSKR1*)	–1.61
14797.0	*Flavin-dependent monooxygenase* (*YUCCA10*)	+2.42
8784.0	*Flavin-dependent monooxygenase* (*YUCCA5*)	+2.52

Table 6. DEGs listed in the 'Phytohormone' category in common between *Ls*Cv vs *Ls*CK and *Ls*Sq vs *Ls*CK.

Cluster ID	Database description	log_2fold change LsCv vs LsCK	log_2fold change LsSq vs LsCK
8434.0	*Transcriptional repressor* (*IAA27/AUX*)	–4.12	–2.29
17059.0	*CIF precursor polypeptide*	–3.17	–2.55
5788.0	*Transcriptional repressor* (*IAA17/AUX*)	–3.09	–1.93
15809.12001	*RALF-peptide receptor* (*CrRLK1L*) – *THESEUS*	–2.48	–2.20
15809.3701	*Brassinosteroid signalling protein kinase*	–2.18	–2.02
18385.0	*RALF-peptide receptor* (*CrRLK1L*) – *FERONIA*	+1.99	+1.78
14748.0	*B-type ARR response activator of cytokinin*	+2.70	+2.32
15809.8656	*PYL/RCAR abscisic acid receptor PYL4-like*	+2.90	+2.34
4711.0	*Auxin efflux transporter* (*PILS7*)	+4.30	+2.79
6996.0	*PNP precursor polypeptide* (*EG45-like*)	+11.00	+6.61

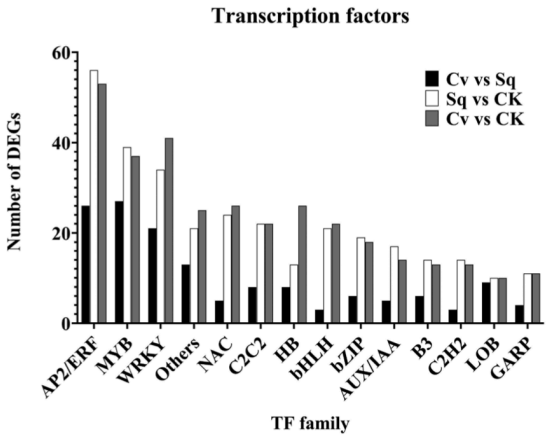

Figure 5. Number of DEGs encoding for TFs found in the three comparisons (*Ls*Cv vs *Ls*CK, *Ls*Sq vs *Ls*CK and *Ls*Cv vs *Ls*Sq).

7.4 Discussion

The use of plant biostimulants as recent eco-friendly approach to promote crop development has caught the interest of researchers due to the variety of ways in which they can improve plant fitness. One of the most promising classes of biostimulants is represented by microalgae extracts containing a plethora of bioactive compounds whose variegated composition could be responsible for the wide range of biological effects exerted on different crops (Deolu-Ajayi et al., 2022). Several manuscripts have been recently published concerning the potential and advantages of using microalgae extracts as biostimulants, especially in undesired conditions (Chiaiese et al., 2018; González-Morales et al., 2021; Deolu-Ajayi et al., 2022; 2022; Franzoni et al., 2022). Overall, they report that the beneficial effects of the algal extracts pass through changes of morphological, physiological, biochemical, epigenomic, proteomic and transcriptomic nature. However, the reprogramming of gene expression can be considered the first step to generate further changes at molecular levels, and for this reason, transcriptomic analysis via RNAseq might be considered the analysis of choice to encompass the interaction between plant and biostimulant extracts. Recently, the transcriptomics of plant biostimulation under stressful conditions has been reviewed revealing that *Ascophyllum nodosum* seaweed extract is widely applied, especially upon leaves of *Arabidopsis* and tomato (González-Morales et al., 2021). Interestingly, the transcriptomic data indicated that *A. nodosum* seaweed extract promotes *A. thaliana* seedlings' growth as well as the induction of genes involved in abiotic stress (Goñi et al., 2016).

Lettuce (*L. sativa*) is one of the most important vegetable crops grown in the Mediterranean region where saline water is frequently used for irrigation (Lucini et al., 2015). Thus, with a view to reduce the use of chemical fertilizers and replacing them with environment-friendly compounds, our previous works were aimed at verifying the influence of both *C. vulgaris* and *S. quadricauda* extracts upon lettuce

morpho-biometric parameters and the related biochemical response (Puglisi et al., 2020*b*, 2022). To elucidate the response of lettuce to microalgae extracts, in this work, we sequenced and *de novo* assembled the *L. sativa* leaf transcriptome to identify differential gene expression, BPs, metabolic pathways, and molecular markers. Our results indicated that the core gene set that responded to microalgal treatments in a species-independent manner includes 1330 clusters, 1184 of which were down-regulated and 146 up-regulated, clearly suggesting that the repression of gene expression is the main effect of algal treatment associable with the stimulating activity upon lettuce seedlings. However, although the total DEGs number between *Ls*Cv versus *Ls*CK and *Ls*Sq versus *Ls*CK comparisons was similar, our results suggested that the extent of transcriptome reprogramming between the treatments under investigation was qualitatively and quantitatively different (Tables 5 and 6). In particular, we enumerated 2439 DEGs specifically deregulated on the basis of the applied algal species (*Ls*Cv vs. *Ls*Sq comparison); this result was also confirmed by both Mapman analysis (Fig. 4), which indicated that a more pronounced response was achieved using *C. vulgaris* extract, and by the RGR values which resulted higher in seedlings treated with *C. vulgaris* extract (Table 1). The analysis of biological pathways provided a comprehensive representation of the most relevant metabolic pathways reprogrammed in lettuce upon algal treatments. Among the most enriched KEGG pathways were 'biosynthesis of amino acids' and 'plant hormone signal transduction' suggesting a key role of algal extract in inducing a deep rearrangement of both hormone biosynthesis, often starting from amino acids functioning as substrates, and the related signal transduction. The analysis of the 'Phytohormone' category clearly indicated that in *C. vulgaris* treated samples the cytokinin biosynthesis and signal transduction were strongly repressed with respect to the *S. quadricauda* treated seedlings, whereas, auxin biosynthesis and homeostasis were activated, thus suggesting that the registered beneficial effects of

both algal extracts (Puglisi et al., 2020b, 2022; Santoro et al., 2022) pass through different metabolic pathways and processes. The fact that a group of genes involved in cell proliferation and differentiation (EPF/EPFL epidermal patterning factor TDL-peptide receptor (EMS1/MSP1), TDIF-peptide receptor, PXY) was found down-regulated in the *Ls* Cv versus *Ls*Sq comparison, that means they are induced by *S. quadricauda* treatment, corroborates this assertion. Moreover, a recent comparative analysis (bio-compounds and fatty acids) of harvested microalgal biomass indicated that *C. vulgaris* and *S. quadricauda* extracts contain a similar amount of carbohydrates (35.10 ± 1.35 and 33.98 ± 2.29 WW−1, respectively). However, *S. quadricauda* extract was richer in both lipids and proteins than *C. vulgaris* extract (Zhang et al., 2023) thus confirming their specific biological composition which can widely justify their different mode of action.

Interestingly, the lettuce response to both algal treatments involved also the deregulation of a huge number of genes encoding hormone-like compounds or molecules related to their signal transduction cascade. In particular, lettuce seedlings perceived the external signals to self-modulate BPs through members of *Catharanthus roseus* receptor-like kinase 1-like (CrRLK1L) proteins with their ligands, rapid alkalinization factor (RALF) peptides. FERONIA (FER), a CrRLK1L member, was initially reported to act as a major plant cell growth modulator in distinct tissues (Zhang et al., 2020). However, as the growth of plants depends on the compromise between cell wall growth and its integrity, *Catharanthus roseus* receptor-like kinase 1-like (CrRLK1L) THESEUS1 (THE1) was previously shown in *Arabidopsis* to trigger growth inhibition and defence responses upon perturbation of the cell wall. In this context, our results show that the deregulation of FERONIA and THESEUS signalling networks might be integrated to support the integrity of the cell wall with the coordination of normal morphogenesis (Zhang et al., 2020). Both algal extracts induced the

expression of PNP precursor polypeptide at a very high level, more in *C. vulgaris* treatment than in *S. quadricauda* (log_2fold change +11.00 and +6.61, respectively). PNPs are a class of systemically mobile molecules involved in several physiological processes ranging from the regulation of stomatal aperture, osmotic-dependent volume changes and responses to plant pathogens (Morse et al., 2004). Nevertheless, understanding of the molecular mechanisms by which PNPs exert their functions is limited by the lack of comprehensive studies reporting sets of proteins they interact with to modulate levels of secondary messengers. In this respect, it has been recently proposed that PNP-A and its PNP-R2 receptor may play an important role in fine-tuning plant immune responses to avoid inappropriate induction of SA-dependent death signals in cells spatially separated from infected or damaged cells, thereby minimizing tissue damage (Lee et al., 2020). Both algal extracts induced the deregulation of many TF families, these include TFs of the APETALA2/ ETHYLENE RESPONSIVE FACTOR (AP2/ERF) family, which have an important role in the regulation of a number of stress responses. They also respond to hormones leading to increased plant survival under stressful conditions. In addition, AP2/ERFs participate in a variety of stress tolerance, allowing them to connect a stress regulatory network (Xie et al., 2019) by interactions and connections with major plant hormones such as ethylene (ET) and abscisic acid (ABA), gibberellins (Gas) and cytokinins (CTK).

7.5 Conclusions

In this work, we evaluated the effect of microalgal extracts on the transcriptomic profile of lettuce leaves. Our results clearly indicate that treatment with *C. vulgaris* induced a qualitative and quantitative deeper response than that obtained using *S. quadricauda* extract. Moreover, although both treatments lead to ameliorated morpho-biometric parameters and share the deregulation of several biological

patterns, the lettuce seedlings' transcriptomic response clearly suggests that *C. vulgaris* actives both the auxin biosynthesis and transduction pathways whereas *S. quadricauda* up-regulates cytokinin biosynthesis pathway, probably because they are rich of different amount of beneficial components. Along the major phytohormones, algal treatments implicate the reprogramming of lettuce metabolic processes through the signal cascade induced by small hormone-like molecules that can act alone or by interacting with major hormones. Most of these molecules are reported to take the field to defend plants in the occurrence of either abiotic or biotic stress, strengthening the plant response against adverse external stimuli. Moreover, this observed de-regulation of genes that are generally categorized as '*stress-responsive genes*', might positively influence plants by exerting a beneficial effect during growth. Consequently, our work produced a comprehensive list of genes that might be the target for genome editing with the aim to genetically improve lettuce allowing a limited or even null use of synthetic fertilizers and pesticides.

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8 Other activity 3

Effect of municipal biowaste derived biostimulant on nitrogen fate in the plant-soil system during lettuce cultivation – Research article

Ferdinando Fragalà[a], Ivana Puglisi[a], Elio Padoan[b], Enzo Montoneri[a], Piergiorgio Stevanato[c], Josè Maria Gomez[d], Natalia Herrero[d], Emanuele La Bella[a], Erika Salvagno [a], Andrea Baglieri[a]

[a] Department of Agriculture, Food and Environment, University of Catania, Italy.
[b] Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università di Torino, Italy.
[c] Department of Agronomy Food Natural Resources Animals and Environment, University of Padova, Italy.
[d] Biomasa Peninsular S.A., Costancia, Spain.

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Abstract

A main concern of agriculture is to improve plant nutrient efficiency to enhance crop yield and quality, and at the same time to decrease the environmental impact caused by the lixiviation of excess N fertilizer application. The aim of this study was to evaluate the potential use of biopolymers (BPs), obtained by alkaline hydrolysis of the solid anaerobic digestate of municipal biowastes, in order to face up these main concerns of agriculture. The experimental trials involved the application of BPs (at 50 and 150 kg/ha) alone or mixed with

different amounts (100%, 60% and 0%) of mineral fertilizer (MF). Three different controls were routinely included in the experimental trials (MF 100%, 60% and 0%). The effect of BPs on lettuce was evaluated by monitoring growth parameters (fresh and dry weights of shoot and root, nitrogen use efficiency), and the N-flux in plant-soil system, taking into account the nitrate leached due to over irrigation events. The activities of enzymes involved in the nitrogen uptake (nitrate reductase, glutamate synthase and glutamine synthase), and the nitrogen form accumulated in the plant tissues (total N, protein and NO_3^-) were evaluated. The results show that the application to the soil of 150 kg/ha BPs allows to increase lettuce growth and nitrogen use efficiency, trough stimulation of N-metabolism and accumulation of proteins, and hence to reduce the use of MF by 40%, thus decreasing the nitrate leaching. These findings suggest that the use of BPs as biostimulant greatly contributes to reduce the consumption of mineral fertilizers, and to mitigate the environmental impact caused by nutrients leaching, according to European common agricultural policy, that encourages R&D of new bioproducts for sustainable eco-friendly agriculture.

8.1 Introduction

Nowadays, the bioeconomy concept requires to exploit sustainable renewable biomasses to produce of fuels, chemicals, and agrochemicals which human population needs. Researchers are trying to valorise biomasses from different sources as alternative feedstocks, focusing these objectives (Sharew et al., 2022). These latter objectives are quite difficult to reach as they are dependent on the availability of biomasses, and the economic aspects related to their collection. So far, most of the R&D work on the valorisation of biomass as renewable feedstock focused on processing plants and crops to be used for fuel production, raising social concerns due to the exploitation of agricultural land for the production of non-food energy crops. On the

contrary, the use of biowastes as feedstock could mitigate the popular discomfort for the environmental impact of the increasing wastes production and current disposal practices.

Municipal biowaste (MBW) is the most available and sustainable potentially renewable feedstock. As two thirds of world population is expected living in urban areas by 2050, and produce more wastes, the cities are crucial to the circular waste-based economy (Paiho et al., 2021). At present time, MBW is a social economic and environmental burden. Its valorization as feedstock producing valued added products would solve both problems. Currently, MBW is processed by anaerobic and aerobic fermentation, yielding biogas, anaerobic digestate and compost. The value of these products does not cover the processing costs. As collection and treatment costs are paid off by citizens' taxes, MBW and its digestate and compost represent negative cost feedstocks (Montoneri et al., 2022a). Converting MBW, digestate and compost to value-added chemicals is potentially the way to improve current MBW treatment plants and turn them into eco-friendly biorefineries producing fuel and new multifunctional value-added biobased products (BPs) for use in the chemical industry, agriculture and waste treatment sectors (Montoneri, 2017).

Recently, the performance of BPs in agriculture as plant growth biostimulants and antifungal agents had been reported (Montoneri et al., 2022b). The BPs, applied to the soil at 50–150 kg/ha, were demonstrated to be more sustainable and efficient plant biostimulants, in comparison to commercial mineral and organo-mineral products (e.g. leonardite), for the cultivation of several ornamental plant species, such as *Euphorbia x lomi* Rauh (Fascella et al., 2015), *Lantana camara* (Fascella et al., 2018), *Murraya paniculate* (Fascella et al., 2021), Hibiscus (Massa et al., 2016), and vegetable species, such as tomato (Sortino et al., 2012), red pepper (Sortino et al., 2013), spinach (Padoan et al., 2022), maize (Rovero et al., 2015), bean (Baglieri et al., 2014), oilseed rape (Jindrichova, 2018). These biobased products

were reported also as potential enhancers of the seed germination process of cress, tomato, and lettuce at low concentrations ranging between 10 and 100 mgL^{-1} (Fragalà et al., 2022). On the other hand, they are also fungicides at 1000–5000 mgL^{-1} concentration against several pathogens as *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Monilia* sp., *Sclerotium rolfsii*, and *Phytophthora nicotianae* (Fragalà et al., 2022).

Moving forward from the above findings, the present work addresses specifically the environmental issues arising from the current agricultural common practice to increase plant productivity using fertilizer doses higher than plant requirements. Exceeding fertilizer amounts accumulate in soil, could be leached into ground water, reach the food chain, and consequently may affect human and animal health (Vanni et al., 2006). Fertilizers are the leading cause for eutrophication, as they contain all the key ingredients for prosperous growth: nitrogen, phosphorous and potassium. Main fertilizers include inorganic NPK and organo-mineral products, such as composts of biowastes from urban, animal, agriculture sources, peat and leonardite hydrolysates (Barone et al., 2019). Compared to nitrates, the most lixiviated nutrients from the soil, phosphates are only moderately soluble and not mobile in soils and groundwater. Phosphates tend to remain attached to soil particles, but erosion can transport considerable amounts of phosphate to streams and lakes. Depending on fertilizers' dosage, soil type, and plant cultivated type, from 70 to 250 kg/ha nitrates leaching may occur. The Council Directive 91/676/EEC requires the reduction of water pollution caused or induced by nitrates from agricultural sources to prevent eutrophication processes (Council Directive, 2023). To protect soil and waters from the negative environmental impact caused by fertilisers, while maintaining plant productivity and crop quality, the most recent EU Fertilizing Products Regulation effective from July 16, 2022 sets out minimum and maximum limits of C, N, K, P and heavy metals for fertilizers (EU, Commission, 2023).

The general goal of the present work was to investigate the effect of BPs on nitrogen metabolism in the plant-soil system, in order to evaluate further possible effects of these new products to reduce nutrient leaching in agriculture, while maintaining the plant productivity. As previous works suggested that the use of BPs may increase plant growth, the present work focuses on BPs effect on nitrogen adsorption and, consequently, on the reduction of nitrate lixiviation trough soil, thus contributing to reduce mineral fertilizers consumption, and to mitigate the environmental impact caused by leaching. To this end, in the present work a new species, lettuce, never tested before with BPs, was taken as case study. Growth parameters as well as plant biochemical response to the treatment were evaluated. The BPs effect on the plants was evaluated by monitoring the nitrogen flux in the plant, determining the activities of enzymes involved in the nitrogen uptake, such as nitrate reductase, glutamate synthase and glutamine synthase, as well as the nitrogen form accumulated in the plant tissues. Nitrate leaching during the cultivation of lettuce in pots was then evaluated.

8.2 Materials and methods

8.2.1 Materials

BPs were produced from the solid anaerobic digestate of MBW provided by the *ACEA Pinerolese Industriale S.p.A*. (Pinerolo, Turin, Italy) waste treatment plant (Montoneri et al., 2020). In brief, the digestate was hydrolysed in water at pH 13 and 60 °C, then separated from the insoluble residue by sedimentation, followed by centrifugation and ultrafiltration. The membrane retentate was dried at 60 °C, and the solid product was dissolved in water at pH 10 (Fragalà et al., 2022). The obtained BPs was characterised for its chemical composition according to previous works (Rosso et al., 2015; Montoneri et al., 2020). Moreover, potentially toxic elements, Cu, Zn, Cd, Hg and Pb in obtained BPs were measured according to Padoan et al. (2020), by

using microwave digestion (HNO_3/H_2O_2 4:1 v/v) on 1.0 g of sample (Milestone Ethos D). Pseudo total contents were then quantified by inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer NexION® 350D). The accuracy was checked using a Reference Materials (NIST SRM 1572, National Institute of Standards and Technology, USA); all recoveries of analysed metals were between 90 and 110%. All measured heavy metals were lower than limit parameters determined by the Regulation (EU) 2019/1009, for Product Function Categories (PFC) 1 A) Solid Organic Fertiliser, PFC 1 B) Organic mineral Fertiliser, and PFC 6 B) Non microbial Plant Biostimulant (Table 1). Finally, the absence of pathogens in BPs is guaranteed by the high pH and temperature treatments subjected to.

Plant material, comply with relevant institutional, national, and international guidelines and legislation, and all methods were carried out in accordance with these relevant guidelines.

8.2.2 Experimental conditions

The agriculture trials were performed in 1 kg soil pots (diameter 20 cm) in greenhouse conditions (27 August 2021 – 03 October 2021), in a farm located in Vittoria (Ragusa, Italy). Soil texture was evaluated using the pipette method, determining the particle size classes which were subdivided into clay, silt, and sand (Violante, 2000). Particles > 2000 μm were not considered. The soil was air dried, sieved at 2 mm and characterized for water holding capacity (WHC), humidity, pH, electric conductivity (E.C.), organic carbon, phosphorus, total nitrogen, potassium, and Cation Exchange Capacity (C.E.C), following the procedures described in Puglisi et al. (2019). Soil characterization is reported in Table 2.

The soil was previously subjected to independent treatments, using two different dosage of BPs (50 kg/ha and 150 kg/ha), based on previous results obtained on other vegetable species (Sortino et al., 2012; Sortino et al., 2013; Padoan et al., 2022; Rovero et al., 2015;

Baglieri et al., 2014). The BPs were used alone or mixed with different amounts (100%, 60% and 0%) of mineral fertilizer (MF), and bured into the soil before transplant. The MF (solid ternary fertilizer NPK made of: NH_4NO_3, KH_2PO_4, and KNO_3) used in the agriculture trials was purchased from a local agricultural supplier. Soils fertilized with MF only and non-fertilized were used as controls. MF 100% corresponds to the amounts used in the regular practice for lettuce cultivation 116.60 kg/ha NH_4NO_3, 162.32 kg/ha KH_2PO_4, and 138.60 kg/ha KNO_3 (Muscolo et al., 2022), MF60% represents a 40% MF reduction with respect to the regular practice (69.96 kg/ha NH_4NO_3, 97.40 kg/ha KH_2PO_4, and 83.16 kg/ha KNO_3), while MF0% means absence of mineral fertilization. Soil N, P, K contents in the different treatments were calculated based on the BPs and MF composition, and the amounts of nutrients supplied to the soil for each treatment are reported in Table 3.

Lettuce seedlings (*Lactuca sativa* var. romana), at four true leaves, were provided by a local nursery, and were transplanted (27 august 2021) in each pot in a completely randomized design composed by three replicas per treatment, and each replica was made of 10 seedlings. The seedlings were regularly grown in the soil treated as above described, and were irrigated every day, to maintain 50% WHC, by dripline sprinkler for 40th days.

In order to simulate raining events naturally occurring, and hence possible phenomena of nitrate lixiviation into groundwater, two full supplemental irrigation treatments, consisting of an amount of water 1/3 greater than that needed to reach the WHC (280 ml), were performed after 8 and 28 days from the transplant. Then, the water lixiviated from pots, was collected and stored at − 80 °C until analyses.

At the end of the experimental period (40 days), lettuces were sampled, separated in root and shoot, and then the morphobiometric parameters were evaluated. The tissues were immediately frozen with liquid nitrogen and stored at − 80 °C until further use.

Soils were sampled, and immediately analysed for enzymatic activity. The remaining sample of soils were stored at − 20 °C until further analysis.

Table 1. Heavy metal contents in BPs used in the experimental trials, and legal limit of Regulation (EU) 2019/1009 (PFC) 1 A): Solid Organic Fertiliser, PFC 1 B): Organic mineral Fertiliser, and PFC 6 B): Non microbial Plant Biostimulant).

Heavy metal	BPs (mg/kg d.m)	PFC 1 A) (mg/kg d.m)	PFC 1 B) (mg/kg d.m)	PFC 6 B) (mg/kg d.m)
Zn	256	800	1500	1500
Cu	202	300	600	600
Cd	<0.5	1.5	3	3
Pb	85	120	120	120
Hg	0.2	1	1	1

Table 2. Physical-chemical properties of the soil used in the experimental trials.

Clay (%)	Silt (%)	Sandy (%)	WHC (%)	Humidity (%)	pH	Electrical conductivity (mS/cm)	Organic carbon (%)	Total Nitrogen (g/kg)	P (mg/kg)	K (mg/kg)	C.E.C (cmols(+)/kg)
13.5	18.3	68.2	0.2	5.97	7.92	2.95	1.57	1.1	10	42	7.59

8.2.3 Morphobiometric parameters of lettuce

Lettuce roots and shoots were separately weighed, in order to obtain the fresh weight of shoot (shoot FW) and root (root FW). Dry weight of lettuce tissues (shoot DW and root DW) was obtained by placing them in a drying oven at 105 °C until constant weight was reached, then allowed to cool for 2 h inside a closed bell jar, and finally the dry weights were calculated. Root lengths were measured with a flexible ruler to the nearest 0.5 mm.

All measurements were performed on 3 plants for treatment and replicates.

Table 3. Nutrient amount of N, P, and K supplied to the soil with the

treatments.

Treatment	N (kg/ha)	P (kg/ha)	K (kg/ha)
BPs150 + MF100%	66.01	37.34	105.37
BPs150 + MF60%	42.00	22.51	65.37
BPs150 + MF0%	6.01	0.34	5.37
BPs50 + MF100%	62.00	37.11	101.79
BPs50 + MF60%	38.00	22.31	61.79
BPs50 + MF0%	2.00	0.11	1.79
MF100%	60.00	37.00	100.00
MF60%	36.00	22.20	60.00
MF0%	–	–	–

8.2.4 Determination of the nitrogen forms in lettuce tissues

The nitrogen chlorophyll content of lettuce leaves, related to the nitrogen status of the plant, was measured, before the second over-irrigation event, using in field condition a portable N-Tester (Konica, Minolta, Japan), as average of three different points of the last expanded leaf of each lettuce plant, for all treatments and replicates (Puglisi et al., 2022). The tool provides a numeric three-digit dimensionless value that is commonly expressed as N-Tester value, and is used for leaf chlorophyll estimation in lettuce (Pennisi et al., 2019).

Total nitrogen was determined in leaves and roots by the Kjeldahl method, by digesting 2 g DW of tissues with concentrated sulphuric acid and selenium catalysis (Baglieri et al., 2013).

Total protein extraction from lettuce tissues (root and leaf) was performed according to La Bella et al. (2021). Briefly, aliquots of lettuce leaves and roots were homogenized using an extraction buffer (1:1.25 w/v ratio) containing: 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM cysteine, and 5 mM HEPES–KOH pH 7.5. Samples were then filtered and centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was recovered, and the total protein content was determined by the Bradford (1976) method, using Bovine Serum

Albumine (BSA) as a standard curve, and expressed as mg protein g^{-1} DW. All measurements were performed on 3 plants for treatment and replicates.

Nitrate (N-NO_3) concentration in leaves and roots, at the end of the trial, has been analysed on the fresh material. For each plant, 100 mg of fresh tissue was ground in liquid nitrogen and suspended in 10 mL of deionized water. Suspensions were incubated for 1 h at 45 °C and then centrifuged at 5,000 rpm for 15 min and filtered. The extract was used for nitrate spectrophotometric (U-2000, Hitachi, Tokyo, Japan) determination using the Griess reaction (Miranda et al., 2001).

8.2.5 Enzymatic activities related to nitrogen metabolism in lettuce tissues

Each enzymatic activity was assayed using an aliquot of the total protein extract, obtained as previously described, containing crude enzyme extract.

Nitrate reductase (NR) activity was measured according to Kaiser et al. method (Kaiser et al., 2001). Briefly, a solution containing 100 mM KH_2PO_4 and 100 mM KNO_3 was incubated at 28 °C for 15 min with the suitable amount of enzyme extract. The mixture was then centrifuged at 500 rpm, the supernatant was recovered, and the activity spectrophotometrically measured at 540 nm (Jasco V-530 UV–vis spectrophotometer, Tokyo, Japan), using a calibration curve, with known concentrations of $NaNO_2$. Activity was expressed as Unit mg^{-1} protein.

Glutamine synthetase (GS) was performed according to Canovas et al. (Canovas et al., 1991). In brief, the assay mixture contained 90 mM imidazole–HCl (pH 7.0), 60 mM hydroxylamine (neutralized), 20 mM KAsO4, 3 mM MnCl2, 0.4 mM ADP, 120 mM glutamine, and the suitable amount of enzyme extract. The enzymatic reaction was incubated at 37 °C for 15 min, then a mixture (1:1:1) of 10% (w/v) $FeCl_3$ $6H_2O$ in 0.2 M HCl, 24% (w/v) trichloroacetic acid, and 50%

(w/v) HCl was added. The activity was spectrophotometrically determined at 540 nm, using a standard curve of γ-glutamyl hydroxamate, and was expressed as μmol-glutamyl hydroxamate mg^{-1} protein min^{-1}.

Glutamate synthase (GOGAT) activity was assayed as described by Avila et al. (1987). Briefly, the assay mixture, containing 25 mM Hepes–NaOH (pH 7.5), 2 mM L-glutamine, 1 mM α-ketoglutaric acid, 0.1 mM NADH, 1 mM Na_2EDTA, and the suitable amount of enzyme extract, was measured spectrophotometrically (Jasco V-530 UV–vis spectrophotometer, Tokyo, Japan), by following NADH oxidation at 340 nm. GOGAT activity was expressed as nmol NAD^+ min^{-1}, mg^{-1} protein, using a molar extinction coefficient of 6220 L mol^{-1} cm^{-1}.

8.2.6 Determination of the nitrogen forms in soil

The determination of nitrate nitrogen (NO_3^-N) was performed following the procedure described by Mulvaney (1996) and Miranda et al. (2001). Soil samples were air dried and sieved at 2 mm. Nitrogen forms were extracted from soil (10 g) with 1 M KCl, under mechanical agitation for 60 min and further centrifugation at 3000 rpm for 10 min. Nitrites were detected in the supernatants, by using Griess solution, which was prepared by mixing 0.1% naphthalene ethylenediamine hydrochloride (NED) and 1% sulfonamide in phosphoric acid. The reaction was developed at room temperature for 20 min, then was spectrophotometrically analysed at 540 nm, using a NO_2^- standard curve. Nitrate was measured by its reduction to nitrite by vanadium (III), and calculating its concentration in the supernatants by subtracting the amount of nitrite previously determined. $N\text{-}NO_3$ was expressed as mg N-form/g dry weight of soil (mg g^{-1} DW soil).

Total nitrogen was determined by the Kjeldahl method, by digesting 5 g of soil samples with concentrated sulphuric acid and selenium catalysis (2013).

8.2.7 Determination of the N-NO_3 in leached water

The nitrate content was determined in leached water after an extraction with 1 M KCl for 1 h, and then determined spectrophotometrically as above described for the soil, using Griess solution (2001).

8.2.8 Nitrogen use efficiency parameters

Nitrogen uptake efficiency (NUpE), nitrogen utilization efficiency (NUtE), and nitrogen use efficiency (NUE) were calculated according to Xu et al. (2012).

In detail parameters were calculated as follows:

Total N accumulation (TNA) = total N concentration x shoot DW (expressed as mg N);
NUpE = TNA/root DW (expressed as mg N g^{-1} DW);
NUtE = shoot DW/N concentration (expressed as g^2 DW mg^{-1} N);
NUE = NUtE x NUpE (gDW).

8.2.9 Statistical analyses

Data were analysed by one-way ANOVA ($p < 0.05$) followed by Tukey's test for multiple comparison procedures using the Statistics package software (version 10; Statsoft Inc., Tulsa, OK, USA) to investigate the effect of the treatment on plant, soil, and water analysis.

8.3 Results

8.3.1 Morphobiometric parameters of lettuce

The morphological traits of lettuce seedlings subjected to the BP treatments were measured, and the results are shown in Table 4. As expected, among controls, MF100% showed, for all the evaluated parameters, values greater than ones for MF60% and MF0% soil. The only exceptions were observed for root FW and DW, for which

MF100% and MF60% registered similar values. At the shoot level, the best performances were obtained in the treatment BPs150+MF100% and BPs150+MF60%, recording for the FW of the edible portion, an increase of around 24% and 22% respect to the control MF100%, respectively. Moreover, also BPs50 + MF100% and BPs50 + MF60% showed significantly higher values than the control MF100% (e.g., shoot FW showed increases of 13% and 10%, respectively). Interestingly, the treatment BPs150 + MF0%, without added MF, recorded values always similar to MF100% in spite of the fact the applied nutrients were 1–2 order of magnitude lower. Similarly, at the root level, the highest values were obtained with the treatments BPs150+MF100% and BPs150+MF60%, recording a root FW 27% and 21% higher than the control MF100%, respectively, and a root length 17% and 19% higher than the control MF100%, respectively (Table 4). All other treatments showed parameters not significantly different from the control MF100%, except for root length, in which the treatment BPs50 + MF0% showed a value similar to the control MF60%, and lower than MF100%.

8.3.2 Nitrogen forms in lettuce tissues

The nitrogen status of the plant was monitored in field using a N-Tester, prior to the second over-irrigation event, as described in the Material and Methods section. The values (Fig. 1) showed that no significant differences were recorded among treatments.

The total N content in leaves (Fig. 2) significantly increased in the treatments with BPs150 + MF100% and BPs150 + MF60%, respect to the control with MF 100% (29% and 26%, respectively). The treatments BPs50 + MF100% and BPs50 + MF60% showed total N values similar to the control MF100%, thus indicating a potential fertilization saving of 40%. The treatments with BPs50 + MF0% and BPs150 + MF0%, showed an amount of total nitrogen lower than MF100%, but not significantly different from the control MF60%. At

the root level, the treatments BPs150 + MF100%, BPs150 + MF60%, and BPs50 + MF100% showed N values similar to the control MF100%, whereas treatments BPs50 + MF60%, BPs50 + MF0%, and BPs150 + MF0% recorded values lower than control MF100%, but not significantly different from the control MF60%.

Figure 3 reports the content of the total proteins extracted from lettuce tissues. The total protein content in leaves was strongly influenced by the treatments, recording a significantly increase in BPs150 + MF100% and BPs150 + MF60%, respect to the control with MF100% (32% and 28%, respectively). The treatments BPs50 + MF100% and BPs50 + MF60% also raised the protein content of the lettuce epigeal part, as compared to the control MF 100% (around 16%). Finally, in both the treatments with the two BPs dosage without mineral fertilizations (BPs150 + MF0% and BPs50 + MF0%), values always similar to MF100% and MF60% occurred. As previously reported for N total, a fertilization reduced of 40% leads to similar protein content as with the regular fertilization. At the root level, all the treatments showed not significant differences respect to the control MF100%, although they showed values higher than MF60%.

The N-NO_3^- content extracted from lettuce tissues is reported in Fig. 4. In leaves, due to the great variability of N-NO_3^- values in the replicates, no significant differences were observed among treatments. In roots a great variability of N-NO_3^- also occurred. However, in both cases, the highest value was recorded for BPs50 + MF100%. This value, although not significantly higher than those for most of the other treatments, was significantly higher than the lowest value recorded for the treatments BPs150 + MF100%, and BPs150 + MF0% and MF0%.

Table 4. Morphological traits of lettuce seedlings subjected to BP treatments. Data are means ± SD. Values in the same column followed by different letters are significantly different ($p < 0.05$).

Treatment	Shoot FW (g)	Shoot DW (g)	Root FW (g)	Root DW (g)	Root length (cm)
BPs150 + MF100%	79.05 ± 3.74 a	6.19 ± 0.31 a	24.10 ± 1.41 a	3.29 ± 0.13 a	17.07 ± 1.74 a
BPs150 + MF60%	77.56 ± 2.22 a	5.82 ± 0.19 a	22.97 ± 2.19 a	3.19 ± 0.30 a	17.43 ± 0.80 a
BPs150 + MF0%	65.53 ± 1.16 c	4.84 ± 0.23 c	19.37 ± 0.86 b	2.91 ± 0.12 b	15.13 ± 2.40 b
BPs50 + MF100%	72.25 ± 5.24 b	5.35 ± 0.46 b	18.27 ± 2.75 b	2.67 ± 0.51 b	15.13 ± 0.94 b
BPs50 + MF60%	69.75 ± 4.24 b	5.09 ± 0.33 b	18.47 ± 1.33 b	2.72 ± 0.14 b	15.07 ± 1.85 b
BPs50 + MF0%	55.99 ± 3.75 e	3.76 ± 0.45 e	18.66 ± 0.97 b	2.75 ± 0.14 b	14.23 ± 0.29 c
MF100%	63.78 ± 2.47 c	4.81 ± 0.49 c	19.00 ± 1.26 b	2.55 ± 0.17 b	14.57 ± 1.82 b
MF60%	59.09 ± 1.48 d	4.23 ± 0.34 d	17.71 ± 1.18 b	2.16 ± 0.25 b	13.97 ± 0.47 c
MF0%	45.33 ± 2.47 f	2.81 ± 0.36 f	14.63 ± 0.88 c	1.75 ± 0.19 c	11.14 ± 1.63 d

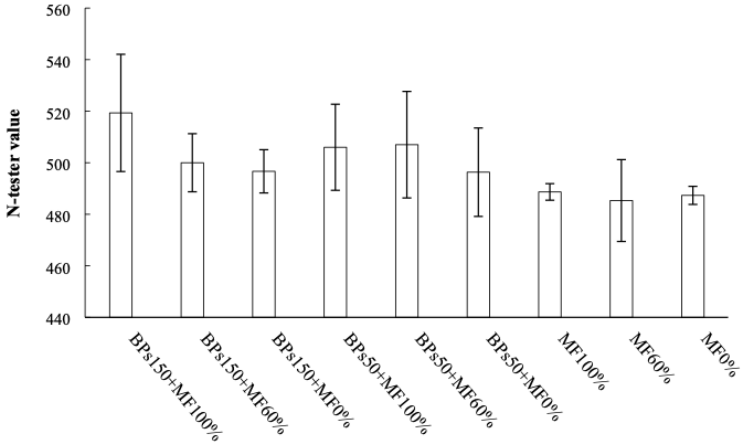

Figure 1. Nitrogen status of the plant during cultivation, before the second over-irrigation event. Values are reported as indices described by N-Tester. Error bars indicate standard deviation ± SD. The absence of letters above the columns shows the lack of significant differences.

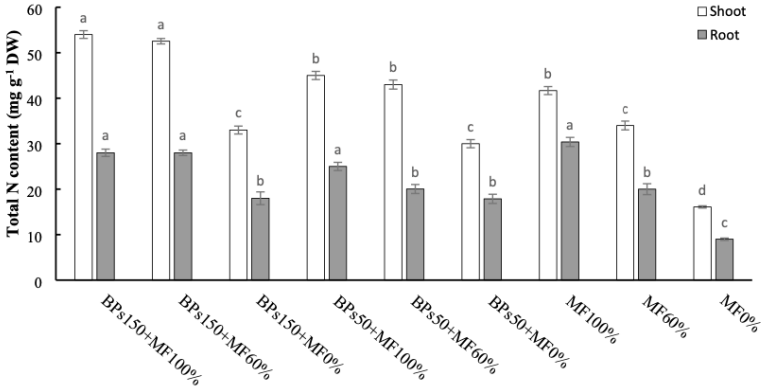

Figure 2. Total nitrogen (N) content in lettuce tissues (shoot and root). Error bars indicate standard deviation ± SD. Values followed by different letters are significantly different ($p < 0.05$).

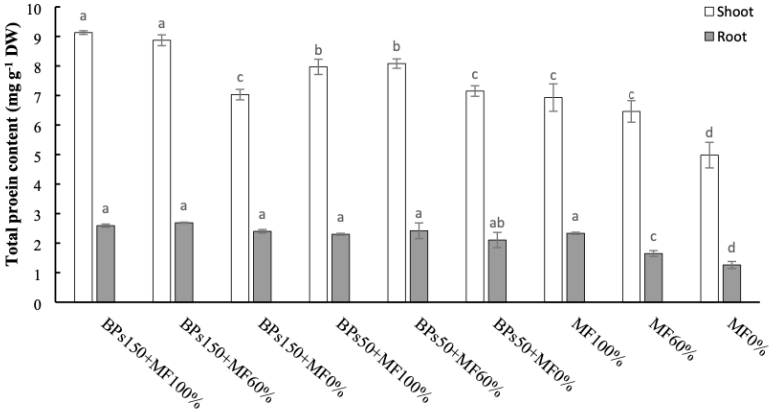

Figure 3. Total protein content in lettuce tissues (shoot and root). Error bars indicate standard deviation ± SD. Values followed by different letters are significantly different ($p < 0.05$).

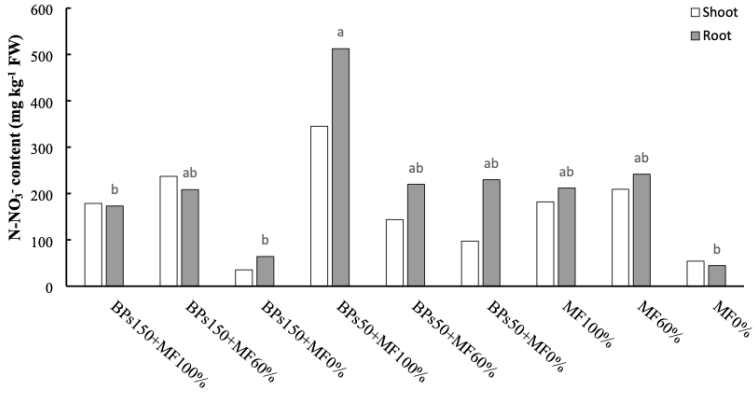

Figure 4. $N\text{-}NO_3^-$ content in lettuce tissues (shoot and root). Values followed by different letters are significantly different ($p < 0.05$). The absence of letters above the columns shows the lack of significant differences.

8.3.3 Enzymatic activities related to the nitrogen metabolism in lettuce tissues

Figure 5 reports the enzymatic activities of nitrate reductase (NR), glutamine synthetase (GS), and glutamate synthase (GOGAT) in the lettuce plants grown on soil subjected to the treatments listed in Table 2.

NR activity, measured in lettuce leaves (Fig. 5A), increased respect to the control MF100% by about 68% under the treatment BPs50 + MF100%, and around 35% under the treatment BPs50 + MF60%. All other treatments showed NR activity values in the shoot similar to the control MF100%. In roots, the treatments BPs50 + MF100%, BPs150 + MF100%, and BPs50 + MF60% rapidly induced the activation of GS, reaching values of activity 43%, 30%, and 44%, respectively, higher than that measured in the control MF100%.

GS activity in leaves was significantly higher in the plants treated with BPs150+MF100% (52%), BPs150 + MF60% (44%), and BPs50 + MF100% (41%) respect to the control MF100%, followed by

BPs50 + MF60% (12% higher than MF 100%), whereas all other treatments showed values of activity always similar to the control. As regard roots, the highest values of activity were recorded in the treatments BPs150 + MF100% (41% higher than MF100%) and BPs150 + MF60% (37% higher than MF100%). The treatment BPs50 + MF100% showed an activity lower than these latter, but higher than the MF100%. All other treatments showed activities similar to the control (Fig. 5B).

GOGAT activity in leaves showed a trend very similar to GS activity, recording the highest values under the treatments with BPs150 + MF100% (57%), BPs150 + MF60% (47%), and BPs50 + MF100% (42%), respect to the control MF 100%. The treatment BPs50 + MF60% showed an activity 25% higher than MF100%, whereas the treatments without MF (BPs150 + MF0% and BPs50 + MF0%) showed values of activity not significantly different from the control. As regard roots, all the treatments showed values of activity similar to the control MF100%, except the treatments BPs150 + MF100% and BPs150 + MF60%, which showed an increase respect to the control of 32% and 28%, respectively (Fig. 5C).

8.3.4 N-NO_3^- in soil

Figures 6 and 7 report the N-NO_3^- and total N measured in soil at the end of the experimental trials. The N-NO_3^- data showed no significant differences between soils treated with the mineral fertilisers only (MF100% and MF60%) or with the MF-BS mixes. All the treatments with MF gave higher N-NO_3^- values than the values measured for the control MF0%. The treatments with BS only (BS150-MF0% and BS-MF0%) resulted not significantly different from MF0%. On the contrary, the soils treated with BPs exhibited the highest total N values, although these resulted not significantly different from values measured for all other treatments.

The total N content in soils, at the end of the experimental trials,

showed that not significant differences among treatments and controls MF60% and MF0% occurred (Fig. 7).

8.3.5 N-NO_3^- content in leached water

Figure 8 reports the N-NO_3^- contents in waters leached during the experimental trials. The data evidenced three groups of values significantly different one from the other. The MF100% and MF60% group showed the highest total average value (838 mg L^{-1}). The second group, including the treatments with the BS-MF mixes, showed the highest total average value (471 mg L^{-1}). The third group, including the BS150%+MF0%, BS50%+MF0% and MF0% treatments, showed the lowest total average value of 50 mg L^{-1}. In terms of reduction of N-NO_3^- leaching relatively to the first group, the second and third group exhibit reduction of 44% and 94%, respectively.

8.3.6 Nitrogen efficiency parameters

Table 5 reports the values of the Nitrogen efficiency parameters measured for the different soil treatments. The plants grown in fertilized soils with the BPs-MF mixtures showed the highest TNA, NUpE and NUE values, always higher than all other treatments. The BPs150 + MF100% treatment exhibited the highest values. The NUE for this treatment showed an increase of 28% respect to the MF 100% treatment, and 158% respect to the control MF0%.

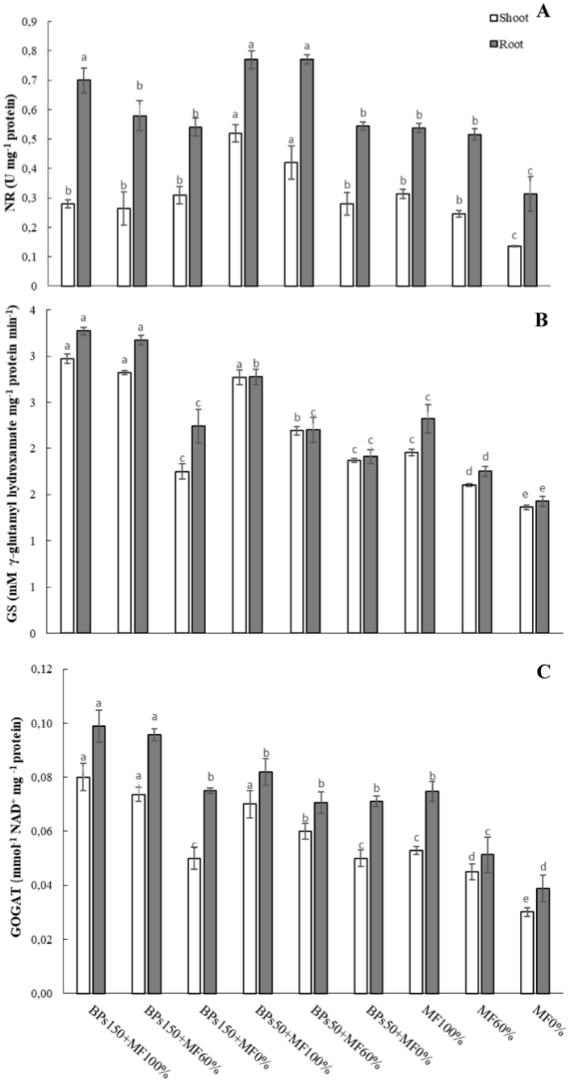

Figure 5. Nitrate reductase (NR) activity (A), glutamine synthase (GS) activity (B), glutamate synthase (GOGAT) activity (C) in lettuce tissues (shoot and root). Error bars indicate standard deviation ± SD. Values followed by different letters are significantly different ($p < 0.05$).

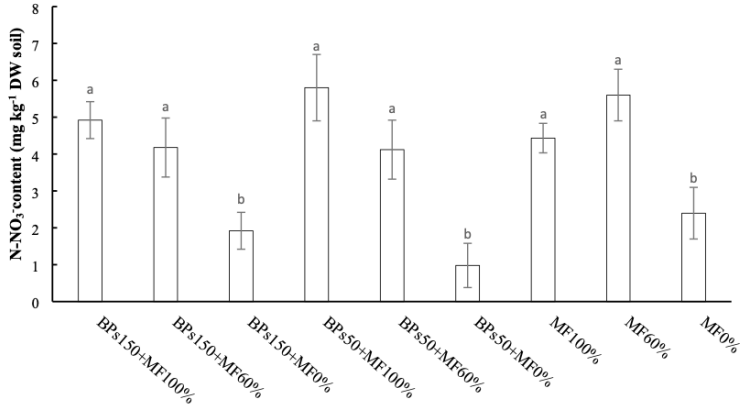

Figure 6. N-NO_3^- content in the soil at the end of the experimental trials. Error bars indicate standard deviation ± SD. Values followed by different letters are significantly different ($p < 0.05$).

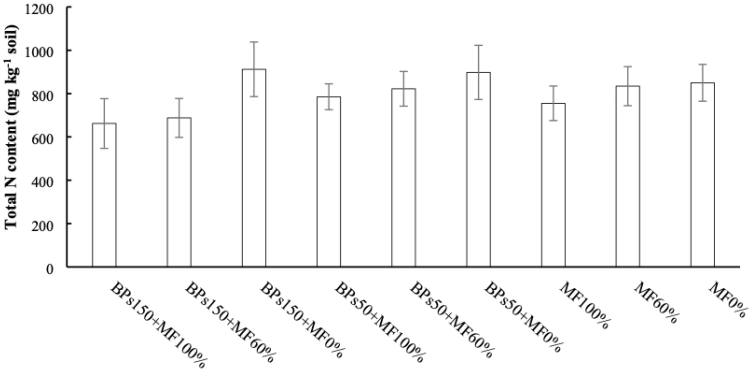

Figure 7. Total nitrogen (N) content in the soil at the end of the experimental trials. Error bars indicate standard deviation ± SD. The absence of letters above the columns shows the lack of significant differences.

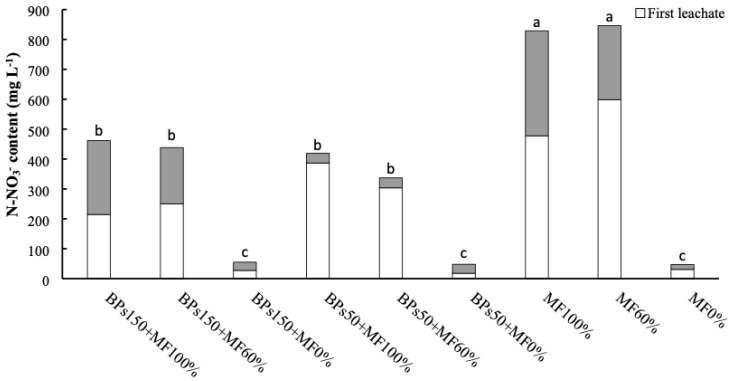

Figure 8. Nitrogen form N-NO_3 in the water leached (First and Second leachate) during the experimental trials. Values followed by different letters are significantly different ($p < 0.05$).

Table 5. Nitrogen efficiency parameters in lettuce seedlings subjected to BP treatments. Values in the same column followed by different letters are significantly different ($p < 0.05$).

Treatment	TNA (mg N)	NUpE (mg N g^{-1}DW)	NUtE (g^2 DW mg^{-1}N)	NUE (g DW)
BPs150 + MF100%	334.26 ± 12.01a	101.60 ± 2.11a	0.11 ± 0.04a	11.65 ± 0.52a
BPs150 + MF60%	305.75 ± 15.72b	95.85 ± 3.42b	0.11 ± 0.03a	10.62 ± 0.39b
BPs150 + MF0%	159.72 ± 11.32e	54.89 ± 5.65e	0.15 ± 0.04a	8.05 ± 0.21d
BPs50 + MF100%	240.75 ± 10.03c	90.17 ± 3.70b	0.12 ± 0.02a	10.72 ± 0.20b
BPs50 + MF60%	218.87 ± 8.50d	80.47 ± 2.25c	0.12 ± 0.01a	9.53 ± 0.42c
BPs50 + MF0%	112.8 ± 9.68f.	41.02 ± 8,98e	0.13 ± 0.01a	5.14 ± 0.53e
MF100%	200.46 ± 11.23d	78.61 ± 3.21c	0.12 ± 0.01a	9.07 ± 0.21c
MF60%	143.82 ± 10.21e	66.58 ± 2.87d	0.12 ± 0.02a	8.28 ± 0.12d
MF0%	45.22 ± 15.39g	25.84 ± 4.56f	0.17 ± 0.05a	4.51 ± 0.25e

8.4 Discussion

Several studies evaluated the biostimulant effect of BPs on a wide range of crops (Montoneri et al., 2022b), but BPs for lettuce cultivation had never been tested. In lettuce at the shoot level, the treatments BPs150 + MF100% (+ 24% respect to MF100%) and BPs150 + MF60% (+ 22% respect to MF100%) determined a relevant increase

of the FW of the edible portion, in accordance with the biostimulant effects observed for other species (Sortino et al., 2012; Sortino et al., 2013; Padoan et al., 2022; Rovero et al., 2015; Baglieri et al., 2014; Jindrichova et al., 2018). Moreover, the treatment with the highest amount of BPs without fertilization (BPs150 + MF0%) showed values of FW of the edible part comparable to MF100%, suggesting that BPs may be useful to ameliorate the use of the residual nutrients into the soil. The highest amount of BPs (150 kg/ha), both with MF100% or MF60%, determined also a positive effect at the root level, recording higher FW values in the treatments BPs150 + MF100% and BPs150 + MF60%, respect to the control (Table 4). Starting from the positive effect on the morphobiometric traits of the lettuce seedlings, the fate of the nitrogen (N) was investigated, as N represents the most important macronutrient in lettuce production for proper foliage growth and good green colour (Havlin et al., 1999). During lettuce cultivation, nitrogen status of the plant was monitored in field, using a non-invasive technique, and the results showed a great variability in the measurements with values not significantly different among the treatments (Fig. 1). N-test readings have been proven to be well correlated with the leaf chlorophyll content and/or leaf N concentration in several cereals such as *Hordeum vulgare* L. (Wienhold and Krupinsky, 1999), *Zea mays* L. (Schepers et al., 1992), *Oryza sativa* L. (Peng et al., 1993), and wheat (Follet et al., 1992). These evidences suggest that, during the experimental trials, chlorophyll content keeps rather constant values. Moreover, according to Pennisi et al. (2019), who reported values of N-tester for lettuce ranging between 300 and 400, lettuce treated with 150 kg/ha BPs reached values ranging between 500–520, thus suggesting the presence of a great amount of chlorophyll in their leaves.

On the contrary, significant differences were observed as regard the different forms of nitrogen accumulated in lettuce tissues at the end of the experimental period. The treatments BPs150 + MF100%

and BPs150 + MF60% greatly affected the accumulation of total nitrogen (N) and proteins at the shoot level of the lettuce (Fig. 2 and 3). This increased protein content is compatible in order to support the enhanced growth of the epigeous part of lettuce (Taiz et al., 2018). However, the increased N absorption efficiency of the plant, on the other hand, may lead to nitrate accumulation (Brewster, 1994). Lettuce leaves can accumulate a wide range of nitrate, varying from 190 to 6600 mg kg^{-1}, depending on different factor such as species, individual plant, cultivation season, age, morphotype, climate, and fertilization (Abu-Rayyan et al., 2004). Risks related to high levels of nitrate are mainly related to methemoglobinemia, a disease affecting infants leading to anoxia or death, toxicity due to carcinogenic and mutagenic nitrosamine compounds, and associated to gastric cancer, due to the ingestion of N-nitroso compounds (Thresher et al., 2020; Mirvish, 1977). Moreover, a high nitrate levels in the edible part of baby leaf lettuce may determine a decrease of vitamins and hence of the nutritional profile (Conversa et al., 2021). Therefore, research is focusing on the use of techniques or treatments increasing N absorptions, but reducing its accumulation under form of nitrate. In Italy the presence of nitrate in lettuce is regulated by EU regulation N. 1258/2011, taking into account EFSA opinions (Panel, 2010), indicating for lettuce cultivated in greenhouse a limit of nitrate corresponding to 4000 mg kg^{-1}, between 1 April—30 September, and 5000 mg kg^{-1}, between 1 October—30 March. Successfully, our results suggest that both the treatments 150BPs + MF100% and 150BPs + MF60% raised the total N accumulation in lettuce leaves (Fig. 2), by increasing the total protein content (Fig. 3), and nevertheless maintaining the levels of nitrate (Fig. 4) similar to those of control plants (MF100%, MF60% and MF0%). Interestingly, the highest value of nitrate, observed in BPs50 + MF100%, showed anyway a value (320 mg kg^{-1} FW) greatly lower than legal limits (4000–5000 mg kg^{-1}).

In plants, nitrate may be metabolized both in shoots and roots,

and the rate of its conversion is dependent on different environmental factors, type and amount of N supply, plant species, and age (Cedergreen and Madsen, 2003). Nitrate reductase (NR) is a cytosolic enzyme that may be considered as the rate-limiting stage of the nitrate assimilation pathway, and it is considered to be a limiting factor for the growth and development of plants. NR, in the cytosol of plant cells, catalyses the reduction of NO_3^- into NO_2^-, and acts as a crucial point in the plant N metabolism (Nemie-Feyissa et al., 2013). Our results showed that, in the soil with MF100%, the treatments with both concentration of BPs, significantly increased NR activities in roots, whereas in leaves NR activities were higher in the treatments with the lower amount of BPs (BPs50 + MF100% and BPs50 + MF60%) (Fig. 5A). These results may be explained by the evidence that higher N accumulation in lettuce correspond to a higher NR activity during the initial stage of plant growth, whereas a decrease of NR activity during the final stage of plant growth may occur (Pinto et al., 2014).

In the primary metabolism involved in N assimilation, the glutamine synthetase (GS) and glutamate synthase (GOGAT) have also been proposed to play a key role through ammonium incorporation into carbon skeletons, by assimilating the cation into an organic form as glutamine and glutamate (Lea, 1993; Gupta et al., 2012). Both GS and GOGAT, significantly increased in treatments BPs150 + MF100% and BPs150 + MF60% (Fig. 5B,C), in accordance with an increased growth of lettuce, and a higher amount of total N and proteins. Supporting these results, the involvement of N metabolism in the enhanced growth of lettuce was also observed using other biostimulant types, such as microalgae-based extracts (Puglisi et al., 2020; Puglisi et al., 2022; La Bella et al., 2021), plant-based preparations containing triacontanol (Ottaiano et al., 2021), l-amino acid-based biostimulants (Navarro-León et al., 2022).

It is well known that nitrogen is distributed into the plant, in the fixed fraction into the soil, and in the leached water (Havlin et al.,

1999). Our results showed that, although the N total of all the soils was quite similar (Fig. 7), significant differences were observed as regard nitrate concentrations (Fig. 6). Interestingly, all the soils subjected to fertilization, both MF100% and MF60%, showed amount of NO_3^- rather similar among them, and always greater than soils not fertilized (BPs150 + MF0%, BPs50 + MF0%, and MF0%). Meanwhile, NO_3^- amounts in leachates significantly decreased in all waters collected from the fertilized soils (both 100% and 60%) subjected to BP treatments (both concentrations) (Fig. 8). All these results taken together, suggest that nitrate keeps constant in the fertilized soils for two different reasons: i) in the control fertilized soils (MF100% and MF 60%), the residual amount of nitrate (Fig. 6), after the plant uptake, may be strictly linked to the loss of NO_3^- by lixiviation (Fig. 8); ii) on the contrary, the plants grown in fertilized soils and treated with BPs (BPs150 + MF100%, BPs50 + MF100%, BPs150 + MF60%, and BPs50 + MF60%) seem to uptake an higher amount of NO_3^- from the soil, in order to support a greater growth of lettuce (Table 4), by increasing total protein content in the edible portion, and hence greatly reducing the amount of leached nitrate in the waters (Fig. 8). This hypothesis is supported by the evidence that, among the mechanisms of action of biostimulants based on humic-like substances, the increased uptake of nutrients such as nitrogen from the soil is one of the main studied processes (Chilom et al., 2013; Puglia et al., 2021; Mghaiouini et al., 2022).

In this context, the nitrogen use efficiency (NUE) is considered a further important parameter, being related to the produced biomass per unit of available N. This parameter takes into account two factors: N uptake efficiency (NUpE), representing the ability of the plant to absorb N from the soil, and N utilization efficiency (NUtE), representing the potentiality of the plant to transfer and utilize N in the biomass production of the different plant tissues (Xu et al., 2012). Our results showed that BPs, in particular BPs150 + M100%, increased the NUE

respect to the lettuce grown in MF100% (Table 4). According to Lemaire et al. (2020), higher NUE improves the yield and quality of the plant, and decreases the environmental impact caused by the lixiviation of excess N fertilizer application. Moreover, in lettuce cultivation, Navarro-Leòn et al. (2022), have recently shown that the use of L-amino acid-based biostimulants improves nitrogen use efficiency (NUE), associated to NO3− and total N accumulation in the plants. Our results hence suggest that 150 kg/ha BPs may be possible candidates to increase the lettuce growth, trough stimulation of N-metabolism, to reduce mineral fertilization, as the treatment BPs150 + MF60% showed results very similar to the treatment BPs150 + MF100%, and finally to decrease the nitrate concentration into groundwater.

Table 6 shows summarises the effects of the different treatments on the measured parameters.

It may be readily observed that, for all measured parameters, the treatments with the BPs-MF mixes rank first and exhibit the highest effects, compared to the treatments with MF only or BPs only, and with the control MF0%. Particularly significant is the $N\text{-}NO_3^-$ in leached water 1575% increase measured for the treatment with MF100% and MF60%, relatively to the BPs150 + MF0% and BPs50 + MF0%, which together with the control MF0% trial exhibited the lowest $N\text{-}NO_3^-$ value in leached water. This prospects that formulates containing both MF and BPs in the proper relative amounts can achieve the highest plant productivity, together with the lowest environmental impact from fertiliser leaching in waters through the soil and the best safest crop quality.

With reference to the goal of lowering the consumption of mineral fertilizers, and the consequent depletion of mineral fossil sources, production on energy intensive N compounds and related GHG production, and finally the European import of mineral fertilisers, by implementation of BPs as alternative/supplementation to commercial

MF, Table 3 shows that, compared to the MF100% and MF60% treatments, the use of BPs150 + MF0% and BPs50 + MF0 implies a strong reduction of mineral fertilisers supplied. Generally, according to Table 3 data, the use of all BPs-MF mixes, except for BPs150 + MF100%, would result in a reduction of N, P, K amounts.

8.5 Conclusions

Considering all the concerns associated with nitrogen fertilization, nowadays it is essential to use new agronomic techniques able to increase NUE by plants and reduce the environmental impact linked to the lixiviation of nitrogen. In this context, the use of biostimulants has the potentiality to address some of the problems related to N fertilization. The present work has shown new evidences about BPs biostimulant properties on lettuce, a new species never tested before with BPs. Our results showed that 150 kg/ha BPs are able to increase lettuce growth, enhance NUE, and in the meantime reduce the loss of N thought lixiviation. In particular, the use of BPs in lettuce cultivation has shown to increase its growth, improve the nitrogen adsorption, thought the stimulation of N metabolism and the protein accumulation, allowing to reduce of 40% the consumption of mineral fertilizers. Moreover, BPs by increasing the N uptake are also effective to reduce the nitrate lixiviation trough the soil, thus contributing to mitigate the environmental impact caused by leaching.

The results of this paper lead the basis for a further sustainable exploitation of biowaste materials, thus contributing to a more circular economy, which allows to better address the nitrogen fate, prospecting a feasible development of new BPs-based farming practices for a more sustainable agriculture.

Plant performance indicator	Ranking order	Δ1%[a]
Shoot FW (Table 4)	BPs150 + MF100% = BPs150 + MF60% > BPs50 + MF100% = BPs50 + MF60% > BPs150 + MF0% = MF100% > MF60% > BPs50 + MF0% > MF0%	74
Shoot DW (Table 4)	BPs150 + MF100% = BPs150 + MF60% > BPs50 + MF100% = BPs50 + MF60% > BPs150 + MF0% = MF100% > MF60% > BPs50 + MF0% > MF0%	120
Root FW (Table 4)	BPs150 + MF100% = BPs150 + MF60% > BPs50 + MF100% = BPs50 + MF60% = BPs150 + MF0% = MF100% = MF60% = BPs50 + MF0% > MF0%	64.7
Root DW (Table 4)	BPs150 + MF100% = BPs150 + MF60% > BPs50 + MF100% = BPs50 + MF60% = BPs150 + MF0% = MF100% = MF60% = BPs50 + MF0% > MF0%	88
Root length (Table 4)	BPs150 + MF100% = BPs150 + MF60% > BPs50 + MF100% = BPs50 + MF60% = BPs150 + MF0% = MF100% > MF60% = BPs50 + MF0% > MF0%	53
Leaves N-test (Fig. 1)	BPs150 + MF100% = BPs150 + MF60% = BPs50 + MF100% = BPs50 + MF60% = BPs150 + MF0% = MF100% = MF60% = BPs50 + MF0% = MF0%	6.6 ns
Leaves Total N (Fig. 2)	BPs150 + MF100% = BPs150 + MF60% > BPs50 + MF100% = BPs50 + MF60% = MF100% > BPs150 + MF0% = MF60% = BPs50 + MF0% > MF0%	223
Roots Total N (Fig. 2)	MF100% = BPs150 + MF100% = BPs150 + MF60% = BPs50 + MF100% > BPs50 + MF60% = BPs150 + MF0% = BPs50 + MF0% = MF60% > MF0%q	250
Leaf total proteins (Fig. 3)	BPs150 + MF100% = BPs150 + MF60% > BPs50 + MF100% = BPs50 + MF60% > BPs150 + MF0% = BPs50 + MF0% = MF60% = MF100% = MF60% > MF0%	67
Root total proteins (Fig. 3)	BPs150 + MF100% = BPs150 + MF60% = BPs50 + MF100% = BPs50 + MF60% = BPs150 + MF0% = BPs50 + MF0% = MF100% > MF60% > MF0%	75
Roots N-NO_3 (Fig. 4)	BPs50 + MF100% ≥ BPs150 + MF60% = BPs50 + MF60% = BPs50 + MF0% = MF100% = MF60% > BPs150 + MF100% = BPs150 + MF0% = MF0%	90
NR activity in leaves (Fig. 5A)	BPs50 + MF100% = BPs50 + MF60% > BPs150 + MF60% = BPs50 + MF0% = MF100% = MF60% = BPs150 + MF100% = BPs150 + MF0% > MF0%	200
NR activity in roots (Fig. 5A)	BPs50 + MF100% = BPs150 + MF60% = BPs150 + MF100% > BPs50 + MF60% = BPs50 + MF0% = MF100% = MF60% = BPs150 + MF0% > MF0%	120
GS activity in leaves (Fig. 5B)	BPs150 + MF100% = BPs50 + MF100% = BPs150 + MF60% > BPs50 + MF60% > BPs50 + MF0% = MF100% = BPs150 + MF0% > MF60% > MF0%	50
GS activity in roots (Fig. 5B)	BPs150 + MF100% = BPs150 + MF60% > BPs50 + MF100% > BPs50 + MF60% = BPs50 + MF0% = MF100% = BPs150 + MF0% > MF60% > MF0%	125
GOGAT activity in leaves (Fig. 5C)	BPs150 + MF100% = BPs50 + MF100% = BPs150 + MF60% > BPs50 + MF60% > BPs50 + MF0% = MF100% = BPs150 + MF0% > MF60% > MF0%	167
GOGAT activity in roots (Fig. 5C)	BPs150 + MF100% = BPs50 + MF100% > BPs150 + MF60% = BPs50 + MF60% = BPs50 + MF0% = MF100% = BPs150 + MF0% > MF60% > MF0%	186
Soil N-NO_3^- (Fig. 6)	BPs50 + MF100% = MF60% = BPs150 + MF100% = BPs150 + MF60% = BPs50 + MF60% = MF100% > MF0% = BPs150 + MF0% = BPs50 + MF0%	510
Soil total N (Fig. 7)	BPs150 + MF0% = BPs50 + MF0% = MF60% = BPs150 + MF100% = BPs150 + MF60% = BPs50 + MF60% = MF100% = MF0% = BPs50 + MF100%	32 ns
N-NO_3^- in leached water (Fig. 8)	MF100% = MF60% > BPs150 + MF100% = BPs150 + MF60% = BPs50 + MF100% = BPs50 + MF60% > BPs150 + MF0% = BPs50 + MF0% = MF0%	1575
TNA (Table 5)	BPs150 + MF100% > BPs150 + MF60% > BPs50 + MF100% > BPs50 + MF60% = MF100% > BPs150 + MF0% = MF60% > BPs50 + MF0% > MF0%	642
NUpE (Table 5)	BPs150 + MF100% > BPs150 + MF60% = BPs50 + MF100% > BPs50 + MF60% = MF100% > MF60% > BPs150 + MF0% = BPs50 + MF0% > MF0%	293
NUtE (Table 5)	BPs150 + MF100% = BPs150 + MF60% = BPs50 + MF100% = BPs50 + MF60% = MF100% = MF60% = BPs150 + MF0% = BPs50 + MF0% = MF0%	54 ns
NUE (Table 5)	BPs150 + MF100% > BPs50 + MF100% = BPs150 + MF60% > BPs50 + MF60% = MF100% > BPs150 + MF0% = MF60% > BPs50 + MF0% = MF0%	158

Figure 6. Ranking of the different treatments in the order of decreasing effects on the measured parameters. [a]% increase of first ranking (or first listed), relatively to last ranking (or last listed) calculated according to 100 (first–last)/last ranking values; ns = not significant.

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9 Other activities: Participation to conferences

Agricultural Chemistry Winter School (ACWS 2021)
DISAFA, Università degli Studi di Torino e Società Italiana di Chimica Agraria (SICA). 8-11 Febbraio 2021, Torino. Poster abstract.

***Chlorella vulgaris* extract used as biostimulant in lettuce seedlings**

Emanuele La Bella[1], Ermes Ivan Rovetto[1], Ivana Puglisi[1], Andrea Baglieri[1]
[1] Dipartimento di Agricoltura, Alimentazione e Ambiente (Di3A), Università degli Studi di Catania

Abstract
The use of natural biostimulants in the modern agricultural is becoming an interesting alternative to the traditional products used to improve the yields of the crops: these substances, applied in small quantities to the soil or on the foliar surface, are able to increase quality and quantity of production and to enhance nutrition efficiency and tolerance to biotic and abiotic stresses. These substances may affect the plant growth as well as the plant metabolism, when applied at root or leaf level. The aim of this work is to evaluate the effectiveness of radical and foliar treatments on lettuce seedlings, using an extract, prepared from *Chlorella vulgaris*, as biostimulant in order to obtain a sustainable cultivation and a reduction in the cost of chemical fertilizers in lettuce cultivation. In order to investigate the response of lettuce plants subjected to the addition (radical or spray) of an extract from *C. vulgaris*, the morpho-biometric parameters, chlorophyll, carotenoid and total protein contents as well as plant enzymatic activities (GS, GOGAT, Citrate synthase, Malate dehydrogenase and PAL) were evaluated. The experiments were carried out on inert substrate (pumice) at room temperature, with a 16-h photoperiod, by performing 2

consecutive treatments (CV radical treatment and CV spray treatment), one week apart, using a concentration of the extract corresponding to 1 mg Corg L^{-1}. The experimental trial was carried out by performing 4 samplings at 1, 4 and 7 days from the 1st treatment and at 7 days from the 2nd treatment. The results showed that the extract of *C. vulgaris* successfully acts both at root and foliar level, determining an increase in dry matter and pigments contents in the leaves. Furthermore, the extracts positively influenced enzyme activities involved in primary (GOGAT, glutamine synthetase, Citrate synthase and malate dehydrogenase) and secondary (PAL) metabolisms of the plants.

3rd Joint Meeting of Agriculture-oriented PhD Programs
Università degli Studi di Catania, Università degli Studi di Foggia e Università degli Studi di Udine, 11-15 October 2021, Giovinazzo.

Multipurpose agricultural reuse of biomasses and their extracts from different sources

Emanuele La Bella[1], Ivana Puglisi[1], Andrea Baglieri[1]
[1] Dipartimento di Agricoltura, Alimentazione e Ambiente (Di3A), Università degli Studi di Catania

Abstract
The pollution of wastewaters with organic and inorganic compounds, such as nitrates, phosphates, heavy metals, etc. is one of the most critical and common environmental problems. The excessive presence of pollutants causes ecosystem problems, subsequent eutrophication of waterbodies and alteration of water system health. Wastewater, for its composition, can provide the essential nutrients for microalgae growth, and some microalgal species are able to efficiently remove the pollutants, in variable percentages, from different origin wastewater. The adoption of microalgae-based treatment of wastewater represents a good alternative to conventional purification methods. The aim of the research project is the development of a new sustainable approach in the management of urban wastewater through "phycoremediation", and the study of microalgae-based products that could be applied for agricultural purposes. Samples of urban wastewater from an already active wetland were collected, and the isolation and cultivation of autochthonous microalgae species at laboratory scale were performed. The morphological and molecular identification of each species is currently ongoing. The remediation performances of isolated species were evaluated in a pilot laboratory-scale open purification system and compared to the action of well-known microalgae species as *Chlorella*

vulgaris and *Scenedesmus quadricauda*. To test multipurpose agricultural applications of microalgae biomasses, for an eco-sustainable chemical-free agronomy, a preliminary test with a *Chlorella vulgaris* extract as biostimulant was performed on lettuce seedlings. The results showed that the *C. vulgaris* extract positively influenced the growth of lettuce seedlings, by increasing the fresh and dry weights, chlorophylls, carotenoids, protein content, and ashes at shoot level. At the root level, the extract increased dry matter, proteins, and ash content. Furthermore, both primary and secondary metabolisms at shoot level, in particular nitrogen metabolism, were positively influenced.

Winter School "Circular Economy for the Sustainable Bio-based Products: from waste to soil"
Dipartimento di Biotecnologie, Università di Verona e Società Italiana di Chimica Agraria (SICA). 15-16 November 2021.

Oral presentation – II session: "Microalgae in the circular economy"

Phycoremediation: a sustainable biotechnology as an alternative system for the wastewater management in farm holiday

Emanuele La Bella[1], I. Puglisi[1], F. Fragalà[1], P.S. Occhipinti[1], G.B. Grillo[1], C.L. Randazzo[1], C. Caggia[1], A. Baglieri[1]
[1] Dipartimento di Agricoltura, Alimentazione e Ambiente (Di3A), Università degli Studi di Catania

Abstract
Managing wastewater is a serious environmental issue in many industrialized countries, mainly due to the excessive presence of organic and inorganic compounds. Various approaches are available for water remediation, but the conventional methods are often expensive. A simple, sustainable, and inexpensive solution could be represented by the microalgae-based wastewater treatment (MBWT): this technology is able to combine water remediation and microalgal biomass production.
The aim of this study was to evaluate the performance of microalgae treatments as an alternative to the secondary treatment of constructed wetland active in a farm holiday in Sicily.
Water samples from the Imhoff tank, corresponding to the phytodepuration system's input, were treated in open plexiglass tanks at laboratory scale with: I) an unknown *Chlorophyta* strain, and II) a microalgal pool (MP), isolated from the free water surface pond of the phytoremediation system of the farm holiday, III) *Chlorella vulgaris* ACUF110 strain, and IV) *Scenedemus quadricauda* ACUF581 strain,

kindly provided by the Algal Collection Federico II of Naples, Italy. The microalgae cultures were inoculated at a final concentration of about 1,5 x 10^9 cells L^{-1}, into 3 L of wastewater samples, and their removal efficacy was monitored at 30, 45, and 60 days after the inoculum for chemical parameters (Total Kjeldahl Nitrogen: TKN; Total Phosphorous: TP; Biochemical Oxygen Demand: BOD_5; Chemical Oxygen Demand: COD; Electrical conductivity: EC), and at 1, 5, 7, 9, 12, 30, 45, 60 days after the inoculum, considering as microbiological indicator *Escherichia coli* and total coliforms.

Results revealed a high diversity in the removal efficiencies of TKN, TP, and in the reduction of EC. *S. quadricauda*, *Chlorophyta* strain, and MP removed the highest amounts of TKN (82-88%) and TP (80-88%). As regard EC, *C. vulgaris* and *S. quadricauda* reduced values of about 24%, while *Chlorophyta* and MP showed higher performance (from 34 to 40%). BOD_5 and COD were strongly reduced (94-97%) by all microalgae species, showing no significant differences among them. Moreover, the MBWT showed an *E. coli* and total coliforms complete remotion after 30 and 45 days, respectively.

Results showed that the process allows an effectiveness phycoremediation process, representing an interesting solution for the secondary wastewater treatment, and microalgal biomass production, in view of a green circular economy process.

XL SICA Congress – Conciliating sUstaiNability, resilIence, and Food qualitY: new challenges for a 2030 agriculture (UNIFY) Department of Agriculture, Food and Environment, University of Pisa, and Società Italiana di Chimica Agraria (SICA). 5-7 September 2022, Pisa. Poster abstract.

Agricultural reuse of microalgae biomasses recovered by wastewater phycoremediation process

Emanuele La Bella[1], Ivana Puglisi[1], Ferdinando Fragalà[1], Andrea Baglieri[1]

[1] Dipartimento di Agricoltura, Alimentazione e Ambiente (Di3A), Università degli Studi di Catania

Abstract

In the agricultural field, microalgae-based products are attracting the interest of researchers and industries, mainly due to their several advantages, related to their ability to act at different levels on plant metabolism, enhancing the growth and improving the nutrient uptake efficiency and tolerance to biotic and abiotic stress conditions. Microalgae can be used in agriculture in different applications, such as seed priming, foliar application, etc. However, the application of these microorganisms is still at the beginning because their production costs are often too high. A cost-effectiveness and environmentally friendly method to produce microalgal biomasses may be represented by the phycoremediation, a strategy using algal metabolism to remediate wastewaters. Because of its composition, wastewater can provide the main nutrients for microalgae growth. The objective of this study was to evaluate the effects on lettuce cultivation of three microalgae species (*Chlorella vulgaris, Scenedesmus quadricauda*, and *Klebsormidium* sp. K39), obtained after the phycoremediation of wastewaters, applied directly to the soil as living cells. Lettuce

seedlings were grown in the microalgae treated soils, and their morpho-biometric parameters, total protein contents, and the plant biochemical response, by evaluating the main enzymatic activities involved in nitrogen metabolism (nitrate reductase – NRA, glutamine synthetase - GS, and glutamate synthase – GOGAT), were monitored, both at root and foliar level. The microalgae employed in these experimental trials were grown on waters from the drainage system of a holiday farm located in Sicily. In the meanwhile, microalgal biomasses and depurated water were obtained. Microalgae were separately collected, and applied directly to the soil, by mixing them, at two different concentrations (50 mg/kg of soil and 500 mg/kg). Results showed that all three microalgae species positively affected the growth of lettuce plants, increasing the dry matter and the protein contents, compared to untreated controls. At biochemical level, in the treated plants an increase of enzymatic activities occurred.

On the basis of our study, the phycoremediation may hence represent an interesting solution to reduce the costs of microalgal biomass production, in view of a green circular bio-economy process, and the effect on plant metabolism confirmed the efficiency of microalgae as biostimulants.