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Chlorella vulgaris and ***Scenedesmus quadricauda:*** diversified applications for environmental and agricultural systems

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Abstract

The topic of this thesis takes as its guiding principle the multifunctionality of microalgae, focusing on the use of Chlorella vulgaris and Scenedesmus quadricauda as decontaminants of agricultural wastewater, biostimulants for plant growth and for soil fertility. The main objectives of this thesis have been organized into four chapters. The first aim was to evaluate the removal capacity of microalgae with respect to organic and inorganic pollutants from agricultural wastewater. Secondly, the goal was to investigate the biostimulating effect of microalgae extracts on plants of agricultural interest, in particular sugar beet and maize. The third objective was to evaluate the effect of microalgal extracts and living cells on soil biochemical fertility. Finally, the last objective of this thesis was to evaluate the effects on growth of microalgae and tomato plants in a co-cultivation of C. vulgaris or S. quadricauda and tomato plants.

The results suggest that it is possible to use cultivation systems of both microalgae to purify agricultural wastewater which contains inorganic compounds and pesticides. The extracts prepared from C. vulgaris and S.quadricauda seem to be promising as biostimulants, both in promoting germination and in the early stages of plant growth in sugar beet. Furthermore, these microalgal extracts were shown to have positive effects on the physiological parameters under investigation of maize plants.

With respect to the biochemical fertility of soil, the results highlighted that metabolites of C. vulgaris and S. quadricauda may induce a higher soil biochemical fertility and simultaneously increase tomato plant growth.

Finally, the results suggested that the associated cultures of microalgae and tomato plants, in an hydroponic system, may be a cheap and useful way of simultaneously producing a greater quantity of microalgal biomass as well as improved tomato plant growth.

Sommario

La multifunzionalità delle microalghe è stato il filo conduttore di questa tesi, centrata sull'applicazione di Chlorella vulgaris e Scenedesmus quadricauda come decontaminanti delle acque reflue agricole, biostimolanti per la crescita delle piante e la fertilità del suolo. I principali obiettivi di questa tesi sono stati suddivisi in quattro capitoli. Il primo obiettivo è stato quello di valutare la capacità delle microalghe di rimuovere contaminanti organici ed inorganici dalle acque reflue agricole. In secondo luogo, lo scopo è stato quello di investigare l'effetto biostimolante di estratti microalgali su piante di interesse agricolo, in particolare la barbabietola da zucchero ed il mais. Successivamente, il terzo obiettivo è stato quello di valutare l'effetto di estratti microalgali e di cellule microalgali vive sulla fertilità biochimica del suolo. Infine, l'ultimo scopo di questa tesi è stato quello di determinare gli effetti sulla crescita di microalghe e di piante di pomodoro in una co-coltivazione di C. vulgaris o S. quadricauda e piante di pomodoro.

I risultati ottenuti suggeriscono che è possibile utilizzare entrambe le microalghe per purificare acque reflue provenienti dall'agricoltura, che contengono inquinanti inorganici ed agrofarmaci.

Gli estratti ottenuti da C. vulgaris e S. quadricauda sembrano essere promettenti come biostimolanti, sia per favorire la germinazione che i primi stadi di crescita di

piante di barbabietola da zucchero. Inoltre, questi estratti microalgali sembrano influenzare positivamente i parametri fisiologici investigati in piante di mais.
Riguardo alla fertilità del suolo, i risultati evidenziano che i metaboliti di C. vulgaris e S. quadricauda possono indurre una più alta fertilità biochimica del suolo e, simultaneamente, incrementare la crescita di piante di pomodoro.
Infine, i risultati suggeriscono che l'associazione di colture di microalghe e piante di pomodoro, in un sistema idroponico, può risultare utile ed economico per ottenere simultaneamente una più elevata biomassa microalgale, così come una maggiore crescita di piante di pomodoro.

Foreword

The definition of algae includes prokaryotic cyanobacteria and a large, diverse group of eukaryotic organisms, ranging from unicellular forms, like the genus *Chlorella*, to multicellular forms, like the genus *Macrocystis*.

Depending on their size, algae are divided into:

- macroalgae, which are macroscopic multicellular organisms, generally associated with marine algae, that can reach lengths of 65 m;
- microalgae, which are much smaller microscopic unicellular or multicellular organisms (from 1 to 50 μm).

Microalgae can be either autotrophic or heterotrophic and some species are mixotrophic.

An organism is autotrophic when it produces complex organic compounds (such as carbohydrates, fats and proteins) from simple substances present in its surroundings. Autotrophs can be photoautotrophs, using light as an energy source, or chemoautotrophs, utilizing electron donors from organic or inorganic sources as a source of energy.

Instead, an organism is heterotrophic when it is unable to synthetize organic compounds from inorganic substances, and therefore needs to feed itself on organic molecules produced by plants or animals. Heterotrophs can be further divided on the basis of the way in which they obtain energy; if the heterotroph uses light for energy, it is considered a photoheterotroph, while if it uses chemical energy, it is considered a chemoheterotroph.

Finally, mixotrophic organisms can use a mix of different sources to obtain energy and carbon, which means that these organisms cannot be classified as either autotrophs or heterotrophs. Autotrophic microalgae are all photoautotrophs. Photosynthesis is the process of

transferring light energy into chemical energy and converting CO_2 and water into carbohydrates and oxygen, which is quite similar in algae and higher plants (Richmond, 2004). The light reactions take place insides chloroplasts, on the thylakoid membranes. Chlorophyll and others pigments are subsumed on thylakoid in units of organization called photosystems. The main photosynthetic pigments are chlorophylls, carotenoids and phycobilins. There are several kinds of chlorophylls, that differ as regards some details of their molecular structure and specific absorption properties. Chlorophyll *a* is in all photosynthetic eukaryotes and cyanobacteria. Plants, green algae and euglenoid algae also include another pigment, chlorophyll *b*, that helps to extend the light interval in photosynthesis. Chlorophyll *c* substitutes chlorophyll *b* in some algae groups, the most important of which are brown algae and diatoms.

Photosynthesis produces carbohydrates that are used as carbon skeletons to form other organic compounds in algal cells.

Most algae are autotrophic, only a small part are heterotrophic such as *Nitschia*, of the diatom species. Heterotrophic algae are not photosynthetic and in order to grow they need organic matter to replace light as their substrate and energy (Kaplan et al., 1986). In these algae oxygen is supplied through aeration during respiration (Griffiths et al., 1960).

Mixotrophic microalgae can grow through both autotrophy and heterotrophy (Richmond, 2004). In mixotrophic algae CO_2 and carbon can be assimilated simultaneously for their growth, but photosynthesis and respiration can influence each other negatively.

The first study concerning mixotrophic microorganisms was that of Pascher (1917). Mixotrophs, which can offer

competitive advantages over phototrophs and heterotrophs, have been observed in various environments from oligotrophic habitats to eutrophic estuaries (Knowlton and Jones, 2000). However, it is only in recent years that mixotrophic algae have received greater attention from scientists worldwide. According to Chen (1996) and Zhang et al. (1999) the mixotrophic culture is a dual limiting process. Low light intensities or low organic carbon substrate concentrations and high light intensities or high carbon substrate concentrations can damage cell growth. Mixotrophic cultures have high rates of biomass production with photosynthetic metabolites (Lee and Lee, 2002).

Chlorella vulgaris is capable of combining both autotrophic and heterotrophic techniques by performing photosynthesis as well as ingesting organic materials such as glucose (Liang et al., 2009; Yeh and Chang, 2012).

Microalgae have been subjected to various studies due to their great adaptability to different nutrient substrates and their ability to grow in different environmental conditions (Singh et al., 2005; Spolaore Cassan et al., 2006; Mata et al., 2010; Safi et al., 2014).

Indeed, when compared with terrestrial crops, microalgae have some advantages. For instance, microalgae are capable of all-year-round production, under favorable conditions, (Schenk et al., 2008) and there is no need to use to herbicides or pesticides during cultivation. Furthermore, they have high protein contents and the ability to synthesize an extraordinary variety of metabolites (Harun et al, 2010).

Nowadays, there are numerous commercial applications for microalgae; they are a very interesting option for biofuel production; they can be used to enhance the nutritional value of human food and animal feed owing to their chemical composition; they play a crucial role in

aquaculture and they can be incorporated into cosmetics. Moreover, they are cultivated as a source of highly valuable molecules.

Microalgae for human nutrition are marketed in different forms such as tablets, capsules and liquids. They can be incorporated into pastas, snack foods, candy, gums, and beverages (Yamaguchi K., 1997; Liang S. et al., 2004). Microalgae have various possible health promoting effects: the alleviation of hyperlipidemia, suppression of hypertension, protection against renal failure, promoting the growth of intestinal *Lactobacillus*, and suppression of elevated serum glucose levels (Yamaguchi, 1997; Liang et al., 2004; Vilchez et al., 1997). For example, *Chlorella* is produced by more than 70 companies; its most important substance is β-1,3-glucan, which is an active immunostimolator, a free radical scavenger and can reduce blood lipids.

However, other effects have also been found: efficacy in cases of gastric ulcer, wounds and constipation, preventive action against atherosclerosis and hypercholesterolemia and antitumor action (Yamaguchi, 1997).

Microalgae are important for animals too, in fact, they can be incorporated into feed for different animals with 30% of microalgae production being sold for animal feed (Becker, 2004).

Additionally, microalgae can be a natural source of new compounds. This is because some microalgae live in complex habitats and are subject to extreme conditions, therefore, they must adapt rapidly to new environmental conditions to survive. As a result, they produce a great variety of metabolites that cannot be found in other organisms, which are generally hard to produce by chemical synthesis. It is possible to find bioactive compounds in

microalgae, which can be discovered due to the coexistence of these organisms in the natural aquatic community, where an inhibitory interaction occurs between producer and competitor in the same habitat (Lordan et al., 2011).
Moreover, they are a considerable source of protein and value-added compounds, which are of importance to the pharmaceutical and nutritional fields (Borowitzka and Borowitzka, 1992; Katircioglu et al., 2006).
It has been shown that cellular extracts, for example from *Chlorella vulgaris* and *Chlamydomonas pyrenoidosa,* have an antibacterial activity, like that observed in the growth medium of various unicellular algae (Singh et al., 2005). In addition it has been shown that it is possible to obtain a wide range of active antifungal activities in vitro from green-algae extracts, diatoms and dinoflagellates. Several strains of cyanobacteria are known for intracellular production and extracellular metabolites, with different biological activities (antibacterial, antimycotic and antiviral). Incubation temperature, soil culture pH, average constituents, incubation time and light intensity are important factors, that influence the production of antimicrobial agents (Spolaore Cassan et al., 2006).
Furthermore, microalgal extracts influence plant growth and development through a great number of substances (Ördög, 1999). These microrganisms benefit plants by supporting growth promoting regulators, vitamins, aminoacids, polypeptides, antibacterial and antifungal substances that exert a phytopathogen biocontrol and polymers such as exopolysaccharides that improve plant growth and productivity (de Mulè et al., 1999). Microalgae extracts are used to increase the growth parameters of many plants thanks to their biofertilization effect (Adam, 1999; Saffan, 2001). Shaaban (2001) investigated the effect of using the

aqueous extract of *C. vulgaris* as foliar feed on the status of nutrients, growth, and yield in wheat plants (*Triticum aestivum* L.). This study found that a concentration of 50% (v/v) extract in a single foliar spray (25 days after sowing) increased the growth yield and weight grain by 140% and 40%, respectively.

Furthermore, another study showed that *C. vulgaris* exerted an influence on the growth parameters and physiological responses of *Lactuca sativa* seeds germinated in culture medium containing microalgae for 3, 6, 9, 12 and 15 days (Faheed et al., 2008). The result was that the addition of *C. vulgaris* to the culture medium or soil significantly increased the fresh and dry weight of the seedlings as well as their pigment content. All the studies were carried out using the liquid extract of *C. vulgaris* as a biofertilizer for plant growth.

Unfortunately, literature concerning the application of microalgae in the agricultural field has focused prevalently on some species, while neglecting the potential of other microalgae, above all *Scenedesmus quadricauda*.

Aim and structure of the PhD study

Microalgae multifunctionality was the guiding principle of the present study. It focused on two microalgal species, *Chlorella vulgaris* and *Scenedesmus quadricauda.*

The first species was chosen for its easy cultivation and because the organism, including its biochemistry, growth cycles and genetics, has been widely investigated (Kebelman, 2012). The second species was selected because it is ubiquitous in water environments and has often been selected for algae-based wastewater treatment (Chen, 2001; Omar, 2002; Ma et al., 2004; Awasthi and Rai, 2005; Mata et al., 2010). Additionally, the two algal species under

examination were chosen because of the differences in recognized size and shape (Bold, 1978).
Firstly, their use was aimed at reducing the environmental impact of agricultural wastewater, enriched by organic and inorganic pollutants, in order to ensure the proper management of the water cycle. However, this application led to the production of a waste by-product, algal biomass, for which literature reports many interesting applications. So, the by-product became a resource and not a waste product.
Human population growth and an unconditional use of chemical compounds has resulted in a need for sustainable agricultural practices. Among the different possible applications for microalgae, the use of microalgal biomass as a source of biostimulants can be considered as the most rational solution. Moreover, compared to the extensive literature that demonstrates the possibility of extracting biostimulants from seaweed, very few studies focus on microalgal extracts, making them more attractive.
Three crops were chosen to evaluate the biostimulating effect of microalgal substances. The first of these, *Beta vulgaris*, was selected for its higher susceptibility to environmental biotic and abiotic stresses, which strongly affect the world yield. The second, *Zea mays*, was chosen because it was a model plant and due to its economic importance worldwide. A further interesting plant to be investigated was the tomato, since it is considered a crop of relevant economic interest locally.
However, the use of a biostimulant cannot neglect the effect that this can have on the soil, especially as regards fertility.
Biostimulants could be effective on plants, but on the other hand they could also be toxic to soil microorganisms, resulting in the reduction and / or destruction of soil fertility.

In addition, it was therefore also verified if the biostimulating effect on plants was exerted even when these substances were applied in a complex sistem as soil.
The final investigation regarded the possibility of co-cultivating microalgae and crops, which could benefit from micro-algal extracellular products with biostimulant effects.

The main objectives of this thesis have been schematized in the following four sections:

- Removal of organic and inorganic pollutants from agricultural wastewater by using *Chlorella vulgaris* and *Scenedesmus quadricauda* (**Chapter I**)
- Studies on the biostimulating effect of *Chlorella vulgaris* and *Scenedesmus quadricauda* extracts on plants of agricultural interest (**Chapter II**)
- Study on the effect of *Chlorella vulgaris* and *Scenedesmus quadricauda* extracts on soil biochemical fertility (**Chapter III**)
- Optimization of production: co-cultivation of *Chlorella vulgaris* or *Scenedesmus quadricauda* and tomato plants (**Chapter IV**).

CHAPTER I

Removal of organic and inorganic pollutants from agricultural wastewater by using *Chlorella vulgaris* and *Scenedesmus quadricauda*

This chapter was pubblished as:
Baglieri A., Sidella S., Barone V., Fragalà F., Silkina A., Negre M., Gennari M. (2016) Cultivating *Chlorella vulgaris* and *Scenedesmus quadricauda* microalgae to degrade inorganic compounds and pesticides in water**. Environ. Sci. Pollut. Res.** 23: 18165-18174

1. Introduction

Agricultural activities produce a considerable quantity of wastewater containing high concentrations of inorganic and organic compounds, in much the same way as do civil and industrial activities. In order to dispose of this waste in soil or in surface water bodies, it must undergo a process of purification to reduce the concentration of chemical compounds that it contains to levels lower than those established by law (DM 31/2001 Italy).

The treatments used to decontaminate wastewaters can be mechanical, chemical, physical and biological. Studies are still in progress into innovative techniques, for exampleusing organo-clay to remove pesticides (Baglieri et al., 2009, 2013), a micelle-clay complex to reduce the concentration of drugs (Khalaf et al. 2013; Qurie et al. 2014) and numerous static and dynamic biological systems (Coppola et al., 2011; De Wilde et al., 2009).

One of the biological techniques under consideration is that of using microalgae to remove organic and inorganic compounds from wastewaters. Given that microalgae are particularly efficient in accumulating nutrients and heavy metals (de Bashan and Bashan, 2010) they can be used to reduce the concentration of contaminants. Moreover, purification plants can then provide agriculture and industry with algal biomass for use as an organic fertilizer or for the production of high-added-value derivatives (Rawat et al., 2011).

Many studies have been published regarding the capacity of microalgae to remove organic and inorganic compounds from wastewaters originating from food (Chi et al., 2011), industrial and agro-industrial processes (Chinnasamy et al., 2010; Mulbry et al., 2009; Rao et al., 2011) as well as from the civil and domestic sectors (Li et al., 2011; Martinez et

al., 2000). Komolafe et al. (2014) considered the possibility of growing several microalgae (Desmodesmus sp., Oscillatoria, Arthrospora) in water taken from a lagoon wastewater treatment plant with the aim of obtaining a biomass for the production of biodiesel and at the same time purifying the culture medium. The authors reported that treatment with the algae brought about a greater reduction in nutrients (nitrogen and phosphorus) than a conventional activated sludge system. All the microalgae used in the test reduced the concentration of total nitrogen, but to different degrees depending on the species (55.4–83.9 %). In the same experiment the concentration of orthophosphate also diminished but to a lesser degree than the nitrogen (30.1–60.0 %).

Of the different microalgae proposed for use in treating wastewaters, studies on species of *Chlorella, Ankistrodesmus* and *Scenedesmus* have proved successful in treating wastewaters from the olive oil and paper-making industries (Pinto et al., 2003; Tarlan et al., 2002). Zangh et al. (2008) reported that a *Scendesmus* genus microalgae was highly efficient in removing inorganic compounds from household effluents.

However, the efficiency of microalgae in removing different inorganic compounds varies. Lau et al. (1996) reported that purification with *Chlorella vulgaris* resulted in an 86% reduction in inorganic nitrogen and a 78% reduction in inorganic phosphates. Instead, Colak and Kaya (1988) reported that *Chlorella* sp. removed 50.2% and 85.7% of these two elements respectively from industrial wastewater.

Rasoul-Amini et al. (2014) compared the ability of five strains of microalgae in removing nitrate and orthophosphate from batch cultures of urban wastewater. Their results indicated that *Chlorella* sp. (YG 1) was the

most efficacious in the removal of nitrogen (84.11 % in 2 weeks) with respect to the other strains, while *Chlamydomonas* sp. (YG 04) and *Chlamydomonas* sp. (YG 05) brought about the greatest reduction in the concentration of phosphate (100 %).

Ruiz-Marin et al. (2010) carried out studies on the growth of C. vulgaris and Scenedesmus obliquus in urban wastewater and their capacity to reduce the concentration of N-NH4 and P-PO4 in the culture medium. Forty percent of nitrogen was removed from the control (no microalgae), but the reduction was greater when microalgae were present. In all the tests, after 40 h of treatment, *S. obliquus* proved the most efficient in removing ammonia (95.4–100 %) as compared to *C. vulgaris* (65.6–80.0 %). The percentages of phosphorus removed by the two microalgae were relatively similar (53.3–85.1 %).

As yet, very few studies have been carried out with regard to microalgae removing organic contaminants such as pesticides. Of these, Cai et al. (2007) studied the degradation of diclofop-methyl by algal cultures of C. *pyrenoidosa*, *C. vulgaris* and *S.obliquus*, discovering that *C. vulgaris* was able to degrade more of the active ingredient than *C. pyrenoidosa* and *S. obliquus*. Tsang et al. (1999) observed that species of Chlorella sp. were able to degrade trybutyltin into less toxic intermediate compounds, while Gao et al. (2011) studied the efficiency of the same species in removing nonylphenol, finding that 70% of the active ingredient was removed.

The wide variety of sometimes discordant data to be found in literature demonstrates how the capacity of microalgae to adsorb, degrade and remove nutritive and pollutant elements in general can vary according to the algal species used and

the chemical parameters of the wastewater to be treated (Komolafe et al., 2014; Cabanelas et al., 2013).

Aim and scope

The aim of this study was to evaluate the capacity of two microalgae species, *Chlorella vulgaris* and *Scenedesmus quadricauda,* to remove organic and inorganic pollutants of agricultural origin. Two particular case studies were considered: one relating to the growth of the two species in wastewater from a hydroponic greenhouse cultivation in order to evaluate the degree of removal of the main inorganic compounds; and the other regarding how the same species of algae were able to degrade five different active ingredients commonly used in agriculture for phytoiatric treatments.

2. Materials and methods

2.1 Microalgae and wastewater

Tests were carried out using two species of microalgae, *C. vulgaris* and *S. quadricauda*, both supplied by Swansea University (Wales, UK). The wastewater was a residue from the hydroponic cultivation of "cherry-type" tomatoes grown in a greenhouse (Noto, Siracusa, Italy). The wastewater was collected and its main chemical components analyzed: total organic carbon (TOC) (UNI EN 1484 1999); nitrates (NO_3^-) and sulfates (SO_4^{2-}) (UNI EN ISO 10304-1 2009); calcium (Ca^{2+}) and potassium (K^+) (UNI EN ISO 14911 2001); and ammonia nitrogen (NH_4^+), nitrites (NO_2^-), soluble phosphorus (sol. P), total phosphorus (tot. P), and iron in the form of Fe^{3+} (APHA, AWWA, WEF 2012). The chemical composition of the wastewater is shown in **Table 1**. The wastewater did not undergo any kind of treatment before the test.

Table 1. Chemical and biological composition of the greenhouse wastewater.

Compounds	Amount in the wastewater (mg L^{-1})
Cl^-	365.0
SO_4^{2-}	1200.0
Ca^{2+}	625.2
Mg^{2+}	55.1
Na^+	194.0
K^+	57.2
NO^{3-}	210.0
NO^{2-}	0.9
NH^{4+}	1.4
Soluble P	3.5
Total P	4.0
Fe^{3+}	0.04
TOC (C)	23.0
Escherichia coli (UFC/100 mL)	8
Salmonella	Absent

2.2 Wastewater decontamination test

The growth apparatus was a plexiglass tank subdivided into nine compartments illuminated by a 3500-lx, average photon flux (PPF) 100-µmol m^{-2} s^{-1} light source (PHILIPS SON-T AGRO 400) with a 12-h photoperiod. Each compartment was fitted with a silicon tube with a terminal diffuser connected to a forced ventilation system.

The test was performed by placing 424 mg *S. quadricauda* or 388 mg *C. vulgaris* into 2000 mL of greenhouse wastewater or artificial medium (BG11) sterilized in an autoclave at 121 °C for 20 min. **Table 2** shows the chemical composition of the BG11 medium. Each test was repeated three times. In alternate weeks, the quantity of water that had evaporated was restored by adding distilled water or the initial substrate (greenhouse wastewater or BG11). In total, 750 mL of greenhouse wastewater or BG11 and 750 mL of

distilled water were added. The microalgae were grown at room temperatures of between 25–30 °C. Every 7 days, 10 mL of algal suspension was sampled to determine the rate of growth.

Table 2. Chemical composition of BG11 medium.

Component	Conc. in final medium (mg L^{-1})
$NaNO_3$	14.97
$MgSO_4 * 7H_2O$	74.93
K_2HPO_4	30.31
$CaCl_2 * 2H_2O$	36.02
Citric acid	6.00
Ferricammoniumcitrate	8.40
Na_2EDTA	0.95
Na_2CO_3	20.04
H_3BO_3	0.06
$MnSO_4 * H_2O$	0.03
$ZnSO_4 * 7H_2O$	0.03
$CuSO_4 * 5H_2O$	0.01
$(NH_4)_6Mn_7O_{24} * 4H_20$	0.02

In previous tests, it was shown that the two microalgae stopped growing after 49 days when they reached the stationary phase. For this reason, the 49th day was chosen as the final day of the test. At the end of the test, the suspension was carefully removed from each tank and centrifuged at 2500 rpm for 10 min. The supernatant was filtered using a Buchner system with WHATMAN fiberglass filters, then analyzed for the same parameters as were taken into consideration at the beginning of the test. In order to determine total algae production, the sedimentary pellet of algal biomass was oven-dried at 70 °C until constant weight was reached.

2.3 Purification test for water containing agrochemicals

A laboratory test was set up to determine whether *C. vulgaris* and *S. quadricauda* were able to promote the degradation of pyrimenthanil, methalaxyl, iprodione, and fenhexamid fungicides and triclopyr herbicide. These particular compounds were chosen because they are widely used in agriculture and can be considered to represent a much wider range of agrochemicals in that they all have different chemical-physical characteristics (**Table 3**).

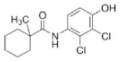
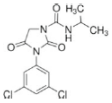
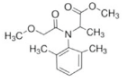
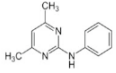
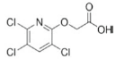
Fifty milliliters of algal suspension, containing 75 mg of cells, and 150 mL of BG11 culture medium containing 5.5 ppm of active ingredient (Sigma-Aldrich, Milan, Italy; 99.0 % purity) were placed in 250-mL flasks, thereby obtaining a final pesticide concentration of 4 ppm.

Contemporarily, the flasks were prepared containing the active ingredients in a sterile substrate. Each test was replicated three times. The flasks were closed with a cotton plug, placed on a mechanical shaker, and illuminated by a light source at a power equal to 3500 lx and average photon flux (PPF) equal to 100 μmol m^{-2} s^{-1} (PHILIPS SON-T AGRO 400) with a 12-h photoperiod. Room temperature was maintained between 25 and 30 °C.

A sample was taken of the suspension (flasks with microalgae) and the solution (control in sterile BG11) as soon as the test began in order to determine zero time. Further, 10-mL samples of the suspension and solution were then taken at different times to determine the concentration of the compounds. Before each sampling, BG11 was added to compensate for the water lost through evaporation.

At the end of the test, the samples containing the microalgae were centrifuged at 2500 rpm for 10 min and the pellet was oven-dried at 70 °C until constant weight was reached. It was then weighed to measure the total biomass.

Table 3. Physical and chemical properties of the agrochemicals studied.

	Fenhexamid (FEX)	Iprodione (IPRO)	Metalaxyl (MET)	Pyrimethanil (PYR)	Triclopyr (TRIC)
Pesticide type	Fungicide	Fungicide	Fungicide	Fungicide	Herbicide
Substance group	Hydroxyanilide	Dicarboximide	Phenylamide	Anilinopyrimidine	Pyridinecarboxylic acid
Chemical formula	$C_{14}H_{17}Cl_2NO_2$	$C_{13}H_{13}Cl_2N_3O_3$	$C_{15}H_{21}NO_4$	$C_{12}H_{13}N_3$	$C_7H_4Cl_3NO_3$
Structure formula					
Molecular mass ($g \cdot mol^{-1}$)	302.20	330.17	279.33	199.11	256.5
Solubility in water, 20°C ($mg \cdot L^{-1}$)	24.0	12.2	7100	121	8100
Log Poct/water (pH 7, 20 °C)	3.51	3.1	1.65	2.84	–0.4
Henry's law costant, 20 °C	5.69×10^{-09}	2.80×10^{-07}	4.70×10^{-09}	7.42×10^{-07}	4.00×10^{-08}

2.4 Determination of growth rate

Growth was measured on a weekly basis using a spectrophotometer (Jasco V-530 UV-Vis spectrophotometer) at a wavelength of 550 nm. The data were expressed in agreement with Vandamme et al. (2012) according to the following relation:

$$Ig = \frac{\text{Abs 550 T}_X - \text{Abs 550 T}_0}{\text{Abs 550 T}_0}$$

where:

Ig: the growth index;
Abs 550 $T_{(x)}$: absorbance of the 550 nm wavelength at time x;
Abs550 $T_{(0)}$: absorbance of the 550 nm wavelength at time 0.

2.5 Determination of agrochemicals

Liquid chromatography (LC) was used to analyze the supernatant which was separated from the algal biomass by centrifugation at 3000 rpm for 15 min, to evaluate the variation in concentration of each agrochemical in the tests over time. Moreover, in order to evaluate how much active ingredient had been adsorbed by the algae by the end of the test, the whole of the biomass obtained was extracted by adding 100 mL of hexane then shaking for 30 min and centrifuging at 3000 rpm for 15 min. The supernatant was filtered over anhydrous sodium sulfate and transferred to a vacuum flask.

The whole process was repeated twice more with extraction times of 15 min. All the supernatants obtained were

collected together and subjected to vacuum evaporation using a Rotavapor (LABOROTA 4000, Heidolph, Milan, Italy). The dry residue was dissolved in 5 mL of acetonitrile and analyzed by LC.

The LC analyses were carried out using a Shimadzu Liquid Chromatograph Mod. LC-10 ADvp, fitted with a UV/vis detector, a 20-µL loop, and a SupelcoSil LC-18 column, eluted with a mobile phase consisting of water acidified to pH 3 with H3PO4 (40 %) and CH3CN (60 %). The analyses were carried out at a flow speed of 1 mL min^{-1}; the wavelengths used for analyzing the active ingredients were equal to 220 nm (fenhexamid, iprodione, metalaxyl, and pyrimethanil) and 230 nm (triclopyr).

3. Results and discussion

3.1 Wastewater decontamination test

At the end of the test, about 25 % less algal biomass of both species was obtained from the greenhouse wastewater than from the control. In the greenhouse wastewater and BG11, the *C. vulgaris* biomass amounted to 3.75 g L^{-1} (corresponding to 67 mg L^{-1} day^{-1}) and 5.15 g L^{-1} (corresponding to 92 mg L^{-1} day^{-1}), respectively; in the greenhouse wastewater and BG11, the *S. quadricauda* biomass amounted to 3.65 and 5.69 g L^{-1} (corresponding to 65.18 and 101.68 mg L^{-1} day^{-1}), respectively.

The yields were in agreement with data reported in literature. In particular, when treating digested animal manure with *Chlorella* sp., Wang et al. (Wang et al., 2010a, b) obtained an 81.4-mg L^{-1} day^{-1} biomass. Data available in literature regarding *Scenedesmus* sp. indicate a biomass production that varies between 6 mg L^{-1} day^{-1} from agricultural livestock wastewater (Kim et al., 2007) to 120

mg L^{-1} day^{-1} obtained from artificial wastewater (Voltolina et al., 1999). Furthermore, Martinez et al. (2000) obtained a yield of 26 mg L^{-1} day^{-1} using *S. obliquus* to treat urban wastewater.

Up to the 49th day of the trial, there was an increase in the growth rate of *C. vulgaris* and *S. quadricauda* over time in both the growth mediums studied. At the end of the test, the growth rate in the experiments that had used greenhouse wastewater as the culture medium was greater for *C. vulgaris* than for *S. quadricauda*. Although both species adapted well to the greenhouse wastewater, both *C. vulgaris* and *S. quadricauda* achieved higher growth rates in the control (BG11) (**Table 4**), in agreement with Hultberg et al. (2013) who observed a greater growth rate for *C. vulgaris* when cultivated in an artificial substrate as compared to when it was grown in residues from hydroponic cultivation.

The initial composition of the greenhouse wastewater in which the algal species were grown was characterized by a higher quantity of nitrous and nitric nitrogen than ammonia nitrogen (211 mg L^{-1} of NO_3^- + NO_2^- and 1.36 mg L^{-1} of NH_4^+). Total and soluble phosphorous were present in quantities equal to 4 and 3.5 mg L^{-1}, respectively. The parameter relating to the total organic carbon (TOC) was 23 mg L^{-1} (**Table 1**).

Table 5 reports the total quantity of the major compounds present in the wastewater during the test, calculated on the basis of the further additions made (2000 mL + 750 mL).

The same table also shows the amount found in the wastewater at the end of the test and the difference between the two values expressed as a percentage.

With regard to the removal of inorganic compounds, both species in the study demonstrated an aptitude for

decontamination. The two algal species showed a preference for nitric nitrogen rather than ammonia nitrogen, contrary to what has been reported in literature where it appears that the microalgae prefer the ammonia type of nitrogen (Kim et al., 2010; Perez-Garcia et al., 2011).
According to a number of authors, if the assimilation of the ammonia type does not lead to a reduction reaction, then it is the most efficient from an energy point of view as it requires a smaller amount of energy in the organication process (Grobbelar, 2004; Maestrini et al., 1986; Wilhelm et al., 2006). Nevertheless, Sidney et al. (2011) showed that if there is a fairly low concentration of ammonia nitrogen in the cultural medium, then the microalgae tend to remove the nitric form.

Table 4. Growth index of *C. vulgaris* (CV) and *S. quadricauda* (SQ) grown in BG11 medium and greenhouse wastewater.

Time (days)	Growth index (IG)			
	CV BG11	CV wastewater	SQ BG11	SQ wastewater
0	0.0	0.0	0.0	0.0
6	1.9±0.2	3.2±0.3	3.2±0.2	3.0±0.2
14	4.9±0.3	6.8±0.4	9.0±0.6	5.3±0.4
21	11.6±1.3	7.7±0.7	14.2±1.4	12.7±0.8
28	16.1±1.1	9.9±1.0	15.3±1.7	15.2±1.4
35	19.7±2.3	14.9±1.7	17.6±0.8	17.4±0.9
42	22.6±2.0	17.3±1.2	27.5±2.2	18.3±1.8
49	34.8±3.1	26.2±2.8	32.7±3.9	20.6±1.4
56	32.0±2.5	21.9±1.8	27.5±1.7	19.4±1.2

In our case, while demonstrating a preference for the nitric forms (99 % removal), *C. vulgaris* also removed about 83 % of the ammonia nitrogen. On the contrary, *S. quadricauda* consumed about 99 % of the nitric nitrogen but only 5 % of the ammonia nitrogen.

Table 5. Total amount of the main compounds present in the greenhouse wastewater throughout the test; total amount recorded at the end of the test; difference espressed in percentage. Nd = not detected. Standard deviation< 10%.

Compounds	Amount in the wastewater (mg)	Amount detected (mg)		Removal efficiency (%)	
		CV	SQ	CV	SQ
Cl^-	1003.8	1181.3	1452.0	+18	+45
SO_4^{2-}	3330.0	2454.0	2934.8	-26	-11
Ca^{2+}	1718.8	767.3	867.8	-55	-50
Mg^{2+}	151.3	189.0	203.3	+25	+34
Na^+	533.5	824.3	994.5	+54	+86
K^+	156.8	36.0	47.3	-77	-70
NO^{3-}	577.5	3.8	3.0	-99	-99
NO^{2-}	2.5	nd	nd	-100	-100
NH^{4+}	3.7	0.7	3.6	-83	-5
Soluble P	9.6	0.6	1.1	-94	-88
Total P	11.0	0.7	1.2	-94	-89
Fe^{3+}	0.1	0.03	0.03	-71	-71
TOC (C)	63.3	25.5	40.5	-60	-36

Franchino et al. (2013) evaluated the capacity of three different algal strains in an agro-zootechnical digestate and observed that *C. vulgaris* removed more ammonium than *S. quadricauda* (99 and 83 %, respectively). On the contrary, Gonzales et al. (1997) observed that *Scenedesmus dimorphus* was more efficient than *C. vulgaris* in removing

ammonium from agro-industrial wastewater, even if only during the initial experimental phases. In our study, both the microalgae removed between 88 and 94% of phosphates. Nevertheless, *C. vulgaris* removed slightly more than *S. quadricauda*, the respective values for the two species being 94 and 88–89 %. Removal of nitrogen and phosphate compounds by *Scenedesmu*s spp. is further confirmed by data in literature provided by Di Termini et al. (2011) and Guo et al. (2013). These works report that algal species belonging to the *Scenedesmus* genus demonstrated an almost 100 % efficiency in removing nitrogen and phosphate nutrients.

The two microalgae were also found to have exerted a positive effect on the reduction in sulfate concentrations, reducing the quantity by 26 and 11 % (*C. vulgaris* and *S. quadricauda*, respectively). Both microalgae reduced the concentration of iron by 71% while potassium went down by 77 % when *C. vulgaris* was present and 70 % when *S. quadricauda* was present.

In the *C. vulgaris* cultures, the reduction in total organic carbon content was 60 %, while for *S. quadricauda*, the reduction was 36 %. Degradation of organic carbon by microalgae is a process related to the heterotrophic/mixotrophic metabolism that they are able to assume in the absence of light and/or when CO_2 is limited (Perez-Garcia et al., 2011). One reason for the difference in removal rates observed when comparing the two species could be the fact that *C. vulgaris* had a higher rate of growth than *S. quadricauda*. Combres et al. (1994) reported that both *C. vulgaris* and *S. obliquus* are able to utilize organic substances under both light and dark conditions. Studies on the mixotrophic and heterotrophic growths of some

microalgae performed by EL-Sheekh et al. (2012) showed that the addition of glucose and soluble organic products derived from wheat bran induced a good growth of *C. vulgaris* and *S. obliquus* under both mixotrophic and heterotrophic conditions. Nevertheless, mixotrophic conditions produced a better effect on the growth of the two microalgae, in particular *C. vulgaris*.

An increase was observed in some inorganic constituents such as chlorides, magnesium, and sodium. It seems evident that more of these elements were added than were consumed by the algae. Fodorpataki and Bartha (2004) found that cultures of *Scenedesmus opoliensis* can acclimate to a high salt concentration (0.5 M NaCl). Under these conditions, the rate of cell division shows only a moderate decrease. A higher tolerance was mainly expressed when high photon lux density is applied suggesting the role of light in supporting the energy demands of a protective mechanism against the abiotic stress. Talebi et al. (2013) demonstrated that among *Chlorella* species, *C. vulgaris* shows signs of adaptation to a high content of salinity. In fact, *C. vulgaris* survived at NaCl concentrations as high as 0.75 M. On the basis of the above, the concentration of salts, in particular NaCl, found of the medium at the end of the test, explains a slight decrease in the growth of the two microalgae. However, this decrease does not affect the purifying activities of *C. vulgaris* and *S. quadricauda* against many other compounds, in particular nitrogen and phosphorus which are of interest due to their role in the eutrophication of water bodies.

3.2 Purification test for water containing agrochemicals

The two species of algae used in the study grew well in the presence of all the active ingredients. As regards *C. vulgaris*, a slight increase in yield was seen when iprodione and triclopyr were present (21 and 42 %, respectively) while the presence of fenhexamid reduced microalgae production by 26 % as compared to the uninoculated control (3.1 g L^{-1}). Pyrimethanil and metalaxyl brought about a reduction in yield of only 10 %, a result which was not statistically significant (**Figure 1**).

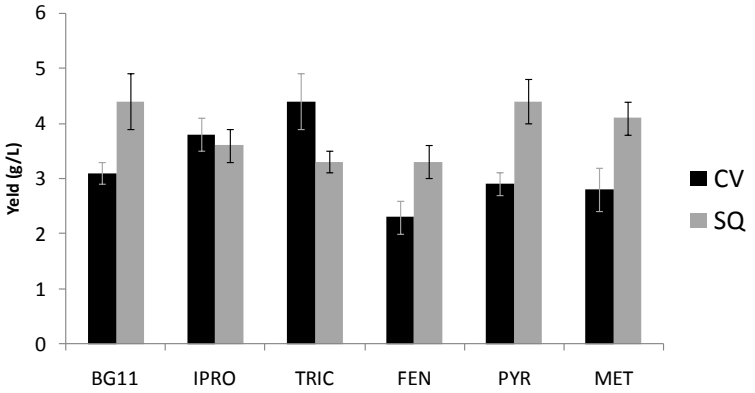


Figure 1. Biomass production of *C. vulgaris* (CV) and *S. quadricauda* (SQ) grown in BG11 medium not contaminated, added with metalaxyl (MET), fenhexamid (FEN), pyrimethanil (PYR), triclopyr (TRIC), and iprodione (IPRO). The bars indicate standard deviation (n = 3).

S. quadricauda grew more efficiently than *C. vulgaris* in sterile BG11 (4.4 g L^{-1}). Pyrimethanil did not influence the quantity of microalgae produced, while there was a reduction in yield of 7, 18, 25, and 25 % in the presence of

metalaxyl, iprodione, triclopyr, and fenhexamid, respectively (**Figure 1**).

Although in some cases the two microalgae showed signs of suffering from a slightly toxic effect, it appears reasonable to affirm that growth is possible in the presence of all the pesticides in the growth medium. The only published data available regard the effect of fenhexamid on *Scenedesmus subspicatus*. It is reported that in a 72-h test carried out in static conditions, the value of EC50 was >26.1 mg L^{-1}, which the authors held to be of little toxicological interest (Byrnes 2001).

The results of the pesticide degradation tests are shown in **Figure 2** and **Figure 3**.

The microalgae proved effective in all cases, but with noticeable differences depending on the added molecule. The test with metalaxyl provided the most interesting result because this molecule dissipated much more quickly in the presence of the microalgae than in the uninoculated control. In fact, 69 % of the added active ingredient was still found in the sterile medium 56 days from the start of the test, while the metalaxyl disappeared from the medium with the two microalgae only 14 days after inoculation (**Figure 2**).

Metalaxyl is very stable to hydrolysis. Sharon and Edgington (1982) found 84% of the compound after 12 weeks of storage in water. Metalaxyl is also stable in sunlight since its absorption maximum is 196 nm (Sukul and Spiteller, 2000). The microalgae also accelerated dissipation of fenhexamid although to a lesser extent than metalaxyl. After 49 days, fenhexamid was no longer present in the medium with microalgae, while 52 % was still present in the uninoculated medium (**Figure 3**). Abbate et al. (2007) studied the degradation of fenhexamid in sterile buffer

solutions and in natural water. They reported that 101 days from the beginning of the test, 4 % of the active ingredient had been degraded at pH 4, 12% at pH 7, and 23% at pH 9.
In natural water, 112 days after contamination, 80 % of the fenhexamid was recovered. These results indicate that this molecule presented a substantial resistance to hydrolysis but a positive correlation with pH. As the pH of the culture medium of *C. vulgaris* and *S. quadricauda* averaged 9.5, it is possible that the accelerated degradation of fenhexamid was partly due to the alkaline conditions of the medium.
In the uninoculated test, as in the tests with microalgae, light radiation may also have contributed to the degradation as it has been reported that fenhexamid might be subject to photo-degradation when exposed to solar radiation (Byrnes, 2001).
The dissipation of metalaxyl and fenhexamid occurred faster in the presence of *C. vulgaris* than *S. quadricauda*. This suggests that with equal conditions the presence of the microalgae was essential in determining the disappearance of the molecules from the aqueous solution. As regards iprodione and triclopyr, while the positive action of the two microalgae in the dissipation process appeared less pronounced, it was without doubt efficacious. This is probably due to the fact that these two molecules are more susceptible to abiotic degradation (**Figure 3**).
Iprodione degrades rapidly in alkaline solution. The DT50 values observed were 37 days, 1.1 days, and 21 min at pH 5, 7, and 9, respectively (Roberts and Hutson, 1998).
As the average value of the pH of the culture broth in the presence of iprodione was 9.2, the alkaline conditions caused by the growth of the microalgae may have

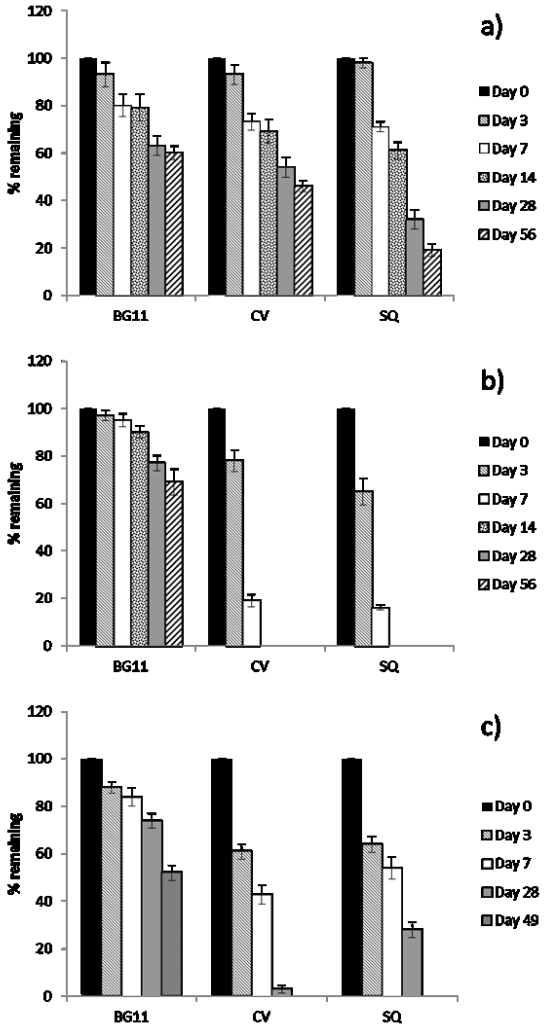


Figure 2. Dissipation of a pyrimethanil, b metalaxyl, and c fenhexamid in culture medium inoculated with *C. vulgaris* (CV) and *S. quadricauda* (SQ) and in sterile medium (BG11). The error bars indicate standard deviation (n = 3).

accelerated the degradation process as compared to the control which had a pH of 7.9.

Triclopyr has little tendency to hydrolyze (DT50 = 270 days) (Linders et al., 1994). Cleveland and Holbrook (Claveland and Holbrook, 1992) investigated the hydrolysis of radio-labeled tricloyr in buffered distilled water at pH 5, 7, and 9. After 1 month, the parent compound represented 97.2 % of the activity remaining in the samples leading to the conclusion that hydrolysis would not be an important mechanism for the dissipation of triclopyr from an aqueous system. On the other hand, Green and Westerdahl (1993) stated that photolysis is the principal degradation pathway for triclopyr in aqueous solution. In river water, the half life of triclopyr was determined to be 1.3 days both in artificial and natural light (Woodburn et al., 1993).

On the basis of the above considerations, triclopyr degradation observed in our study was probably caused mainly by light radiation even if the two microalgae contributed to enhancing the rate of degradation independently of variations in the pH of the medium.

Pyrimethanyl behaved differently from the other pesticides, proving more resistant to dissipation in the presence of *C. vulgaris* and *S. quadricauda*. Nevertheless, also in this case, the two microalgae reduced the active ingredient content in solution as compared to the sterile control. This occurred principally in the presence of *S. quadricauda* which after 56 days growth reduced the pyrimethanil content of the initial dose, while in the sterile BG11 the residual content was 56 % (**Figure 1**). The pesticide extraction tests performed on the biomass produced with organic solvent highlighted that there was no physical adsorption except in the case of pyrimethanil. In this case, extraction from the biomass with

organic solvent highlighted the proportion of active ingredient that was not present in solution.
A similar result was obtained by Bizaj et al. (2011) in liquid cultures containing *Saccharomyces cerevisiae*.

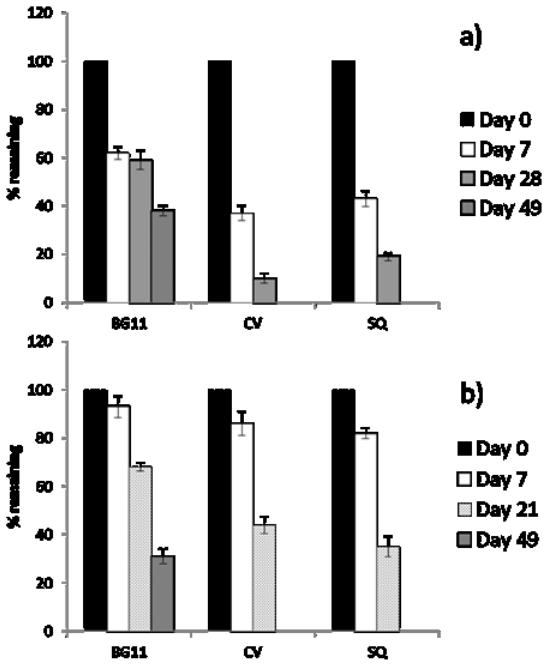


Figure 3. Dissipation of a iprodione and b triclopyr in culture medium inoculated with C. vulgaris (CV) and S. quadricauda (SQ) and in sterile medium (BG11). The error bars indicate standard deviation (n = 3).

In fact, they did not observe significant differences in the removal of pyrimethanil between viable and non-viable cells, concluding that pyrimethanil was not degraded by metabolically active cells.

It is therefore interesting to note that while in the sterile control the molecule was partially degraded, no degradation took place in the presence of the microalgae but there was a transfer from the liquid phase to the solid phase with a consequent risk of producing contaminated biomass. The degradation of pyrimethanil in the control samples could not have been due to hydrolysis because it has been reported that the compound is stable in sterile buffer solutions at pH 5, 7, and 9 (EFSA, 2006). In addition, pyrimethanil does not seem to undergo direct photolysis while it seems possible that it could be indirectly photodegraded in the presence of photosensitizers (EFSA, 2006).

On the other hand, in the tests involving the other active ingredients, the biomass did not contain any residues. In the case of fenhexamid, our result differs from that obtained by Bizaj et al. (2011), who found that fenhexamid, like pyrimethanil, was absorbed by *S. cerevisiae* cells.

The results from this study suggest that it is possible to use a *C. vulgaris* and *S. quadricauda* cultivation system to purify wastewater from farming which contains inorganic compounds and pesticides. There are numerous methods for cultivating microalgae (Sharma et al., 2011). However, a suitably modified open system, like the one described in the "Materials and methods" section, could be a simple, inexpensive, easy-to-use solution even for small farms.

CHAPTER II

Studies on the biostimulating effect of *Chlorella vulgaris* and *Scenedesmus quadricauda* extracts on plants of agricultural interest

Part of the data in this chapter was pubblished as: Barone V., Baglieri A., Stevanato P., Broccanello C., Bertoldo G., Bertaggia M., Cagnin M., Pizzeghello D., Moliterni V.M.C., Mandolino G., Fornasier F., Squartini A., Nardi S., Concheri G. (2017) Root morphological and molecular responses induced by microalgae extracts in sugar beet (*Beta vulgaris* L.). **J. Appl. Phycol.** 1-11. DOI:10.1007/s10811-017-1283-3

1. Introduction

In the last years, the expanding population coupled with increased consumption of dairy-based foods and meat, have represented a considerable pressure on Earth's resources.
In addition, the global climate change has heavily reduced harvests in many areas of the world.
All these reasons have led orientation of research activity in the matter of agricultural systems to intensify yield, without considering the quality of the products and the rational use of resources.
Increase in crop production has been made possible through the use of commercial man-made fertilizers. The sharp increase in the use of chemical fertilizers caused environmental problems, such as deterioration of soil quality, surface water and groundwater, as well as air pollution, reduced biodiversity and suppressed ecosystem function (Socolow, 1999).
Worldwide, the application of sustainable agricultural practices is an important target to reach as soon as possible.
Moreover, cultivation management pays more attention to the reduction of production costs by lowering inputs. In fact, it is not always true that high nutrient availability corresponds to higher quality of the products. On the contrary, excessive fertilization, and especially high nitrogen supply, stimulates vegetative growth with a higher susceptibility to pathogens (Liebman and Davis, 2000).
An optimal solution was represented by biostimulant, defined as a wide spectrum of compounds able of enhancing plant growth and development if applied in small quantities

to the soil or on the foliar surface directly (Ertani et al., 2009).
Paradikovic et al. (2011) stated that the application of biostimulants could be considered as a good production strategy for obtaining high yields of nutritionally valuable vegetables with lower impact on the environment.
In the following years, the word biostimulant is increasingly used by the scientific literature, expanding the range of substances and of modes of actions (Calvo et al., 2014; Halpern et al., 2015).
Biostimulants belong to four major groups: humic substances, aminoacid containing products, microbial inoculants (mycorrhizal fungi and rhizobacteria) and seaweed extracts (du Jardin, 2015).
Many researchers have stated that amounts of auxins and gibberellins capable to affect planth physiology are contained in small quantities of humic substances and seaweed extracts (Nardi et al., 2002, 2007; Canellas et al, 2002; Zhang and Ervin, 2004; Quaggiotti et al., 2004).
Many years before, a study investigating the effects of algal extracts on seed germination demonstrated a faster germination and growth of rice seeds (Skukla and Gutpa, 1967). Similar results were obtained using seaweed extracts on tomato and wheat seeds (Kumar and Sahoo, 2011; Kumari et al., 2011; Hernández-Herrera et al., 2013).
Moreover, it was shown that the application of seaweed extracts improved germination, root development, leaf quality, general plant vigor as well as resistant to pathogens (Khan et al., 2009).
A possible interesting alternative could be represented by microalgal extracts. In recent years interest in the use of

microalgae has increased due to their versatility and potential application in many different sectors.
According to the literature, they can be utilized as animal feed, food, fuel and fertilizers (Metting 1990). Since microalgal biodiversity is very high, they represent a resource worth exploring (Norton et al. 1996).
As well as seaweed, microalgal cells containing bioactive substances may influence plant growth. In literature is reported that these microorganisms contain plant growth promoting substances such as auxins, cytokinins, betaines, aminoacids, vitamins and antifungal compounds (Spolaore et al. 2006).
Since the identification and chemical synthesis of biologically active compounds are difficult and expensive processes, the realization of extracts from microalgal biomass is the most easy and cheap solution to obtain them.
The application of microalgae extract enhances plant development, increases crop production and preserves plants from abiotic and biotic stresses. These aspects are particularly crucial given that environmental stresses can limit crop production up to 70% (Boyer 1982).
Garcia-Gonzalez and Sommerfeld (2016) recently observed a faster germination on tomato seeds treated with living cells of *Acutodesmus dimorphus* and their extracts; noteworthy aqueous extracts induced a positive effect on plant growth.
Higher biomass accumulation and enhanced absorption of nutrients have been observed in lettuce (Faheed and Abd El-Fattah, 2008).
Many studies have already been done on morphology, composition and production of *C. vulgaris*. Its composition made it usable in different fields. Due to the high protein

concentration, carbohydrates (15-55% dry weight) and lipids (5-40% dry weight), it is utilized in animal feed, human nutrition, biofuels, cosmetics and as bio-fertilizer. Given this wide variety of uses, its annual production has reached 2000 t (dry weight).

Shaaban (2001) evaluated the effect of different concentrations of water extract of *C. vulgaris* (25, 50, 75 and 100%) on the yield of wheat (*Triticum aestivum* L. cv. Giza 69) in a greenhouse. It was found that dry mass of the shoot treated previously with 50% algal extract showed a 81.4% weight increase when compared with the control. The increase can be a reflection of the increased nutrient uptake. This concentration of microalgal extract also led to a more than 40% increase in weight of 100 grains when compared with the control.

Fewer studies have been conducted on *Scenedesmus quadricauda*, a colonial green microalga belonging to the Chlorophyceae class. Like *C. vulgaris*, *Scenedesmus* cultures are easy to cultivate and have a rapid growth rate (Huang et al. 1994). In optimal growth conditions *S. quadricauda* can reach 9-16% of lipids (dry weight), mainly composed of large amounts of long-chain hydrocarbons (Rocha et al. 2015). *S. quadricauda* has been applied for wastewater management such as biosorption of heavy metals and the production of biofuels due to its triacylglycerols accumulation under stress (Devi et al. 2012), but it has never been tested as a biofertilizer.

Purpose of work

Sugar beet (*Beta vulgaris* L.) represents the main crop for sugar production in Europe and it is often affected by many abiotic stresses. Low temperatures, salinity, heat and water

deficiency are some of the major constraints (Biancardi et al. 2010).
Water and nutrient stresses are particularly severe in the Mediterranean area, where sugar yield losses of 1 t ha^{-1} are estimated (Jones et al., 2003). The biostimulant application could be very useful to help farmers to overcome these abiotic stresses.
On the other hand, maize is considered a reference plant because of its wordlwide economic relevance and because of its importance as one of the best model plants to combine physiological and agronomic studies (Hirel et al., 2001).
Therefore the object of this study was to investigate, after characterization of the extracts, the potential agricultural applications of *Chlorella vulgaris* and *Scenedesmus quadricauda* extracts both in *Beta vulgaris* L., as high sensitive to biotic and abiotic stresses cultivation, and *Zea mays* L., as best model plant. For the agronomic importance of the two species, analysis were focused in sugar beet and maize around the root apparatus and leaves, respectively. In *Beta vulgaris* were evaluated their effect on seed germination, root morpho-physiological changes and molecular response, aimed at studying their biostimulant effect on seeds and roots. Some physiological parameters of leaves such as dry weight and SPAD as well as the enzymatic activities amylase and invertase in *Zea mays* were evaluated.

2. Materials and methods

2.1 Preparation of microalgal extracts

Tests were conducted using two species of microalgae, *Chlorella vulgaris* and *Scenedesmus quadricauda*, both supplied by Swansea University (Wales, UK) and cultivated

for 30 days in a growth chamber using standard BG11 algae culture medium (Stanier et al. 1971), bubbled with air and illuminated by a 3500-lx, average photon flux (PPF) 100 μmol m^{-2} s^{-1} light source (PHILIPS SON-T AGRO 400) with a 12 h photoperiod.
The biomass of each species (referred to as CV and SQ for *C. vulgaris* and *S. quadricauda* respectively) was harvested by centrifugation and freeze-dried.
After lyophilization, the biomass was washed with distilled water (up conductivity < 200 μS cm^{-1}).
The final pellets obtained were added to methanol to lyse the cell wall and obtain the intracellular extracts.
After centrifugation and evaporation of organic solvent, the extract was collected with distilled water (microalgae extracts stock solution, referred to as CVextr and SQextr from *C. vulgaris* and *S. quadricauda* respectively). The extracts were also lyophilized for subsequent chemical analyses.

2.2 Chemical, spectroscopic and biochemical characterization of microalgal extracts

The chemical and spectroscopic characterization involved both lyophilized microalgae biomasses (CV and SQ) and the obtained extracts (CVextr and SQextr).
Total carbon and nitrogen content was determined on samples using combustion analysis (Elementar vario MACRO CNS, Elementar Analysensysteme GmbH, Hanau, Germany).
The FT-IR spectra were performed with a Perkin-Elmer FT-IR 2000 spectrometer (Perkin Elmer Italia, S.p.A., Milan, Italy), equipped with an IR source, KBr beam splitter and DTGS KBr detector. For each sample, 64 scans were

recorded with a 4 cm^{-1} resolution over a 4,000 to 400 cm^{-1} range using pellets obtained by pressing a mixture of 1 mg organic freeze-dried sample and 400 mg dried KBr.

Solid-state ^{13}C MAS NMR spectra were recorded, fully proton-decoupled, on a BrukerAvance II 400 MHz instrument (9.4 T) (Bruker Corp., The Woodlands, TX, USA) operating at 100.63 MHz. Rotors with a 7 mm diameter were filled with about 50 mg of the freeze-dried sample; spinning rate was 8,000 Hz s^{-1}. The experimental parameters adopted were: spectral width 20,000 Hz, data points 2 K, 100,000 scans, 5 μs, 90 degrees of excitation pulse and 4 s of relaxation delay. The HPDEC pulse sequence was used with a decoupling power of 300 W (9H). The FID were zero-filled and processed with 5 Hz line broadening.

Lastly, in order to exclude any residual enzymatic activity in the microalgae extract eleven hydrolytic enzymes involved in the principal nutrient cycles were determined in CVextr and SQextr.

These were: (i) C-cycle: α (α-gluc) and β-glucosidase (β-gluc), glucoronidase (glu), xylosidase (xyl), nonanoate esterase (nona); (ii) N-cycle: leucine-aminopeptidase (leu), (iii) P-cycle: acid (acP) and alkaline phosphomonoesterases (alkP), phosphodiesterase (diP), pyrophosphate-phosphodiesterase (piroP); (iv) S-cycle: arylsulphatase (aryS).

All potential enzymatic activities were measured in duplicate from all the samples. An aliquot of each algal extract was dispensed into 384-well white microplates with the appropriate buffer to fluorometrically quantify the enzymatic activities using fluorogenic, 4-methyl-umbelliferyl- (MUF) and 4-amido-7-methyl-coumarine

(AMC) substrates. All measurements were done in duplicate and the activities were expressed as nanomoles of MUF (or AMC) h^{-1} mL^{-1}.

2.3 Bioassay for germination test in sugar beet

The sugar beet hybrid "Shannon" used in this study was provided by "Department of Agronomy, Food, Natural resources, Animals and Environment (DAFNAE) of University of Padova (Italy).

Germination was observed daily over a period of 7 days. Four replicates of 100 seeds for each treatments were tested, according to methods of the Association of Official Seed Analysts (AOSA, 2005).

The study was conducted in a completely randomized block design.

Before treatments, seeds were sterilized by soaking in 76% ethanol for 5 min and rinsed with sterilized water.

After, they were placed on testing solutions-moistened filter paper.

Six concentrations were tested for each microalgal extract (CV and SQ): 0 mg Corg/L (Untreated), 0,1 mg Corg/L (C1), 1 mg Corg/L (C2), 2 mg Corg/L (C3), 5 mg Corg/L (C4) and 10 mg Corg/L (C5).

Seeds were incubated in a growth chamber in the dark at 25 °C. Germination was considered if at least 2 mm of the radicle had emerged.

Germination indices were calculated as follows:

- $\boldsymbol{GP(Global\ Percentage)} = \left(\frac{\text{Number of germinated seeds}}{\text{total number of seeds}}\right) \times 100$

- $\boldsymbol{FGP(Final\ Germination\ Percentage)} = \left(\frac{\text{Number of germinated seeds at the last day of measurement}}{\text{total number of seeds}}\right) \times 100$

- $\boldsymbol{GI(Germination\ index)} = \sum \frac{\text{Number of seeds germinated on day}}{\text{Number of days}}$

- $\boldsymbol{SE(Speed\ of\ emergence)} = \left(\frac{\text{Number of germinated seeds at the starting day of germination}}{\text{Number of germinated seeds at the final days day of measurement}}\right) \times 100$

- $\boldsymbol{GE(Germination\ energy)} = \left(\frac{\text{Percentage of germinated seeds at the starting day of germination}}{\text{Total number of seeds sets for bioassay}}\right) \times 100$

- $\boldsymbol{CGR(Coefficient\ of\ the\ rate\ of\ germination)} = \left[\frac{(N_1+N_2+N_3+\cdots+N_n)}{(N_1\times T_1)+(N_2\times T_2)+(N_3\times T_3)+\cdots+(N_n\times T_n)}\right] \times 100$

- $\boldsymbol{SVI(Seedling\ vigor\ index)} = \left(\frac{\text{seedling lenght (mm)} \times \text{Germination percent}}{100}\right)$

Gp and FGP were global methods, while GI was determinate as described by the AOSA (1983). SVI was estimated according with Islam et al, (2009), instead SE and GE was modified from Islam et al., (2009) and Ruan et al., (2002), respectively. Finally, CRG was calculate according to Bewley and Black (1985) and Chiapusio et al., (1997).

2.4 Bioassay for growth test in sugar beet

Pre-soaking seeds were grown in a Hoagland solution (Hoagland and Arnon 1950) and kept in a climatic chamber at 25 °C. Hoagland solution had the following composition:

40 μM KH_2PO_4, 200 μM $Ca(NO_3)_2 \cdot 4H_2O$, 200 μM KNO_3, 200 μM $MgSO_4 \cdot 7H_2O$, 10 μM FeNaEDTA$\cdot$ $3H_2O$, 4.6 μM H_3BO_3, 0.036 μM $CuCl_2 \cdot 2H_2O$, 0.9 μM $MnCl_2 \cdot 4H_2O$, 0.09 μM $ZnCl_2$, 0.01 μM NaMoO $\cdot$ $2H_2O$, 200 μM $MgCl_2 \cdot 6H_2O$, 200 μM $CaCl_2 \cdot 2H_2O$, 200 μM KCl.
After 5 days of growth, 2mL/L (referred to as concentration C2, corresponding to 1 mg Corg/L) and 4mL/L (referred to as concentration C3, corresponding to 2 mg Corg/L) of the two microalgal extracts stock solutions were added to the Hoagland's solution. These concentration were chosen because they exerted the higest biostimulant effect in gernmination test. Roots were sampled 36 h after treatment.
Samples of Hoagland solution were collected before and after adding microalgal extracts and analyzed using inductively-coupled plasma (ICP-OES, Spectro, Kleve, Germany). Treatments were arranged in a complete randomized block design and replicated three times. The experiment was repeated twice. At the end of the experiment, fresh roots were harvested and analyzed immediately, while part of the plant material was stored at –80 °C for further molecular analyses.
Root morphological parameters (total root length, root surface area and total number of root tips) were determined by computerized scanning (STD 1600, Regent Instruments, Quebec, Canada) and analyzed using WinRHIZO software (Regent Instruments).

2.5 Gene expression analysis involved in growth of sugar beet

To extract total RNA were used 100 mg of root tissues applying a EuroGold TriFast TM Kit (Euro Clone, Italy) following the manufacturer's recommendations. RNA

quantification was done with a Qubit RNA HS Assay Kit on a Qubit Fluorometer (Thermo, USA).
FastGene 55-Scriptase (Nippon Genetics, Japan) was used to reverse transcribe 1 µg of total RNA. The expression level of 53 sugar beet genes, coming from a previous experiment of RNA-seq and present in multiple biological pathways, such as lipid metabolism, stress response, protein folding and signal transduction, as illustrated schematically in **Figure 1**, were tested by means of a QuantStudio 12K Flex Real-Time PCR System (Life Technologies, USA). The reaction mix, prepared for gene expression analysis, was composed of 2.5 µL of 2× TaqMan Open Array master mix (Life Technologies, USA) and 2.5 µL of cDNA. The comparative cycle-threshold method was used for quantification of gene expression with normalization to reference genes (*Tubulin; GAPDH; Histone H3*) using the $2^{-\Delta Ct}$ formulas. The sequences of primers and TaqMan probes designed for the Real-time PCR experiments are reported in **Table 1**. The relative quantitation of gene expression between the samples was calculated using the comparative threshold (CT) method (Heid et al., 1996). The comparative expression level of each single gene was given by the formula $2^{-\Delta\Delta CT}$ where ΔΔCT was calculated by subtracting the baseline's ΔCT from the sample's ΔCT and where the baseline represents the expression level of the control treatment. Data were subjected to ANOVA using Statistica 8.0 (StatSoft, Inc. Tulsa, OK, US). Differences between groups were considered significant at $P < 0.01$.

2.6 Bioassay for growth test for maize

The present research was investigated using *Zea mays* plants. After soaking in distilled water for a night, maize

seeds were surface-sterilized using sodium hypochlorite 5% (v/v) for 10 minutes and germinated on distilled water-wetted filter paper. Germination was performed in the dark at 25°C.

Clusters of 10 seedlings were grown hydroponically in a 3 L beaker containing an aerated Hoagland solution (Hoagland and Harnon, 1950) with the following composition: 40 μM KH_2PO_4, 200 μM $Ca(NO_3)_2 \cdot 4H_2O$, 200 μM KNO_3, 200 μM $MgSO_4 \cdot 7H_2O$, 10 μM FeNaEDTA· $3H_2O$, 4.6 μM H_3BO_3, 0.036 μM $CuCl_2 \cdot 2H_2O$, 0.9 μM $MnCl_2 \cdot 4H_2O$, 0.09 μM $ZnCl_2$, 0.01 μM NaMoO · $2H_2O$, 200 μM $MgCl_2 \cdot 6H_2O$, 200 μM $CaCl_2 \cdot 2H_2O$, 200 μM KCl.

Nutrient solution was renewed every other day. Plants were kept in a growth chamber with 16 h of light at 25°C and 60% relative humidity and 8 h of dark at 18°C and 80% relative humidity. Three days after transplant, maize plants were treated adding inside beakers C. vulgaris and S. quadricauda extracts at different concentrations: 0 mg C/L (untreated), 1 mg C/L (C2) and 2 mg C/L (C3). Two days later, plants were randomly collected from three pots per treatment. A part of the plant material was frozen with liquid nitrogen and kept at −80 °C for physiological analyses.

Figure 1: Categories of main functions (%) of genes analysed by Real-time PCR.

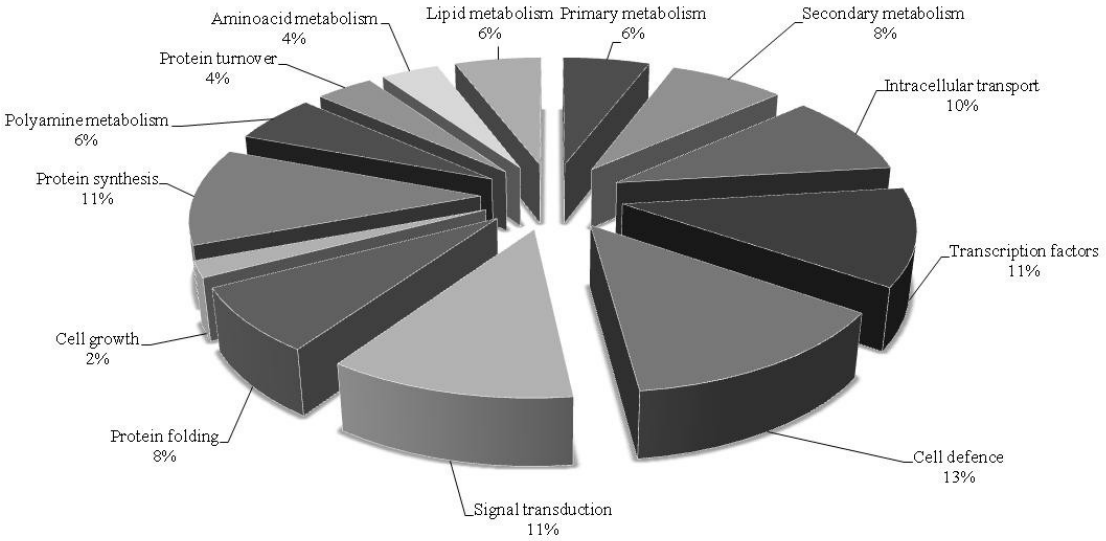


Table 1. Sequences of primers and TaqMan probes designed for the Real-time PCR experiments.

Gene name	Assay ID	Forward Primer Seq.	Reverse Primer Seq.	Reporter 1 Seq.	Gene function
Bv_05920_sapz	AI0IYVP	GGATGAGGAGTATGAAGCAGTTGTG	ACAGCTGTAGTAACATGAGTATCTTTGT	CCAGCCCAAAGTCTCC	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1
Bv9_210120_xaea	AI0IYVQ	CCACCCACTCCTTCATTTCACATAG	GATGAAGGGCTATTGCAAACAGTTT	AAGCGGAGAATTTG	Putative pentatricopeptide repeat-containing protein At1g19290
Bv3_065290_srwc	AI1RW1X	AGCATATGGTTCAGTAAAATGGTGGAA	TGGAATGAAAGAAGTGGTACTGCAA	AAGTGCAACAAATCTG	Auxin efflux carrier component 1
Bv9_216450_jzsj	AI1RW1Y	ACTCTATGGTGGGTCTAGCTCTTTT	GGATCAGCACCAACAATCAATGC	CCTGCCCCATCTCCA	Chalcone synthase
Bv5_119750_ywjf	AI20U75	GGAGCTTCAGAGGATCAGATCAAAA	TCGCTTCCTCATTTCCTTGATTCTT	CTTCAACGCAAGCTTC	DnaJ homolog subfamily B member 11
Bv5_104850_fzej	AI20U76	AAAGCTGCTGTCAAGAAGATGGA	GTGATAAACACGGGTCAAAACCTTT	CTCAGCAAGAAATTCT	Serine/threonine protein kinase
Bv2_038430_dqyh	AI39TED	GAGAAAGAGGAGTAACATCAAGAAGCA	CAGCTATCTTGAGTGGTGAAGGAAA	AAGGTGCAGGATCTTC	Heat shock cognate 70 kDa protein 2
Bv2u_047030_nuxd	AI39TEE	AGCTCTTTCATTGGAATCTTCAACT	GCCAAAAGAAGGAGATGGTGCTT	AATCCATGCCAATCTC	Leucine-rich repeat (LRR) protein
Bv3_052080_ijtm	AI5IRKL	CCTTAACAGCACCCTCGATCTT	AGAACCAAGTGAATGACAAGGACAA	CTGGCTGACAAGCTAG	Luminal-binding protein
Bv6_152730_nyjy	AI5IRKM	CAGACCAAAGAGAAAGATTGACATTTTCA	TCTGTCTCCAGTATAGTTCTCCTACTCT	CCAGTGACAGAATTAG	Vesicle transport v-SNARE 12
Bv8_185010_swpc	AI6RPQT	AAAGCGGATTAGCCTGAAGGT	GCATTACCCTCTCCTCGAACTCT	CTGCCAGCATCAACAC	Chaperone protein dnaJ
Bv8_196710_fwxw	AI6RPQU	GAGCCTGTTATACCTGGGCTTAATAAT	TCACCACCACCAACAACAACA	CCGCCACCGAAGCAG	Polyadenylation factor I complex, subunit FIP1, Pre-mRNA 3'-end-processing factor FIP1
Bv_34780_rqdw	AI70NW1	CAAAGGTGGTAAAGACGATTCTAGTAGT	TTTTGGTCGAACGTCTTCAATGC	CACCCCACTCGACTACG	S-adenosylmethionine decarboxylase proenzyme Short=AdoMetDC;
Bv9_225110_xpzj	AI70NW2	ACCTTGTAGAGTCTCCCCAAATAGG	TCAGACGATCTCGTTTATTGTTTGGAT	ATGACCGCACATTGTC	Apoptotic ATPase,Putative disease resistance RPP13-like protein 1
Bv9_228150_kecu	AI89L29	GAAGTTAAGGATTGCCGAGTTTCC	CACCTTCAACACACCAGAAATGG	AACGCCGCCACGTCGT	Dehydration-responsive element-binding protein 3
Bv_30700_jwmm	AI89L3A	TCACGTTGTTACAGAATGCATGGA	CTCAGGTAAACCGAGACTGCAA	TCAGCTTGCAATTTGT	MLO-like protein 11
Bv3_055540_hfjn	AIAA082	GCTTCACTCCTCCCATCTTTTCTAT	CAGACGTGGTCGTGATAGGAT	ACCCCTGTCATTATCC	Methyltransferase-like protein 1
Bv1u_019170_oqom	AIAA083	GGGCAAAAGCTCATCAAATTCATCA	AACAACAACAAAGCCAGCATCAA	CCGCCTGACATATAAT	Endoplasmic reticulum glucose-regulated protein (GRP94/endoplasmin), HSP90 family
Bv8_184830_opag	AIBJZFA	ACCCAGGTTAATGAACATGGAACAT	GCCAACTCATCACTGCCTTTG	CTGCCGCATACTCTTC	Auxin response factor 3
Bv3_067750_fztq	AIBJZFB	TGGTACTGATCCAAAAGATGCAGAAT	CCTGCATCTGACAGCTCTACTG	CAGGGTACATATTCCC	Heat stress transcription factor A-5
Bv3_064750_fpmx	AICSXLI	GATGGCAGCAGAACCAAAGTTT	TGAACTCTTGAGAAGTCTTTCAATAAGTTTTCT	CATCAACAGCTAAAACATG	Two-component response regulator ARR9
Bv8_201480_rddu	AICSXLJ	CGCTCTAAGAAAAGCTCGAAACTTG	TCTACAAGGTTTATCATAGGAGGGCTAA	CATCCCCTGCATCATC	Predicted E3 ubiquitin ligase
Bv1_001950_imjw	AID1VRQ	AAGGTGATCCAATTCCAGGAACTG	TCGAGGCTATGCCTTCAGGATA	CAGACACAGAGAATTC	dsRNA-specific nuclease Dicer and related ribonucleases
Bv6_145280_vezp	AID1VRR	CCTGCCATTGTGGAATATGTACTCA	CTCACACCAGAAAAGGAAAATGCT	TCAAGCTGCTAATTCC	Transcriptional coactivator p100
Bv9_209120_pzcq	AIFATXY	AGCAGTAGTATGAGGTATGATCAGAGTTT	GTCTCCAGACATGGCTTTGGT	CCCACCAAAAGCACC	BEL1-like homeodomain protein 9
Bv7_157840_przp	AIFATXZ	GTTTATTCATTTGTAATTTACTCTTTTACCCTAAACACT	GTGGGAAATTCATGTCCTGTTACAC	CACATCGTGCAATTAT	Mitochondrial import inner membrane translocase
Bv1_004580_xnrs	AIGJR36	TGCCTTTGTTTCCGGTCTCTTA	AGGTTGGGATATCATCAGGAGCTAA	AAGGGCAGCAACTGTG	Cysteine synthase, chloroplastic/chromoplastic, O-acetylserine sulfhydrylase;
Bv3_054270_kkpk	AIGJR37	GTATCACATAGTGCTCTAAAGTTGGGTAA	AGTAAAATATGCCCAACTATCATCAAGCTTT	TTGCTCACAAATTTTC	Calmodulin-binding transcription activator 5
Bv2_035240_udsn	AIHSQAE	CCCTTCCATTGGTCTAACAGAGAA	TGAAGCAAGAAGAGGGTACAATCC	CACCGACAAATTCA	Heat shock transcription factor
Bv9_209300_pjcz	AIHSQAF	GTAAAATGAAAGGGATTGCATTCATAAAGG	CAGCAGCAACATTTCCAATTGTCTT	CTGATGGCTACTGGATTGA	Glyoxalase
Bv3_051290_uonp	AIIt1GM	GGAAGAAAAGAAGGAAGAGGAACCA	ACATCCAACACAATGCAAGTCTACA	CCGCAACCGCCACCAC	Copper chaperone
Bv7_157460_rcod	AIIt1GN	GGTGTTTTGACTCGAAGACTTGAC	GCTGTTTGGAGAATGACCAATACCA	CTCGATCACAGATTCC	2-Cys peroxiredoxin BAS1, chloroplastic
Bv6_126170_qykn	AIKAMMU	CACAAGAGGCAGGGAACGT	GAAAGCTCTTCCACCTCTCTAACAA	CACGTCCTGTGGATTC	CDK5 activator-binding protein
Bv3_063630_mpup	AIKAMMV	GAACCTCAATTTAGCCTAGCCGTTA	TGATTTTGTTTTCTATCCTTTGGACTTCAC	CCAGTAGGGCAAACAA	K00384 thioredoxin reductase (NADPH)
Bv6_137080_rjqn	AILJKS2	AGGCTAAATATGATGAATACCAAATTG	GCTGAGGGAATCCATTGAAGCA	CAGGACGGCCATTCCA	4-alpha-glucanotransferase, chloroplastic/amyloplastic
Bv3_049600_wxxy	AILJKS3	TGGCGGAAAGTGGGTAGTC	AACCCTTTGCTTCTTTCGATTTTGG	CCAATCCCAAATCCG	high affinity inorganic phosphate transporter
Bv7_156890_eowm	AIMSIZA	TCCTGATTTTAAGTTAGCAGTGAACCAT	GTGAAAGCGCGAGACTCTTCT	CAGGACGGCTCTTCCA	3-ketoacyl-CoA synthase 17
Bv5_119310_kswa	AIMSIZB	CCTCCTAGTTTCTCCTCAGCTATCA	CATAAGTGTCAGCCCATAGACCTTT	TCGGCCCAACTTGTC	Inorganic phosphate transporter 1-7
Bv7_176160_uqiw	AIN1GSI	CATCAGAGTTATTTGCAGACTATGTCTCT	GGCGAGGAGTAATACCATTTGTCT	ATGGCCTAACAAGTTCC	Glycogen phosphorylase
Bv5_120330_zdrp	AIN1GSJ	CTGCATATTCTTCAGCATAACCCATTTT	CAGTTCATGTAAATGACATAGAATGGGATCA	CAGTGCTTCCAACTTC	Stachyose synthase
Bv5u_123730_dagi	AIPAFBQ	CACAATAACTCTGCCCAAATAATCTTT	CACCCATCCAGGTCTACTCAGA	CTTCCGCGACAAATT	Beta-Amylase 3702.AT4G17090.1
Bv8_183530_vxcq	AIPAFBR	CACCCCGACAATGTTAAGGATGAT	CTATGTTTTCAAGTGGACTACCCTTCT	CCACCCACCACCCTC	Cellulose synthase A catalytic subunit 8 [UDP-forming]
Bv3_062700_tcwq	AIQJDHY	AGCTTCCGTATGTTCATGTTCGT	CGTTCAGCTAAGCTATGGCTAT	CAGTCGCTTGACCTCGTC	Transcription factor bHLH48 AltName:
Bv8_199730_kxjp	AIRSBN6	GAGTTGCTTAGCAAGTTCAATGGAA	GTGAGCTACAACTTAGGAGGAATGG	ACCCCGGCACTACATC	3-ketoacyl-CoA synthase 11
Bv8_203060_pggz	AIS09UE	GGCATTTGCAAAAGAGTCTATGGAA	CAATCTTTAACTGTGCATTTCGAACCA	CTGTCCCACATGACCC	Acyl-CoA synthetase
Bv7u_180460_dcmt	AIT970M	GCCTTTCTGCTTCTTCAACTGTAC	TTACAACAGGGAGACTGATAGGAGTAG	CACAAACCCAAAACCC	28 kDa ribonucleoprotein, chloroplastic Short=28RNP;
Bv_24910_jato	AIVI56U	GCAGCAAACAAACGGTTGGAAA	TCTCAAAAACATCACAGGAAACAGGAA	ATCCACAGGAAACACC	Aspartic proteinase-like protein 1 EC=3.4.23.-; Flags: Precursor;
Bv3_057000_nenr	AIWR4C1	GGGATGAAGAGAGAGTGAACAATGAA	TCATGCGTCTTGCACATTTGTTTT	ACATCCTTCAATCCCC	Glutamate/leucine/phenylalanine/valine dehydrogenases
Bv_36700_auze	AIWR4C2	AGGGCTTTGGCATTACTTCATCAAT	TGAGTTGATTTTGAAGGAGGTAGTGTTC	CTGGGACAAGGTTTTC	Inositolphosphorylceramide-B C-26 hydroxylase
Bv4_079980_yozf	AIX02I9	CTGCAATCTCGTCGTTGGTAAC	TTCACACCGTCCTCATCTCAAC	CCAGCACGATGAAACT	S-adenosylmethionine synthetase 1, AdoMet
Bv2_044340_tafw	AIX02JA	ACTTGTCAATACTACACAATTTTATAATTTCATCAATAACATTTT	GGCGGAATTTGTGGGATGTC	TCATCCACAGAAAATG	Sulfotransferase
Bv2_025660_etua	AIY90PH	GTTGGCAAGACAAAGGAGACATTG	AACCAATCATGTAAAGGTCCACAGAA	TCGAAGCAGCAAACAT	Laccase
Bv6_137840_uaap	AIY90PI	CCTCACAAATTTGCCCTCTCCTA	CAACCCGTTTCCTTGATGGATAAC	ACCCGTCGAGTCTCT	Flavonol sulfotransferase-like
BV2_037220_rayf	AIQJDHZ	CTATGCATCTTCACTTTGAAACAGTTTT	CTAGAAGAGGCTGACGAGAAAGAAG	ATGGTGCTACGAGCTTC	Tubulin (Housekeeping)
BV5_107870_ygnn	AIRSBN7	GAGCAAGACAATTGGTGGTACAG	CACGAATACAAGTCAGACCTTCACA	CTGGCATTGGAAACAA	GAPDH (Housekeeping)
BV6_127000_pera	AIS09UF	GTTCCTGGCCGGAATCTATGG	GCCAAGAAAGCAATTAGCAACAAAA	CCGGAGCTGATTTACG	Histone H3 (Housekeeping)

2.7 Physiological parameters and enzymatic activities in maize plants

For dry weight measurement, plants were divided into roots and leaves, and weighed separately. The samples were placed in a drying oven for 2 d at 70 °C and allowed to cool for 2 h inside a closed bell jar, then the dry weight was measured per plant.

SPAD index is influenced by the absorption of light due to the presence of chlorophylls; therefore, it can be used for an evaluation of the chlorophyll content itself. Leaves are crossed by two light beams, with a wavelength of 650 nm (absorption peak of chlorophyll) and 940 nm (absorbance of the chlorophyll none). A photodiode collects the transmitted energy by measuring the transmissibility of the leaf lamina in these two spectral bands. The ratio of the energy transmitted in the two spectral regions is linearly proportional to the total chlorophyll content present in the leaf. The measurement of the SPAD index was performed using the SPAD-502 Leaf Chlorophyll Meter (Minolta Camera Co., Ltd., Osaka, Japan) on three points of plants leaves.

Maize leaves were frozen in liquid nitrogen, powdered by mortar and pestle, and, successively extracted with 10 mL of (w/v 1:10) 0.1 M KH_2PO_4 extraction buffer (pH 5.0 and pH 7.0 for invertase and amylase activity, respectively) and homogenate for 90 sec. Samples were filtered and centrifuged at 15,000 rpm for 20 min at 4°C, then, the surnatant was recovered and tested for invertase and amylase activity.

Amylase activity was evaluated using an aliquots (100 µL) of enzyme extract mixed to the activity buffer (0.2 M sodium acetate containing 1% starch pH 5.4), then samples

were incubated at 37°C for 1 h (Pizzeghello et al., 2002). Reaction was stopped with Sumner reagent (10 mL NaOH 8 % (w/v) 0,5 g 3,5 dinitrosalicilic acid, 15 g Na-K tartrate, 25 ml H_2O milliQ) and 8 mL H_2O. In the blank the enzyme extract was replaced with H_2O milliQ.
Invertase activity was evaluated according to Pizzeghello et al., (2002); briefly an aliquots (200-300 μL) of enzyme extract was mixed to the activity buffer (0.1 M potassium acetate containing 0.1 M sucrose pH 4.0) and incubated at 30°C for 30 min shaking. Reaction was stopped with 1 mL Sumner reagent (10 mL NaOH 8 % (w/v) 0,5 g 3,5dinitrosalicilic acid, 15 g Na-K tartrate, 25 mL H_2O milliQ) and heat bath.
Finally, the absorbance of samples was measured at 540 nm and the concentration of glucose hydrolyzed from sucrose and starch was determined from a standard calibration curve using glucose.

2.8 Statistical analysis

Data were analyzed by one-way ANOVA ($P < 0.05$) followed by the Tukey's test for multiple comparison procedures.

3. Results and discussion

3.1 Chemical, spectroscopic and biochemical characterization of microalgal extracts

Distribution of the diverse forms of carbon designed according to the areas of the different NMR spectra regions, as indicated by Baglieri et al. (2014) (aliphatic, 0 to 45 ppm; N and O alkyl, 45 to 95 ppm; aromatic, 95 to 160 ppm; carboxyl, 160 to 195 ppm) are reported in **Table 2.**

The biomass spectra for CV and SQ are very similar in shape (**Figure 2**) and distribution of the various carbon forms. Conversely, some differences are recognized between the two extracts. Indeed, the CVextr spectrum has three visible peaks 31, 128 and 182 ppm, whereas only two main peaks 31 and 128 ppm are detected in SQextr (**Figure 2**).

The latter peak occurs within the aromatic carbon region, however, it could also be produced by the characteristic double bond resonance from triacylglycerides (-C=C-), from 125 to 135 ppm, in some species of microalgae (Akhter et al. 2016). These compounds may be due to the reserve material extracted from whole cells. In fact, in microalgae, triacylglycerides are stored in the vacuoles, while the phospholipids and glycolipids are components of the cell membrane (Olofsson et al. 2012). The N and O alkyl carbon calculated from the integration of the signal in the 45 to 95 ppm region is lower in CVextr than SQextr, vice versa for the carboxyl carbon (from 160 to 195 ppm; 9.37% and 4.42% for CVextr and SQextr respectively).

Overall, distribution of various forms of carbon showed that the extracts from both species have a greater amount of alkyl and aromatic carbon than algal biomass. In contrast, extracts (CVextr and SQextr) showed a lower distribution of carbohydrates and proteins carbon (N and O alkyl carbon) compared to biomass sources (**Table 2**).

Similarly, in agreement with Akhter et al. (2016), what was observed for the carboxyl carbon may be attributable to protein molecules and/or lipids. Consequently, both extracts showed a higher degree of hydrophobicity (HB/HI) than that observed for the respective biomass from which they were extracted. The degree of hydrophobicity was determined

according to Baglieri et al. (2014) for humic substances as: HB/HI = [(0-45)+(95-160)/(45-95)+(160-195)].

Table 2. Distribution of C intensity of ^{13}C NMR of biomass of *Chlorella vulgaris* and *Scenedesmu squadricauda* (CV and SQ, respectively), and extracts (CVextr. and SQextr., respectively).

	Alkyl	N and O-alkyl	Aromatic	Carboxyl	HB/HI
	0-45*	45-95*	95-160*	160-195*	
CV	40.91	33.66	10.85	14.58	1.1
CV estr	63.39	4.75	22.5	9.37	6.1
SQ	41.75	32.01	11.28	14.97	1.1
SQ estr	55.23	16.25	24.09	4.42	3.8

***ppm**

The degree of hydrophobicity for CVextr resulted as much higher than that calculated for SQextr, CVextr thus being more apolar than SQextr (**Table 2**).

The FT-IR spectra (**Figure 3**) showed the most pronunced differences between the biomass of microalgae (CV and SQ spectra - solid line) and their extracts (CVextr and SQextr spectra - dotted line). Assignment of the bands in the FT-IR spectra was performed according to Baglieri et al., (2012) and Duygu et al. (2012). Each peak was assigned to a functional group. Protein spectra were characterized by three strong features at 1665, 1556 and 1406 cm^{-1} in CV and SQ biomass. These bands were due primarily to C=O stretching vibration in amide I (signal at 1665 cm^{-1}), N-H bending and C-N stretching vibration in amide II (signal at 1556 cm^{-1}), CH_2 and CH_3 bending of methyl for the latter signal. However, this band may also be attributed to C-O

stretching of the carboxylate groups of the carboxylic acids or to ($N(CH_3)$) bending of methyl of lipids (Sigee et al., 2002). Lipids spectra were characterized by two sets of vibrations,
C-H stretching of methane at 2965 cm^{-1}, CH_2 stretching of methylene. In CV and SQ biomass, carbohydrate absorption bands, due to C-O stretching of polysaccharides and complex sugar ring modes at 1162 cm^{-1} and at 1050 cm^{-1}, are clearly visible. Finally, in the spectra of biomass sources two other peaks are detectable. The first broad at about 3300 cm^{-1} (precisely at 3296 cm^{-1} in CV and 3303 cm^{-1} in SQ) attributed to O-H stretching of water or to N-H stretching of the amide A, and the second at 1260 cm^{-1} assigned to the >P=O stretching of phosphodiesters of nucleic acids (Figure 2).
In microalgal extracts only two strong signals attributable to the proteins at 1556 cm^{-1} - more intense in CVextr than SQextr - and 1406 cm^{-1}were observed, whereas the signal at 1665 cm^{-1} was very weak (**Figure 3**). These results are in agreement with data of ^{13}C NMR, which showed a lower protein content in the microalgae extracts compared to the respective biomass.
The bands attributed to lipids in the CVextr and SQextr spectra have similar intensities to those observed in the CV and SQ biomass, respectively. CVextr and SQextr also showed two additional bands, at 3019 cm^{-1} and 1742 cm^{-1}, also attributable to lipids. The first is assigned to C-H stretching of methane and the second one to C=O stretching of esters of fatty acids. As observed for proteins, a higher lipid content in microalgae extracts than that recorded in CV and SQ biomass was observed in ^{13}C NMR spectra (**Figure 2**).

In addition, the signal of carbohydrates at 1050 cm^{-1} (**Figure 3**) in the extracts was evident, while the signal at 1162 cm^{-1} disappeared. Also the signal (at 1260 cm^{-1}) assigned to nucleic acids was very weak, whereas the one attributed to stretching of the O-H water was moved approximately to 3200 cm^{-1} (precisely 3221 cm^{-1} in CVextr and 3194 cm^{-1} in SQextr).

Table 3. Elemental composition (%) of biomass of *Chlorella vulgaris* and *Scenedesmus quadricauda (*CV and SQ, respectively), and extracts (CVextr. and SQextr., respectively).

	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
CV estr	62.2	1.37	0.24	0.37	0.51	0.05	0.01	0.52	3.87
SQ	52.9	7.94	0.34	0.47	0.54	0.65	0.24	0.06	0.26
SQ estr	62.8	1.17	0.36	0.41	0.53	0.15	0.01	0.35	2.42

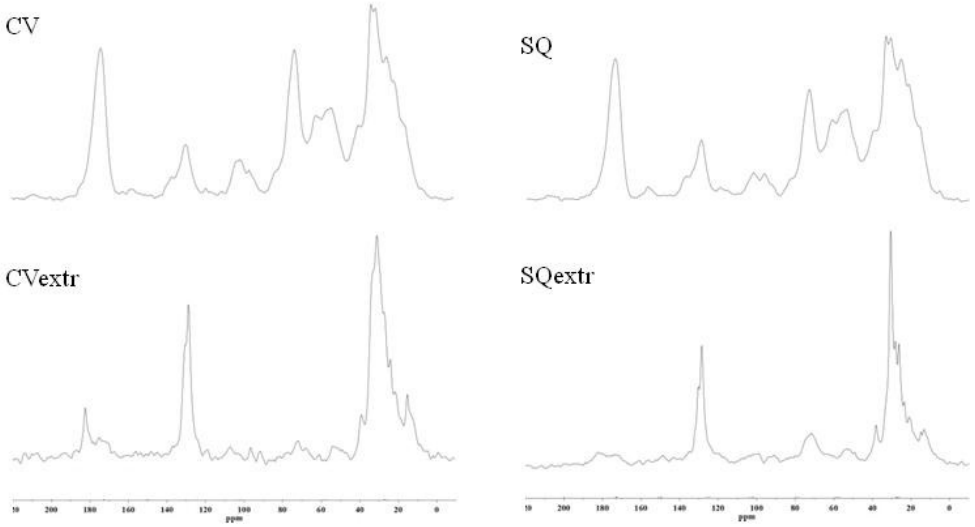


Figure 2.[13C] NMR spectra of biomass of *Chlorella vulgaris* and *Scenedesmus quadricauda* (CV and SQ respectively) and extracts (CV extr and SQ extr respectively).

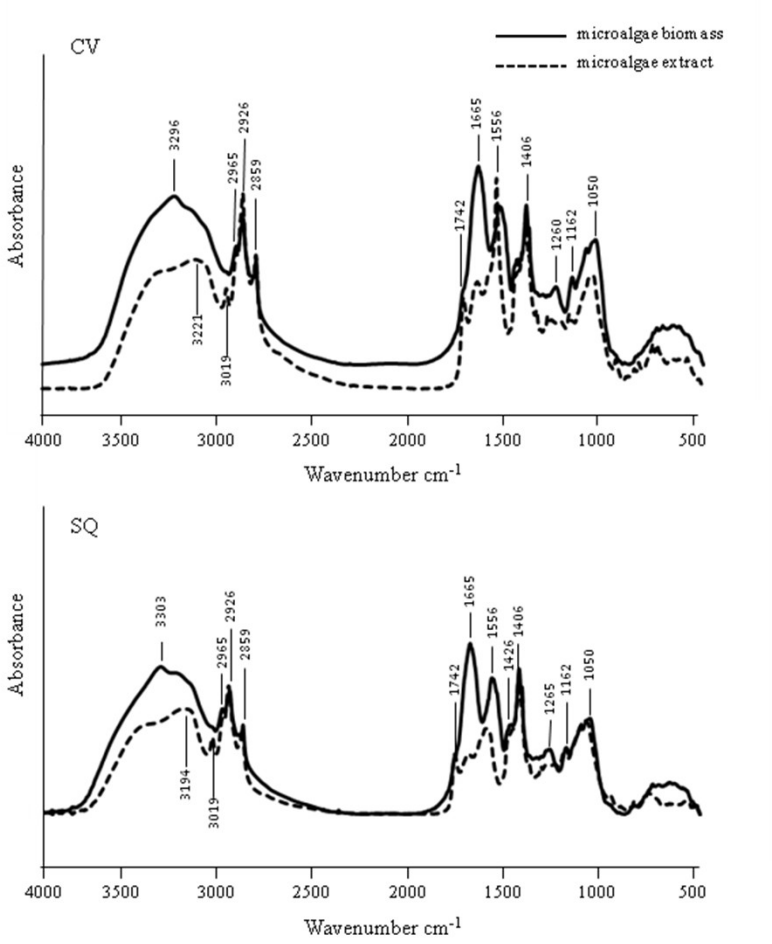


Figure 3. FT-IR spectra of biomass of *Chlorella vulgaris* and *Scenedesmus quadricauda* (in solid line) and extracts (in dotted line).

Table 3 shows the element composition of CV and SQ biomass and their respective extracts. The C and N contents were very similar in CV and SQ biomass, as well as their extracts. Our results showed that C and N content was higher in SQ (52.9% and 7.94% respectively) than that found in other species of the same genus by other authors.
In fact Di Caprio et al. (2015) found that in a strain of *Scenedesmus* sp., grown for 31 days in BG11 medium, the content of C and N was 40.5% and 5.1% respectively. Similar results (46.54% C and 4.52% N) were also obtained by Makarečienė et al., (2012) for *Scenedesmus* sp. cultivated for 30 days in the same medium. However, González-Fernández et al., (2010) found a value of total N corresponding to 11% in *S. almeriensis*, after 10 days of breeding in Mann and Myers culture medium (Mann and Myers 1968). This confirms that quite a variability in C and N content occurs in *Scenedesmus* sp.
The C and N content found in CV biomass was in accordance with the results reported by Bumbak et al., (2011), who determined values of C ranging between 51% and 72% and values of N between 6% and 8% in *Chlorella* spp. biomass, depending on the availability of N in the culture medium used (M-8). As regards the N content in this species, comparable values (between 6.2% and 7.7%) were found by Crofcheck et al., (2012). For single microalgal species, carbon content was higher in extracts than biomass (CVextr carbon>CV carbon; SQextr carbon>SQ carbon). By contrast, nitrogen content was much lower in CV and SQ biomass than CVextr and SQextr (**Table 3**). These data are in agreement with the results obtained by the ^{13}C-NMR analysis. In extracts from the starting biomass, an increase in alkyl and aromatic carbon as well as a reduction of N and

O alkyl carbon was detected in both species (**Table 2**). In fact, the percentage of carbon in unsubstituted aliphatic and aromatic compounds was higher than that registered in substituted compounds. Percentage contents of P, S and Mg were similar between extracts and biomasses, for each single species (**Table 2**), however their contents in SQ species were higher than those measured in CV. In CV, S and Mg contents were in agreement with Bumbak et al., (2011) and Crofcheck et al., (2012), while P content in this species was much lower than that reported in the literature (0.2% *vs* 1.2%) (Bumbak et al., 2011).

The Ca and Fe contents were higher in biomass than extracts for both species (Ca: 0.50% in CV and 0.05% in CV extract, 0.65% in SQ and 0.15% in SQ extract; Fe: 0.13% in CV and 0.01% in CV extract, 0.24% in SQ and 0.01% in SQ extract). Interestingly, in both extracts the content of these elements was similar, whereas in biomass it was always higher in SQ than CV (Ca: 0.50% in CV and 0.65% in SQ; Fe: 0.13% in CV and 0.24% in SQ), suggesting that in extracts these elements may be influenced by the extraction procedure (**Table 3**).

Furthermore, Fe content in CV (0.13%) was in agreement with data reported in the literature (0.04% / 0.55%) (Crofcheck et al., 2012), while Ca content was higher than that found in the same species by Crofcheck et al., (2012) (between 0.005 and 0.08%).

The K and Na contents in extracts of both species were always higher than those measured in the starting biomass. In particular Na content increased by about 9 times in CVextr and SQextr if compared to the corresponding starting biomass (**Table 3**).

Interestingly, the elements involved in osmosis and ionic balance (such as K) and pH regulation (Na) (Ferreira et al., 2004; Sanudo-Wilhemy et al., 2014) recorded the highest increase in concentration in both extracts, suggesting that the extraction procedure may in some way affect their contents. Lastly, no enzyme activity was detected in CVextr and SQextr meaning that only free amino acids could be present in both solutions.

3.2 Effect of C.vulgaris and S. quadricauda extracts on germination of sugar beet seed

In all treatments, but with noticeable differences in seeds for treatments, germination started after 3 days.

This process is the result of a large number of metabolic activities mainly consisting of increased respiration and protein synthesis ending up in embryo activation and radicle emergence. Success or failure of this process are closely related to environmental factors, that play a crucial role in determining the final amount of germinated seeds.

In **Figure 4** are reported the germination percentage (GP) of sugar beet seeds treated with the different concentration of microalgal extracts. Results showed a greater increase in GP for seed treated with CV extracts with respect to untreated seeds. Data indicated that all concentrations of CV extracts increased germination percentage (GP) respect to the untreated seeds, obtaining the best results for C3 (2 mg Corg/L) (**Figure 4**).

GP increased in seeds treated with the five SQ extracts concentrations until day 5 also, but only C2 (1 mg Corg/L) keeps its effect much longer than the other four.

In **Figures 5, 6, 7, 8** are reported the Final Germination Percentage (FGP), Germination index (GI), Speed of

emergence (SE), Germination energy (GE) and Coefficient of the rate of germination CRG of sugar beet seeds treated with the different concentration of microalgal extracts.
Increments of about 27% of FGP than the control are observed in seeds treated with C3 and C2 of *C. vulgaris* and *S. quadricauda* extracts, respectively (**Figure 5)**.
Much lower percentage increments values of FGP than the untreated seeds (about 7%) was found by Aly et al., (2008) in sugar beet seeds pre-soaked in *C. vulgaris* culture filtrates. The same authors suggested a dilution of the culture before applying as seed pre-soaking treatment. Probably, the combination of the extraction technique, the use of the extract C3 at 2 mg Corg/L concentration and the much longer time of contact of seeds with these substances (7days) contributed positively to obtaining these results.
Results for FGP, particularly for SQ extracts, are in accordance with observations from Sivasankari et al (2006) in *Vigna sinensis*. The authors found that the seeds soaked in seaweed extracts at lower concentrations determined greater FGP than control (seeds soaked in water), while extracts at higher concentrations determined less germination. CV C3 (2 mg Corg/L) was the best treatment for everyone germination indexes calculated (GE, SE, GI, CRG, SVI) (**Figure 5**, **6, 7**, **8**) and root length also **(Figure 8).**
The higher increments of GI, SVI and root length are instead observed in C2 (1 mg Corg/L) for SQextr **(Figures 6 and 8)**. Interestingly, in both treatments the differences are more evident in data obtained at 7 days after soaking whereas are rather constant at 5 days after soaking.

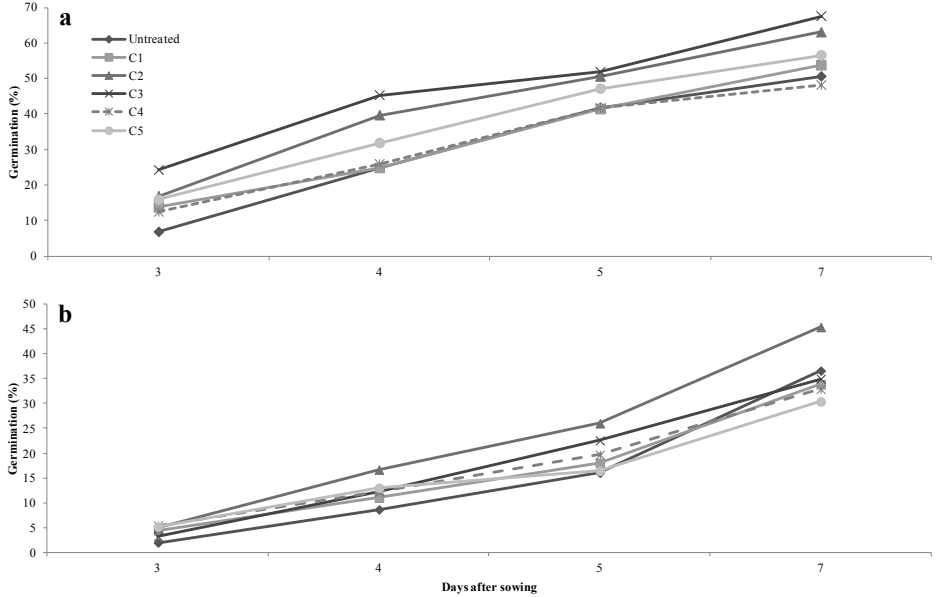


Figure 4. Germination percentage (GP) of sugar beet seeds treated with *C. vulgaris* (a) and *S. quadricauda* (b) extracts at concentrations tested.

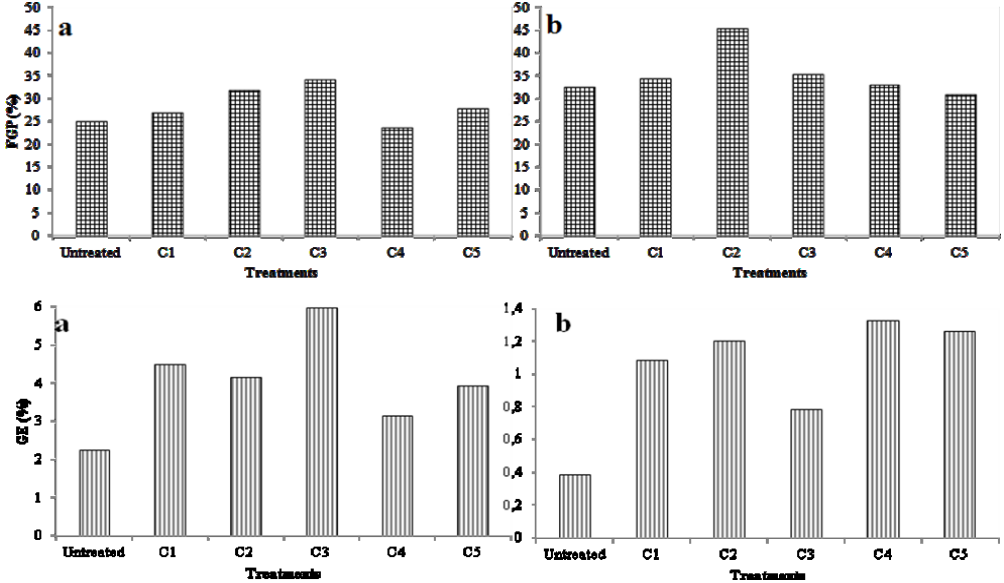


Figure 5. Final Germination Percentage (FGP) and Germination Energy (GE) of sugar beet seeds treated with *C. vulgaris* (a) and *S. quadricauda* (b) extracts at 5 concentrations tested.

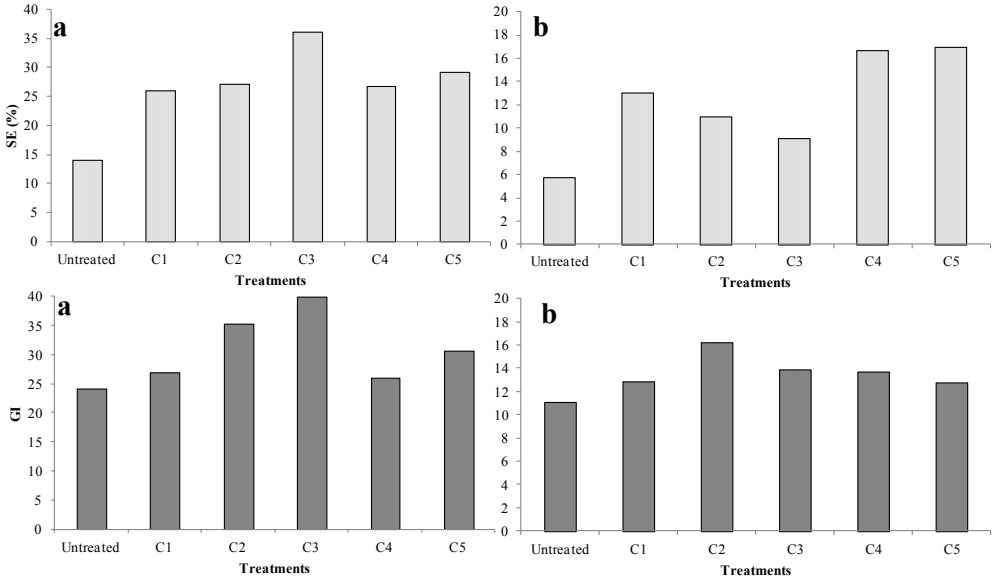


Figure 6. Speed of Emergence **(**SE) and Germination Index (GI) of sugar beet seeds treated with *C. vulgaris* (a) and *S. quadricauda* (b) extracts at 5 concentrations tested.

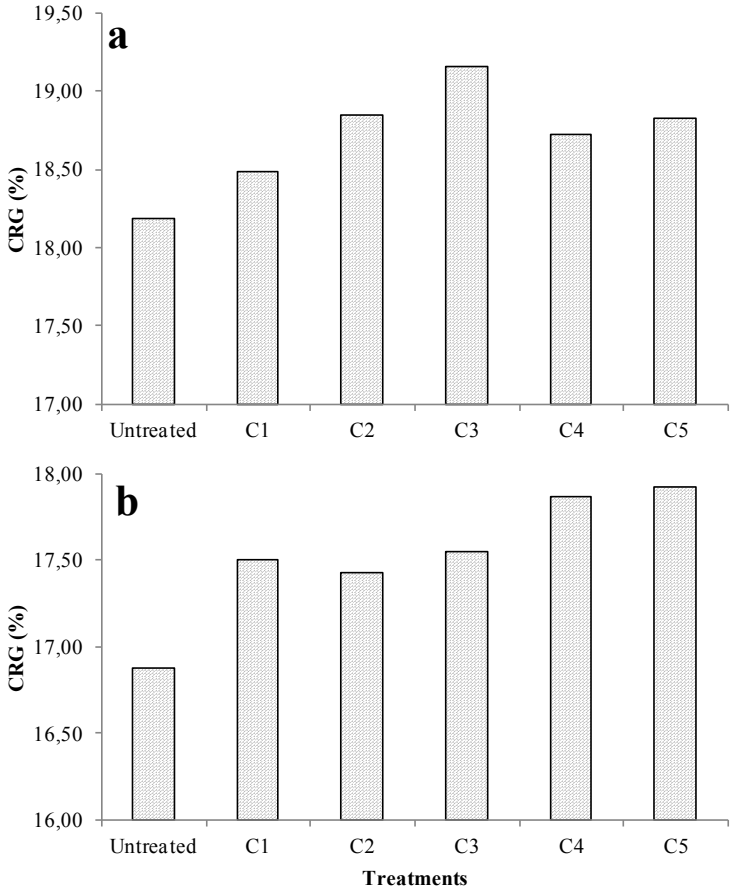


Figure 7: Coefficient of the Rate of Germination (CRG) on sugar beet seeds treated with *C. vulgaris* (a) and *S. quadricauda* (b) extracts at 5 concentration tested.

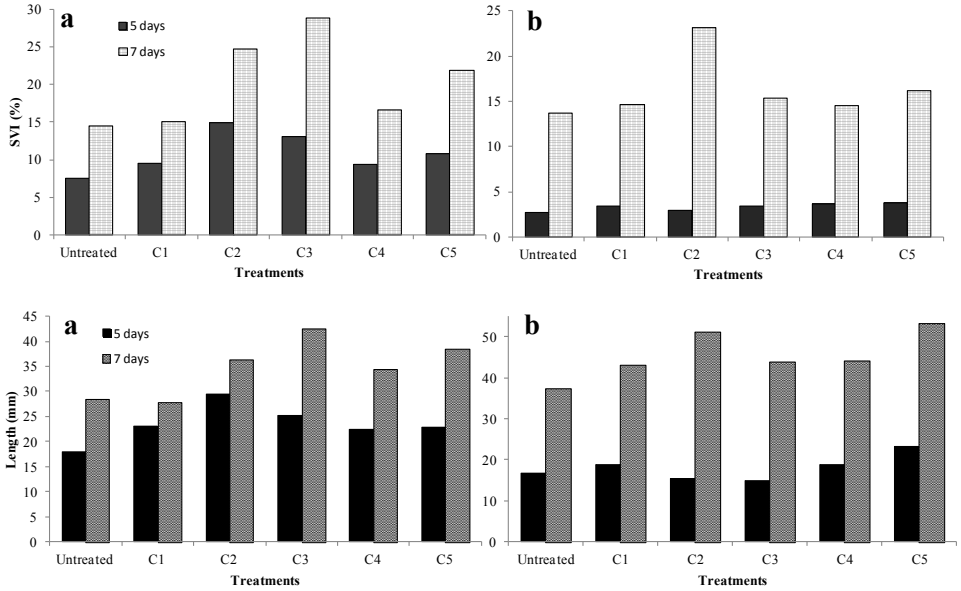


Figure 8. Effect of *C. vulgaris* (a) and *S. quadricauda* (b) extracts on root length and Seedling of Vigor Index (SVI) 5 and 7 days after soaking at 5 concentrations tested.

3.3 Effect of C. vulgaris and S. quadricauda extracts on growth test in sugar beet

Agricultural biostimulants include different types of bio-active compounds such as micro-organisms, plant growth regulators, enzymes, macro and micro algae extracts. Biostimulants are known to act on plant physiology improving resistance to abiotic and biotic stresses, increasing yield and crop vigour. These products are able to modify root growth and architecture (Lucini et al., 2015).

In this study, root apparatus was positively influenced by microalgae application as revealed by WinRhizo analysis. Total root length, root surface area and number of root tips significantly ($p<0.01$) increased in plants treated with the two doses of microalgae (**Figure 9**). In particular plants treated with SQ showed a higher number of root tips compared to those treated with CV.

No difference between the two doses was revealed, meaning that even a minimal concentration can produce a significant difference in root morphology, as previously observed by Bulgari et al., (2015). Another study conducted on tomato revealed improvements in root traits using a dose of 2mL/L (Petrozza et al., 2014).

ICP-OES profiles have not revealed significant changes in the ionomic profile of Hoagland's solutions treated with the two microalgae extracts with respect to the untreated solution.

3.4 Gene expression involved in growth of sugar beet

In order to study the role of CVextr and SQextr on plants genetic expression, we analysed the transcript level of 53 genes related to nutrient acquisition on treated and untreated plants. **Figure 10** shows the heat map of the expression pattern of the 53 genes in plants not treated (untreated) and

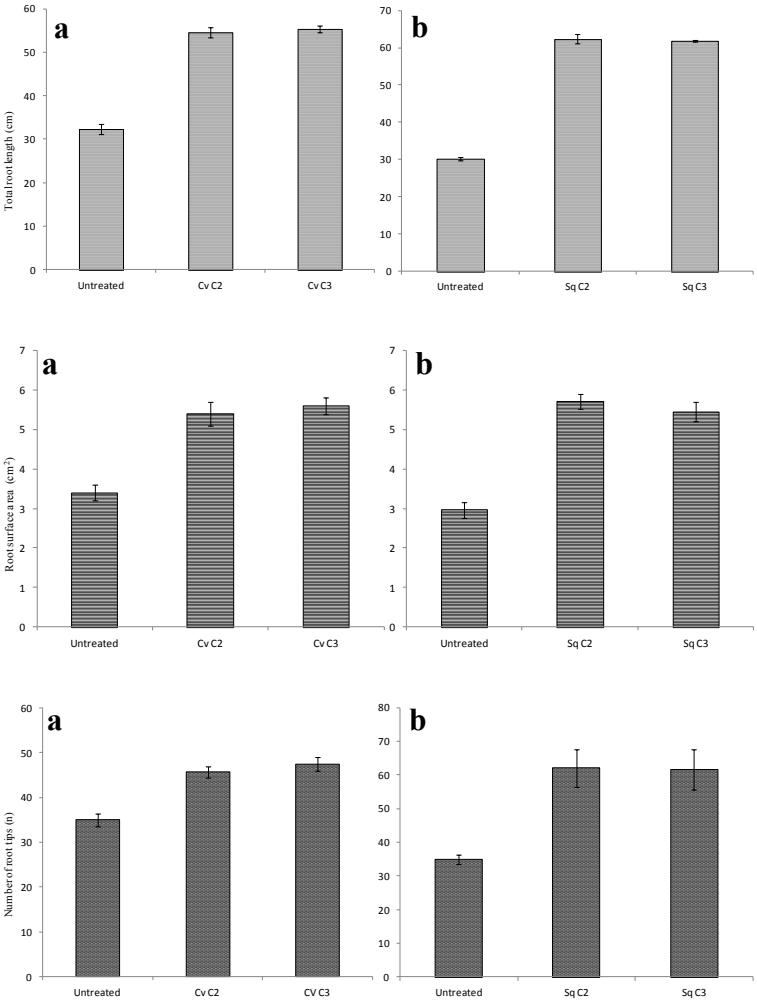


Figure 9. Total root length, root surface area and number of root tips of sugar beet plants, treated for 36 hours with *Chlorella vulgaris* (a) and *Scenedesmus quadricauda* (b) extracts at two different doses (C2 and C3).

treated with two different doses (C2 and C3).
The expression levels of all genes increased in response to the treatment. Red and green colours mean high and low levels of expression, respectively. In **Figure 11** are shown the expression level of analysed genes (as indicated in material and methods). Both SQ extract concentrations applied, induced a similar response in the biochemical metabolism of the plant. Noteworthy, all genes analyzed increased their expression respect to the control, reaching a higher expression ranging from 3 to 6 fold times.

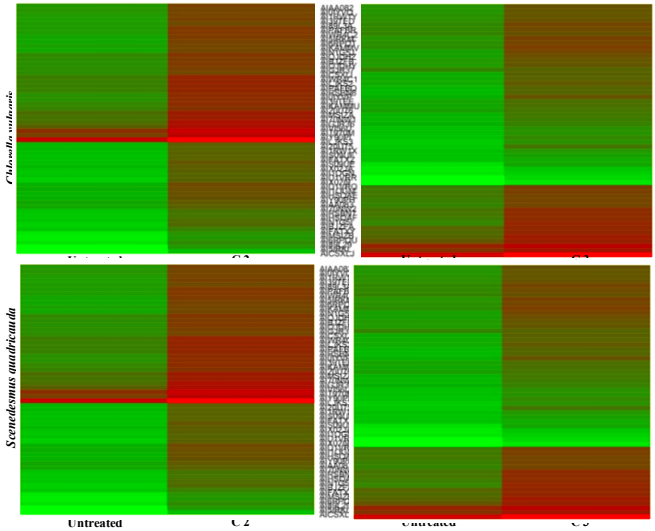


Figure 10. Heat map exhibiting the expression pattern of 56 sugar beet genes evaluated in the roots of untreated seedlings and treated with 1 mg C org/L (C2) and 2 mg C org/L (C3) of the two different microalgae extracts. Colours ranged from green to red; low expression: green, high expression: red.

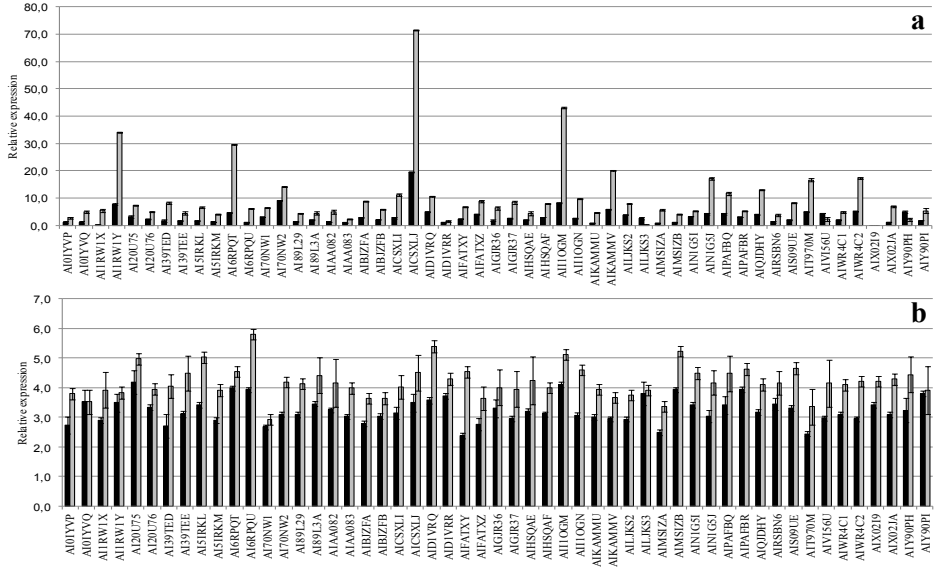


Figure 11. Relative expression value of 53 genes in plants treated with 1 mg C org/L (C2, black) and 2 mg C org/L (C3, grey) of *C. vulgaris* (a) and *S. quadricauda* (b). Data are representative of three independent experiments and reported as mean ± ES.

Interestingly, CV induced a different expression pattern respect to SQ, showing that different biochemical events occurred in response to the extract effect on the plant.
The expression level of genes involved in the response of the plant resulted to be much increased in the presence of the higher concentration of CV extract.
In fact the gene coding for E3 ubiquitin ligase in plants treated with the higher CV extract concentration is expressed around 70 times higher than untreated plants, followed by chaperone proteins (40 fold) and chalcone synthase (35 fold). Furthermore two genes coding for a chaperone protein DNAJ and a thioredoxin reductase showed a level of expression 30 and 20 fold higher than untreated plants, respectively.
An hypothesis may be that E3 ubiquitination activity may also display a secondary unrelated function. This could lead to its increased intracellular concentration and the stimulation of secondary function (Ardley et al., 2005). This property may be particularly relevant when the higher concentration of CV extract was applied. In this case the activated metabolism in plants, lead to the overespression of genes involved in the secondary metabolism (calchone synthase) as well as enzymes involved in the manteinance of redox sistem of the cell (thioredoxin system). These results are particularly interesting since the overexpression of these genes are associated to an increase in root growth. In fact although many bioactive compounds that prime plant immunity have been found so far, only a few practical plant activators have been developed, since these compounds that constitutively activate defense responses are often associated with arrested growth and reduction in yield (Noutoshi, 2012). The application of CVC2 extract after 48 hours may activate a secondary plant metabolism leading to

a greater protection from stress or oxidative damage characterized at the same moment by an increase in root growth and lenght, coupling simultaneusly two positive effects.

There are evidences that biostimulants help plants to overcome different biotic and abiotic stress situations (Joubert and Lefranc, 2008; Ertani et al., 2013b). Several alfalfa-based protein hydrolysate (EM) responsive genes identified via microarray were implied in detoxification and oxidative stress resistance. Tomato plants treated with EM showed increased TAC of ROS that are usually generated at high levels under stress (Ertani et al., 2017). Among the genes with a key role in mitigating oxidative stress, the main represented were glutathione peroxidase (GPX), glutathione reductase (GR), GST, peroxidases, thioredoxins, and DHAR. Interestingly, most of these genes are implied in the glutathione/ascorbate detoxifying cycle, thereby suggesting that this pathway may be an important target of the biostimulant mode of action of CV extract. Few experiments have been done on gene expression analysis of plants treated with seaweed as biostimulant. In *Brassica napus* L., *Ascophillum nodosum* extracts have been tested revealing a biostimulant activity on plant growth, nutrient uptake and translocation, without changing the mineral composition of the nutrient solution (Billard et al., 2014). To the best of knowledge, this is the first report showing a correlation between the promoting effect of root traits and the overexpression of genes in plants treated with microalgae. The listed genes could therefore be considered as markers not only of nutritional status but also associated to root development.

3.5 Physiological parameters in maize plant

The effect of *C. vulgaris* and *S. quadricauda* extracts on maize plant growth is reported in **Table 4**.

Table 4: Root and leaf dry weight of maize seedlings treated with microalgal extracts compared to untreated seedlings (100%). Values followed by different letters are significantly different (P< 0.05).

Treatment	Roots		Leaves	
	(mg)	(%)	(mg)	(%)
Untreated	4 ± 0.3 ***c***	100	24 ± 1.8 ***b***	100
Cv C2	7 ± 0.8 ***a***	175	32 ± 2.0 ***a***	133
Cv C3	5 ± 0.4 ***b***	125	35 ± 2.1 ***a***	146
Sq C2	6 ± 0.5 ***a***	150	22 ± 1.6 ***b***	92
Sq C3	7± 0.6 ***a***	175	32 ± 2.5 ***a***	133

All treatments stimulated root weight. In specific, lower *C. vulgaris* concentration C2 determined the best root stimulation (+75%), while the highest concentration C3 caused an increase (+25%). C2 and C3 *S. quadricauda* extracts also upgraded root dry weight (+50% and +75%, respectively).

Data showed that both *C. vulgaris* doses enhanced leaf dry weight (+33% for C2 and +46% for C3). Higher *S. quadricauda* concentration increased leaf dry weight also (+33%). Similar results both in roots and leaves were observed in other studies, after short treatment of maize plants with humic substances (Vaughan and Malcom, 1985; Nardi et al., 2002; Ertani et al., 2009).

Table 5 shows that SPAD index increased for each treatment compared to untreated shoots. The same gain (+29%) is observed in both *C.vulgaris* doses and at higher *S. quadricauda* concentration, while it is smaller (+19%) at lower *S. quadricauda* dose.

Table 5: Effect of microalgal extracts on SPAD index expressed as percentage respect to the untreated seedlings (100%). Values followed by different letters are significantly different (P< 0.05).

Treatment	SPAD	(%)
Untreated	33.80 ***b***	100
Cv C2	43.26 ***a***	128
Cv C3	43.72 ***a***	129
Sq C2	40.18 ***a***	119
Sq C3	43.58 ***a***	129

Several studies have shown that biostimulants increase the content of chlorophyll pigments in different plant species. Increments of chlorophyll content and dry weight of maize plants are also obtained by Shaaban (2001) after soil application of *C.vulgaris.* Higher chlorophyll content and activity of net photosynthesis is also found after *Chlorella* sp. application in maize (Grzesik and Romanowska-Duda, 2014), while other study observed a pigment content increase in *Lactuca sativa* seedlings grown in fertilized soils with *C.vulgaris* (Faheed and Adb-El Fattah, 2008). Increments of 19% were observed by Khan et al., (2012) in grape wines after application of seaweed extract and aminoacids, while Spinelli et al., (2009) noticed a 12% increase after seaweed extract treatment in "Fuji" apple. Recently, chlorophyll content increase was observed in *Salix viminalis*, after biofertilization using cyanobacteria and green algae (Grzesik et al., 2017).

3.6 Enzymatic activities in maize plant

Biostimulants may change both the level and percent distribution of sugars in maize leaves, by affecting enzyme activities involved in carbohydrate metabolism (Canellas and Olivares, 2014).

According to Merlo et al., (1991), in most plants, starch and glucose are the principal end products of photosynthesis. The leaf starch content decreased in plants treated with substances with biostimulant action, such as humic substances, whereas the level of soluble sugar (maltose and glucose) concomitantly increased (Merlo et al., 1991). In the cytosol, these soluble sugar are consumed in the glycolytic pathway.

Consequently, the treatment of plants with biostimulants determines a decrease of starch coupled with an enhanced activity of amylase, as well as the activity of invertase in the leaves.

Alpha-amylase is one of the most important enzymes to starch degradation. In fact, it is an endo-hydrolase that is able to rapidly degrade the starch into soluble substrates for other enzymes to attach (Beck and Ziegler, 1989).

Invertase is a key enzyme in carbohydrate metabolism; it is an hydrolase, that cleaves sucrose into glucose and fructose irreversibly. Therefore, its prevalent role is probably to supply glucose for cell energy production (Karuppian et al., 1989).

C. vulgaris and *S. quadricauda* extracts increased amylase activity in plants when compared to the control. The increase was more pronounced for the *C. vulgaris* treatments **(Figure 12)**. Particularly, the maximum value of amylase was recorded in plants treated with the lower concentration of *C. vulgaris* (+299%) C2 compared to untreated plants, while increased by 188% at higher concentration C3 of the same microalgal species.

S. quadricauda extracts enhanced amylase activity up 161% when treated with C2 (1 mg of Corg/L) and 139% with C3 (2 mg of Corg/L).

C. vulgaris treatments enhanced the invertase activity **(Figure 13)** if compared to untreated-plant, with a increase of 10% and 15% in C2 and C3, respectively.
Unlike to this, lower concentration of *S. quadricauda* treatment showed an increase of invertase activity of 26% respect to the control, while C3 produced an increase of 15% respect to untreated plants.
Previous studies demonstrated that invertase activity increased in response to hormones such as auxins in *Phaseolus vulgaris* (Morris and Arthur, 1984), gibberellins in *Pisum sativum* (Wu et al., 1993) or cytokinins in *Chenopodium rubrum* (Eheness and Roitsch, 1997).
These fundings may suggest that the increases observed could be attributed to the presence of hormone-like substances in the microalgal extracts.
It was demonstrated that existing several physiological functions of invertase; it could be involved in cell elongation, through maintenance of cell turgor (Pfeiffer and Kutschera, 1995), or could be an important regulators of gene expression (Koch, 1996). Particularly, last author stated that this enzyme could be indirectly involved in the control of cell differentiation and plant development. This hypothesis could explain the reason why the invertase activity increase did not determine a simultaneous gain in leaves dry weight after treatment with *S. quadricauda* extract at the lower concentration. Probably in this treatment a delay of the biostimulating action of the extract may occur, not yet visible as biomass produced. Obviously, gene expression analysis would be needed to support and/or confirm this hypothesis.

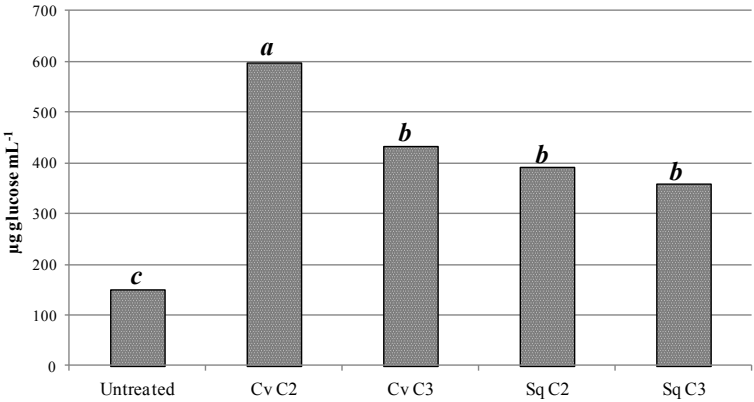


Figure 12. Effect of microalgal extracts on amylase activity. Values followed by different letters are significantly different (P< 0.05).

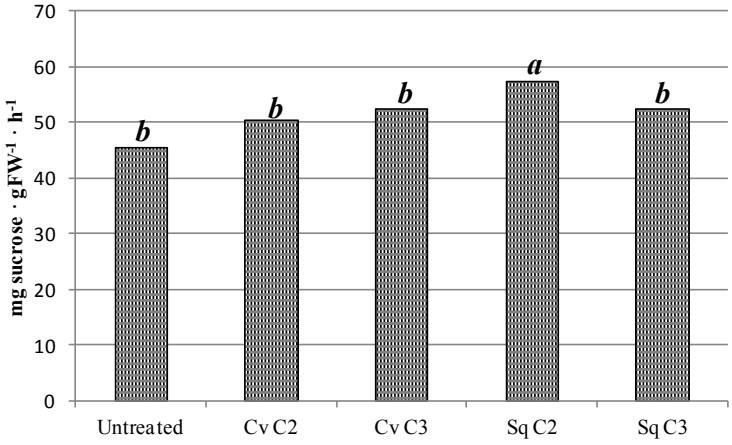


Figure 13. Effect of microalgal extracts on invertase activity. Values followed by different letters are significantly different (P< 0.05).

4. Conclusion

In conclusion, data indicate that the extracts from *C. vulgaris* and *S. quadricauda* were found to be promising in possessing biostimulating activity, both in the promotion of germination and in the early stages of plant growth in sugar beet. Hence, this simple and eco-friendly practice may be recommended to the growers for attaining better germination and root growth, as confirmed by the overexpression of root traits and genes related to nutrient acquisition in sugar beet.

Simultaneously, these microalgal extracts showed to increase dry weight, also if to different values, and SPAD as well as stimulate carbohydrate metabolism in maize plants, both increasing amylase and invertase activities.

These results suggest that the biostimulant effect was also exerted in leaves of reference plants with a worldwide economic relevance and an important agronomic culture, such as maize and beta.

CHAPTER III

Study on the effect of *Chlorella vulgaris* and *Scenedesmus quadricauda* extracts on soil biochemical fertility

1. Introduction

Soil plays a central role in the functioning and long-term sustainability of ecosystems. It is a living-dynamic, non-renewable resource and its condition influences food production, environmental efficiency and the global ecological balance (Dick, 1997; Doran and Zeiss, 2000; Alkorta et al., 2003).

Soil quality depends in part on its natural composition, but also on the changes caused by human use and management (Pierce and Larson, 1993). Human activities that influence the soil environment can be divided into two categories, those resulting in soil pollution and those aimed at improving its productivity (Gianfreda et al., 2005). A soil is biologically active when biological processes occur rapidly; such as a lot of metabolites being produced within a defined period of time (Schaller, 2009).

There are many methods of measuring soil biological activity, but these methods are not appropriate for producing generally accepted results. Despite this, they do give information relating to the ecological status of soil ecosystem (Burns, 1982; Frankenberger and Dick, 1983).

The soil enzymatic activity assay is only one way of measuring the ecosystem status of soils. Baldrian (2009) proposed a variety of methods for measuring enzymatic activities in soils. These techniques are quite simple, but they often differ in their mode of detection (spectrophotometry, fluorescence, radiolabelling), reaction conditions (temperature, use of buffers, time of reaction) and/or in their use of a variety of reaction substrates to measure enzyme activity, even for a single enzyme (Tabatabai 1994; Alef and Nannipieri 1995; Gianfreda and Bollag 1996; Schinner et al., 1996, Burns and Dick, 2002).

Unfortunately, generally accepted standard procedures still do not exist (Baldrian, 2009).

Soil enzymes are produced by the typical habitants of the soil and they continuously play an important role in maintaining soil ecology, physical and chemical properties, fertility, and soil health. These enzymes have key biochemical functions in the overall decomposition process of organic matter in the soil system (Sinsabaugh et al., 1991). They are important in catalyzing several vital reactions necessary for the life processes of microorganisms in soils and the stabilization of the soil structure, the decomposition of organic wastes forming organic matter, and nutrient cycling, hence playing an important role in agriculture (Dick et al., 1994; Dick 1997).

All soils contain a variety of enzymes that determine the soil metabolic processes (McLaren, 1975) which, in turn, depend on the physical, chemical, microbiological, and biochemical properties of the soil.

Enzymes are present in two general locations in soils: intracellular enzymes, which are associated with viable cells, and extracellular enzymes. The function of intracellular enzymes is obvious with their central role in the innumerable life processes of cells. Extracellular or abiotic enzymes as defined by Skujins (1976) as those living outside of the cells. An inherent difficulty in studying soil enzymes is that only small amounts of the total enzymes found in the soil can be extracted. Strong extractants generally denature proteins such as enzymes by disrupting the stereospecific structure of enzymes that is necessary for biochemical reactions.

Consequently, most investigations on soil enzymes are performed by measuring their activity directly in the soil.

This has a number of implications for interpreting and understanding the role of enzymes in soils.

The first consideration is the assay itself. Enzyme assays are carried out by adding a substrate solution of known concentration to a known amount of soil and measuring the rate of conversion of substrate to product. The assay is carried out under a strict set of conditions that includes temperature, buffer pH and ionic strength. Thus the results are operationally defined and any change in these conditions will alter the activity measured. Nevertheless, the specificity and integrative nature of soil enzyme activity provide a potential basis for using it as an indicator of certain functions in soils.

In this way, soil enzymes act as important soil indicators. One common indirect method of measuring the microbial activity in soil is by means of fluorescein diacetate hydrolysis activity (FDA). In this assay, fluorescein diacetate can be used to measure microbial activity in soils due to a number of different enzymes such as protease, lipase and esterase which can hydrolyze this substrate (Green et al., 2006).

Furthermore, easy, well-documented assays are available for a large number of soil enzyme activities (Dick et al., 1996; Tabatabai 1994a,b; Von Mersi and Shinner, 1991; Tabatabai and Bremner, 1969; Eivazi and Tabatabai, 1977; Kandeler and Gerber, 1988). These include dehydrogenase, urease and phosphatases.

Dehydrogenase enzyme activity is commonly used as an indicator of biological activity in soils (Burns, 1978). This enzyme is considered to exist as an integral part of intact cells but does not accumulate extracellularly in the soil. Dehydrogenase enzyme is known to oxidize soil organic

matter by transferring protons and electrons from substrates to acceptors. These processes are part of the respiration pathways of soil microorganisms and are closely related to the type of soil and soil air-water conditions (Kandeler, 1996; Glinski and Stepniewski, 1985). Since these processes are part of the respiration pathways of soil microorganisms, studies into the activities of dehydrogenase enzyme in the soil are very important as they may give indications of the potential of the soil to support biochemical processes which are essential for maintaining soil fertility as well as soil health.

Urease is an enzyme that catalyses the hydrolysis of urea into CO_2 and NH_3 with a reaction mechanism based on the formation of carbamate as an intermediate (Tabatabai, 1982). This results is a rapid loss of nitrogen to the atmosphere through NH_3 volatilization (Simpson et al., 1984; Simpson and Freney, 1988). Soil urease originates mainly from plants (Polacco, 1977) and microorganisms found as both intra- and extracellular enzymes (Burns, 1986; Mobley and Hausinger, 1989). On the other hand, urease extracted from plants or microorganisms is rapidly degraded in soil by proteolytic enzymes (Pettitet al., 1976; Zantua and Bremner, 1977). This suggests that a significant fraction of ureolytic activity in the soil is carried out by extracellular urease, which is stabilized by immobilization on organic and mineral soil colloids.

In most cases this enzyme is an extracellular enzyme representing up to 63% of total activity in the soil (Martinez-Salgrado et al., 2010)

Phosphatase enzymes are also good indicators of soil fertility and play a key role in the soil system (Eivazi and Tabatabai, 1977; Dick et al., 2000).

These enzymes are believed to play critical roles in P cycles (Speir and Ross, 1978).

Organic phosphorus is abundant in soils and can contribute to the P nutrition of plants and microbes following hydrolysis and the release of free phosphate (Condron et al., 2005). This process is catalyzed by phosphatase enzymes, which are actively secreted into the soil by many plants and microbes in response to a demand for P, or passively released from decaying cells (Quiquampoix and Mousain, 2005).

In soil, phosphomonoesterases have been the enzymes most frequently studied, probably because they are active under both acidic and alkaline conditions, according to their optimal pH, and because they act on low molecular P-compounds, including nucleotides, sugar phosphates and polyphosphates (Makoi and Ndakidemi, 2008); thus they can be used as soil quality bioindicators.

Understanding the dynamics of enzyme activities in these systems is crucial for predicting their interactions as their activities as far as soil health is concerned may, in turn, regulate nutrient uptake and subsequent plant growth.

Since these enzymatic activities are closely linked to fertility and soil health, finding biostimulants that can increase them could be of fundamental importance.

In previous studies it was observed that soil health can be enhanced by applying seaweeds and seaweed extracts, by improving moisture holding capacity (Moore, 2004) and by promoting the growth of beneficial soil microbes (Khan et al., 2009).

Soil structure and exoenzyme activity could also be improved by a great number of substances produced by cyanobacteria (Zaccaro, 2000). Caire et al., (2000) observed

that cyanobacteria biomass and their exopolyssaccharides incorporated into soil, promoted the growth of other microorganisms and increased the activity of soil enzymes that participate in liberating the nutrients required by plants. These results are in agreement with those of Mahmoud et al., (2007) who observed that cyanobacterial inoculation generally enhanced soil biological activity.
Other studies have stated that biostimulants obtained from different organic materials by hydrolysis reactions are directly absorbed by soil microorganisms, as well as plants (García-Martínez et al., 2010a,b).

Aim and scope
One limitation of the results of many previous studies regarding soil biostimulants was that they concentrated only on the soil or on the plant, omitting the interactions that occur in the soil-microorganism-plant system.
In the previous chapters of this thesis too, the effects of microalgal extracts were evaluated only on plants. But what happened when the same biostimulating substances were distributed in the soil?
The goal of this work was, therefore, to determine the soil biochemical response after adding *C. vulgaris* and *S. quadricauda* extracts or living cells, assessing whether the effects obtained in soil without vegetation are comparable to those of the same soil covered by plants.

2. Materials and methods

2.1 Experimental conditions and soil characterization

The experiment was conducted under laboratory conditions, using an agricultural top soil. Before use, the soil samples were air dried, sieved at 2 mm and characterized according

to Violante (2000), for pH, texture, organic carbon, phosphorus, cations Mg^{2+}, Ca^{2+}, Na^{+} and K^{+} content.
Soil pH was determined in water using a 1:2.5 soil/solution ratio.
The texture of the soil was performed using the pipette method determining the particle size classes subdivided into clay (particles < 2 μm), silt (2 to 63 μm), and sand (63 to2000 μm). Particles > 2000 μm were not considered.
Organic Carbon was determined by the oxidation-titrimetric method. Soil was boiled under a cold-finger condenser with 25 mL of a 0,4N $K_2Cr_2O_7$ mixture for 1 hour. The excess of dichromate was then titrated with 0.4N $Fe(NH_4)_2(SO_4)_26H_2O$.
Phosphorus was determined by the Olsen method. A 2.5 gram soil sample and 50 milliliters of 0.5 M sodium bicarbonate (pH 8.5) solution were shaken for 30 minutes. The mixture was then filtered through Whatman filter paper and the ortho-phosphate in the filtered extract was determined colorimetrically at 630 nm by reacting it with ammonium molybdate using ascorbic acid as the reducing agent. Results were reported as parts per million (ppm) phosphorus (P) in the soil.
Cations in the soil were determined by atomic absorption spectrometry.
A complete characterization of the soil used in all the experiments is reported in **Table 1**.
For each replicate, 1 kg of soil was placed into a plastic pot (15x15x10 cm) and maintained at 50% water holding capacity (WHC) for the entire duration of the experiment.
After two days of acclimatization, plots of soil were treated with *C. vulgaris* and *S. quadricauda* extracts. The extracts were added to the soil to obtain a final concentration

corresponding to C2 (1 mg Corg/L) and C3 (2 mg Corg/L) in the free water of the soil.

Simultaneously, soil plots were supplemented with fresh microalgae obtained after centrifugation, in order to separate the cells from the growth medium. The quantity of microalgae biomass added to the soil (68 mg and 55 mg in biomass of fresh *C. vulgaris* and *S. quadricauda*) corresponded to the amount necessary to obtain an extract concentration of 1.5 mg Corg/L (intermediate quantity of extract concentrations used in the other theses).

Homogeneous soil samples were collected from each pot at 0, 3, 6, 13 and 20 days post-acclimation of the soil (t0, t3, t6, t13 and t20 respectively) and stored at −80 °C for further enzymatic analysis. T3 samples correspond to 24 hours after treatment.

Every treatment was repeated on four independent plots of soil. Furthermore, four replicas were planted with 4 young (four-leaf stage) tomato plants (*Solanum lycopersicon*, cultivar Missouri), immediately prior to treatment.

Table 1 Characterization of the soil.

Soil properties	
Sand (%)	73.2
Silt (%)	18.5
Clay (%)	8.3
pH (H_2O)	7.7
Organic Carbon (%)	0.6
P (Olsen)(%)	0.26
Ca (%)	1.24
K (%)	0.45
Mg (%)	0.18
Na (%)	0.57

2.2 Fluorescein diacetate hydrolytic activity (FDA)

FDA activity was assayed according to Green et al, 2005. Briefly, 1 g of soil and 0.50 mL of FDA lipase substrate solution, in 50 mL of a sodium phosphate buffer were incubated for 3h at 37°C. Hydrolysis reaction was stopped by adding 2mL of acetone. The absorbance of the filtered supernatant was then measured at 490 nm after centrifuging about 30 mL of soil suspension for 5 min at 8,000 rpm. The concentration of fluorescein hydrolyzed during the reaction was calculated from a fluorescein standard calibration curve.

2.3 Dehydrogenase activity (DHA)

Dehydrogenase activity was determined by mixing 1g of soil with [2-(p-iodophenyl)-3-(p-nitrophenyl)-5- phenyl tetrazoliumchloride] solution (INT). After incubating for 2h at 40°C, a mixture of ethanol and dimethylformamide was used to extract the reduced iodonitrotetrazolium formazan (INTF). The measurement was performed photometrically at 464 nm using the method described by von Mersi and Shinner (1991). The concentration of INTF in the samples was calculated from an INTF standard calibration curve.

2.4 Acid and alkaline phosphomonoesterase activity (ACP, ALP)

Acid and alkaline phosphomonoesterase activity was determined using a modified version of the two original methods (Tabatabai and Bremner, 1969; Eivazi and Tabatabai, 1977). Soil samples were incubated at 37°C for 1h after adding p-nitrophenylphosfatase solution. Phosphomonoesterase activity released p-nitrophenol. The latter was treated with sodium hydroxide and the resulting colour was determined photometrically at 400 nm. The

concentration of p-nitrophenol released in the samples was calculated from a p-nitrophenol standard calibration curve.

2.5 Urease activity (URE)

To determine urease activity, soil samples were incubated for 2h at 37°C, after adding a buffered urea solution. A potassium chloride solution was used to collect the ammonium released, that was determined using a modified Berthelot reaction (Kandeler and Gerber, 1988). Under alkaline pH conditions, a green-coloured complex was formed as the result of reactions between NH_3 and sodium salicytate in the presence of sodium dichloroisocyanurate. Sodium nitroprusside is used as a catalyst and increases the sensitivity of the method about tenfold. Urease activity was expressed as nitrogen released in the reaction and was determined photometrically at 690 nm, calculated from a NH_4CL standard calibration curve.

2.6 Potential biochemical index of soil fertility (Mw)

Potential biochemical index of soil fertility (Mw) was calculated as proposed by Kalembasa and Symanowicz (2012) to include acid and alkalin phosphomonoesterase activity, dehydrogenase activity and urease activity, as well as organic carbon content, using the following relation:

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

where: ACP means acid phosphomonoesterase activity, ALP means alkaline phosphomonoesterase activity, DHA means dehydrogenase activity, URE means urease activity and C means organic carbon.

2.7 Physiological parameters in tomato plants

For dry weight measurement, tomato plants were divided into roots and leaves, and the leaves were weighed separately. The samples were placed in a drying oven for 2 d at 70 °C and allowed to cool for 2 h inside a closed bell jar, then the dry weight was measured per plant.

The measurement of the SPAD index was performed using the SPAD-502 Leaf Chlorophyll Meter (Minolta Camera Co., Ltd., Osaka, Japan) on three points of the last expanded leaf of each tomato plant for all the replicas.

2.8 Statistical analysis

Data were analyzed by one-way ANOVA ($P < 0.05$) followed by the Tukey's test for multiple comparison procedures.

3. Results and discussion

3.1 Fluorescein diacetate hydrolytic activity

Soil enzyme activity is frequently used to evaluate the metabolism and decomposition process of organic compounds in soils. These experimental tests have shown that the addition of *C. vulgaris*, *S. quadricauda* and their extracts led to the biochemical properties of the soil being greatly modified, only a few days after treatment. The rapid response of the microbial community is an indication of the high sensitivity of enzymatic activity to the changes occurring in the soil.

FDA hydrolysis is largely accepted as an accurate and simple method for measuring total microbial activity in a range of environmental samples, including soil. Colourless fluorescein diacetate is hydrolyzed by both exoenzyme and membrane bond enzyme (Stubberfields and Shaw, 1990),

releasing fluorescein, a coloured end product. The ability to hydrolyze FDA is frequently found, particularly among bacteria and fungi, the main decomposers (Shnürer and Rosswall, 1982). Since more than 90% of the energy in a soil passes through microbial decomposers, it is possible to state that a laboratory test that measures microbial decomposer activity will provide a good estimate of the total microbial activity (Adanm and Ducan, 2011).

Fluorescein diacetate hydrolysis activities and dehydrogenase are related to the oxidative process of organic molecules and reflect the metabolic state of the soil (Nannipieri et al., 1983; Trasar-Cepeda et al., 2008; Fernández et al., 2009).

Figure 1 shows the FDA activity in this study. Only 24 hours after treatment (t3), FDA activity increased to a different degree in all the test samples, except for the one treated with *C. vulgaris* cells which drastically increased FDA activity 4 days after treatment (t6), reaching the highest values both in naked soil or with plants. In these samples, FDA activity increased up to 2 times in soil alone and 2.7 times with plants, when compared to the untreated soil. Increments two times greater than the control were obtained in response to adding organic-mineral compost (80 g kg^{-1}) after 28 days of soil incubation in a 2014 study (Oliveira and Ferreira, 2014). Clearly the effect of the biostimulants tested in this study must be very efficient, since similar results were obtained in only 2 days and with much smaller amounts.

Furthermore, FDA activity increased in all other treatments 4 days after treatment (t6) and maintained high levels for one week; only after this time did it progressively decrease until the end of the experimental period, nevertheless

maintaining values similar to or greater than the control. It is interesting to note that after 11days of biostimulation (t13) a great increase was observed in soil treated with fresh *S. quadricauda;* probably this microalgal species needs more time to release its biostimulant substances. The value obtained in this case was only 1.3 times greater than those obtained in the control soil.

Results seems very similar in soil with plant cover. In this case, it is worth noting the effects of treatment with the lower concentration of *S. quadricauda*, that increased FDA activity at the beginning of the test and maintained its effect for the whole experimental period. Also in this case, at the end of the experimental period FDA activity remained at higher values than the control, apart from the SqC2 treatment. It is possible that the reduced effect on FDA activity induced by the highest concentration of Sq extract may be due to the plant-soil interaction, since this reduction was not observed in the corresponding experiment with the naked soil.

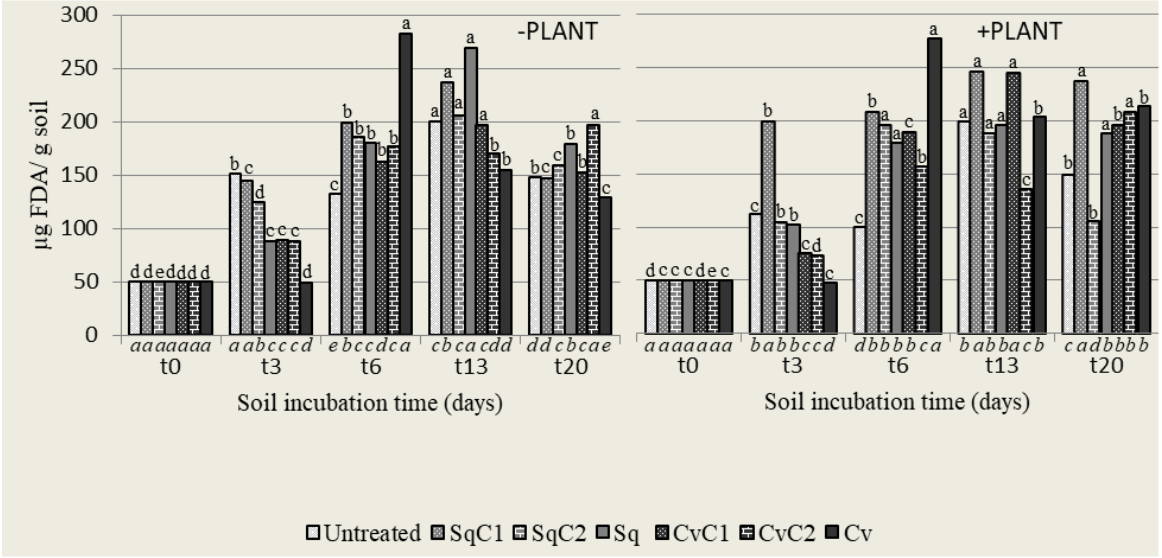


Figure 1 FDA activities (µg FDA per g of soil) in soils without (left) and with tomato plants (right) treated with *C. vulagaris* and *S. quadricauda* extracts and live cells. The values are means of data from three replications. Values of the same treatments, in block letters, followed by different letters are significantly different ($P < 0.05$). Values of the same sampling period, in italic, followed by different letters are significantly different ($P < 0.05$).

3.2 Dehydrogenase activity

Soil dehydrogenases are the major representatives of the oxidoreductase enzymes class (Gu et al., 2009). Among all enzymes in the soil environment, dehydrogenases are one of the most important, and are used as an indicator of overall soil microbial activity (Quilchano and Marañon, 2002; Gu et al., 2009; Salazar et al., 2011), because they occur intracellularly in all living microbial cells (Moeskops et al., 2010; Zhao et al., 2010; Yuan and Yue, 2012). Moreover, they are closely linked with microbial oxidoreduction processes (Moeskops et al., 2010). Dehydrogenases play a significant role in the biological oxidation of soil organic matter by transferring hydrogen from organic substrates to inorganic acceptors (Zhang et al., 2010). Soil dehydrogenase activity increased quite similarly in soils with and without plant cover (**Figure 2**). All treatments stimulated dehydrogenase activity during the whole experimental period, when compared to the control. As observed in FDA activity, the highest values were obtained at t6. At this point the treatment with fresh *C. vulgaris* cells proved to be the best treatment showing a percentage increase of 73% and 68% as compared to the control (fresh *Chlorella vulgaris* cells in naked and covered soils, respectively). Furthermore, dehydrogenase activity maintained high levels until t13 and decreased at the end of the experimental period, always maintaining, in this case too, values similar to or greater than the control.

It is very interesting to observe that the best treatment was the one with *C. vulgaris* fresh cells, both as regards dehydrogenase and FDA activities, probably because both these enzymatic activities are involved in the oxidative processes of organic molecules (Oliveira and Ferreira,

2015). Putatively, living cells of *C. vulgaris* may release some substances into the soil that are involved in these processes and which are present in a smaller quantity in the corresponding extract. These results are very interesting, considering that, in its attempt to survive in the soil, *C. vulgaris* may produce new substances or higher concentrations of them thus inducing a higher microbial activity in the soil.

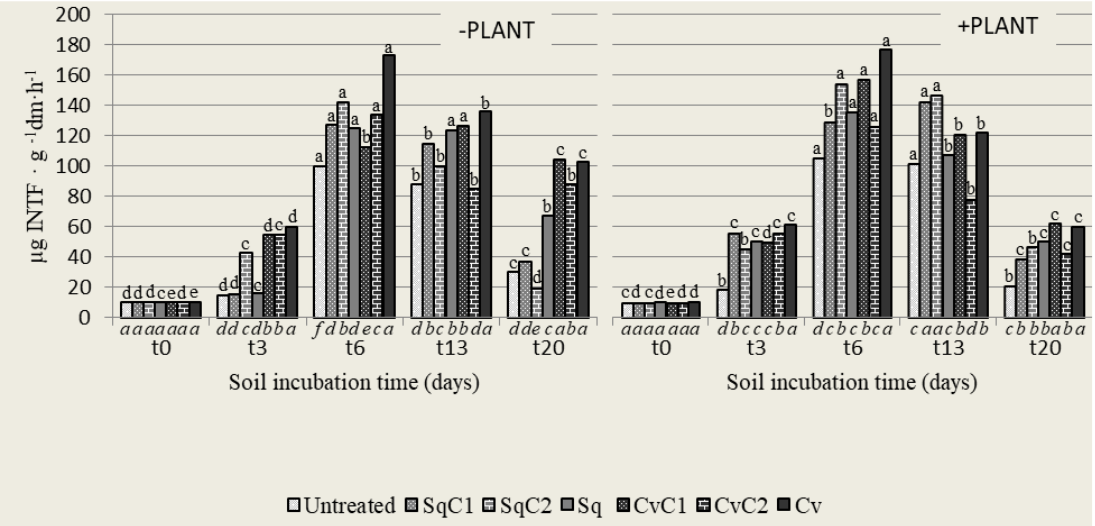


Figure 2 Dehydrogenase activity (µg INTF per g of dry matter in 1 h) in soils without (left) and with tomato plants (right) treated with *C. vulagaris* and *S. quadricauda* extracts and live cells. The values are means of data from three replications. Values of the same treatments, in block letters, followed by different letters are significantly different ($P< 0.05$). Values of the same sampling period, in italic, followed by different letters are significantly different ($P< 0.05$).

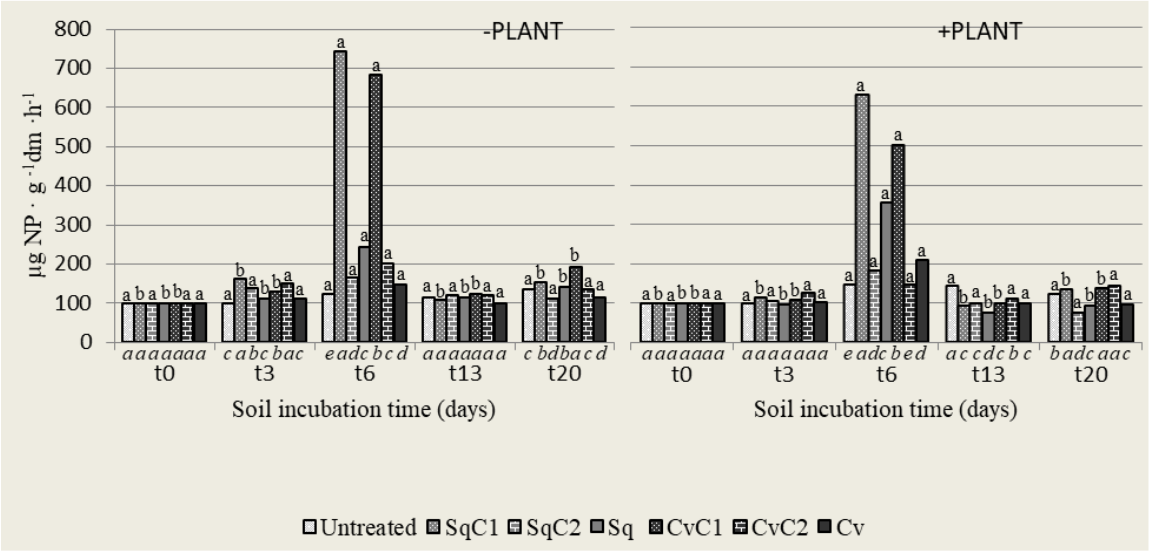


Figure 3 Acid phosphomonoesterase activity (µg NP per g of dry matter in 1 h) in soils without (left) and with tomato plants (right) treated with *C. vulagaris* and *S. quadricauda* extracts and live cells. The values are means of data from three replications. Values of the same treatments, in block letters, followed by different letters are significantly different ($P< 0.05$). Values of the same sampling period, in italic, followed by different letters are significantly different ($P< 0.05$).

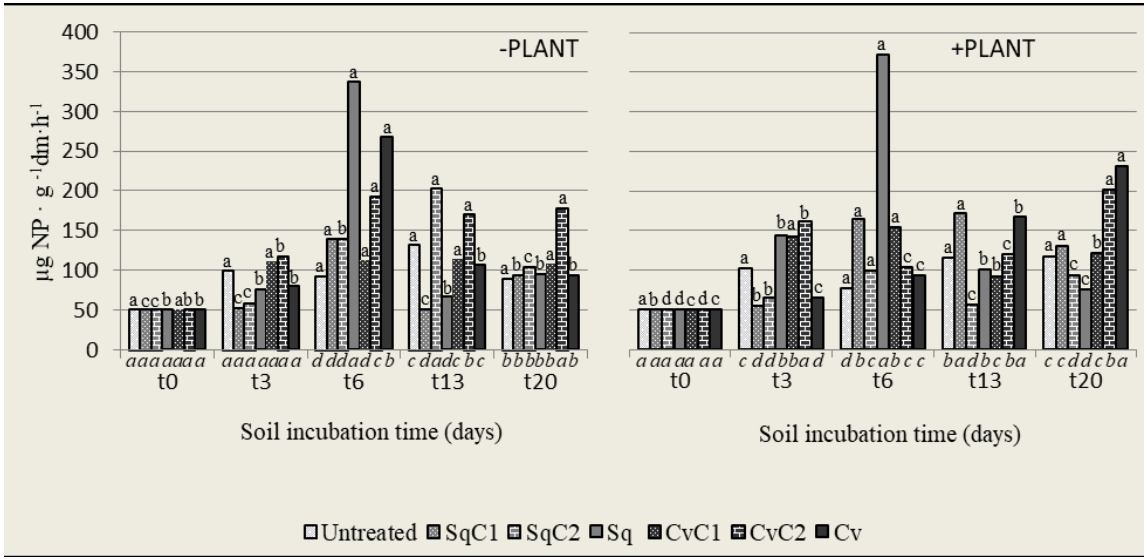


Figure 4 Alkaline phosphomonoesterase activity (µg NP per g of dry matter in 1 h) in soils without (left) and with tomato plants (right) treated with *C. vulagaris* and *S. quadricauda* extracts and live cells. The values are means of data from three replications. Values of the same treatments, in block letters, followed by different letters are significantly different ($P< 0.05$). Values of the same sampling period, in italic, followed by different letters are significantly different ($P< 0.05$).

3.3 Acid and alkaline phosphomonoesterase activity

Phosphatases catalyse the hydrolysis of ester–phosphate bonds, leading to the release of phosphate (P), which can be taken up by plants or microorganisms in the soil (Cosgrove, 1967; Halstead and McKercher, 1975; Quiquampoix and Mousain, 2005). Phosphatase enzymes, involved in the cycling of P, are also highly sensitive to changes in the soil properties due to their relationship with the content and quality of soil organic matter (Masciandaro et al., 2004). It has been shown that the activities of phosphatases depend on several other factors such as soil organism interactions, plant cover, the presence of inhibitors and activators (Speir and Ross, 1978). Acid and alkaline phosphomonoesterases hydrolyze monoester bonds including mononucleotides and sugar phosphates.

In **Figure 3** and **4** are shown acid and alkaline phosphatase activities in soils with and without tomato plants subjected to the different treatments. Acid phosphatase activity began to increase slightly after the treatments (t3) in all the samples examined, but with different intensities (**Figure 3**). Both in soil with and without plants the highest value of acid phosphatase activity occurred 4 days (t6) after treatment with the lower concentrations (C1) of *S. quadricauda* and *C. vulgaris* extracts, respectively. Maximum values (in naked soil with SqC1) were up to about 6 times higher than the untreated soil. One week later, acid phosphatase activity decreased rapidly. At the end of the experimental period, no differences were observed. All other treatments showed values quite similar to the control both in soils with and without plant cover, except for the treatment with living cells of *S. quadricauda* on soils with

plants at t6. In this latter case, the acid phosphatase activity was 2.4 times higher than the control soil.
These results are in agreement with those of García-Martínez et al., (2010a) who observed the highest enzymatic activities 2 and 4 days after biostimulant treatments, but their maximum value was 21 times higher than the control. In another study, García-Martínez et al., (2010b) obtained the highest enzymatic values, nearly 10 times greater the control soil after 24 h. Differences in intensity are obviously dependent on the soil (with its specific microbial component) and biostimulant (both in terms of composition and concentration) tested.
Alkaline phosphatase activity (**Figure 4**) showed a quite similar trend to that observed for acid activity. However, in this case the best activator proved to be the treatment with *S. quadricauda* after 4 days (t6) both in soils with and without plants. Conversely, the treatment with *C. vulgaris* at t6 induced a higher activity only in naked soils.
Overall, it is possible to underline that the acid phosphatase activity reached higher values than that measured for alkaline activity.

3.4 Urease activity

Urease is an extracellular enzyme representing up to 63% of total activity in the soil (Martinez-Salgrado et al., 2010). In **Figure 5** are shown urease activities in soils with and without tomato plants subjected to different treatments. Urease activity slightly increased in all the samples (**Figure 5**). However, similarly to other enzymatic activities, the most relevant increase was observed at t6, in soil without tomato plants treated with *C. vulgaris* extracts, at lower concentration. Interestingly, in naked soil treated with living

cells of *C. vulgaris*, urease activity reached a maximum value 24 hours after treatment, maintaining similar values to the control for the following experimental period. This finding is in contrast with all the other activities measured, which showed values which were always greater at t6 for treatment with *C. vulgaris*. However, it is equally true that the variability of enzymatic activities in soil changes mainly due to the availability of the substrate. Interestingly, in plant-covered soil a rapid increase was observed mainly at the beginning of the test (t3) using *S. quadricauda* extract at the lower concentration (SqC1), whereas the greater urease activity increments at the end of the experimental period (t20) resulted from the treatments with living *C. vulgaris* and its respective extract at lower concentration (CvC1).

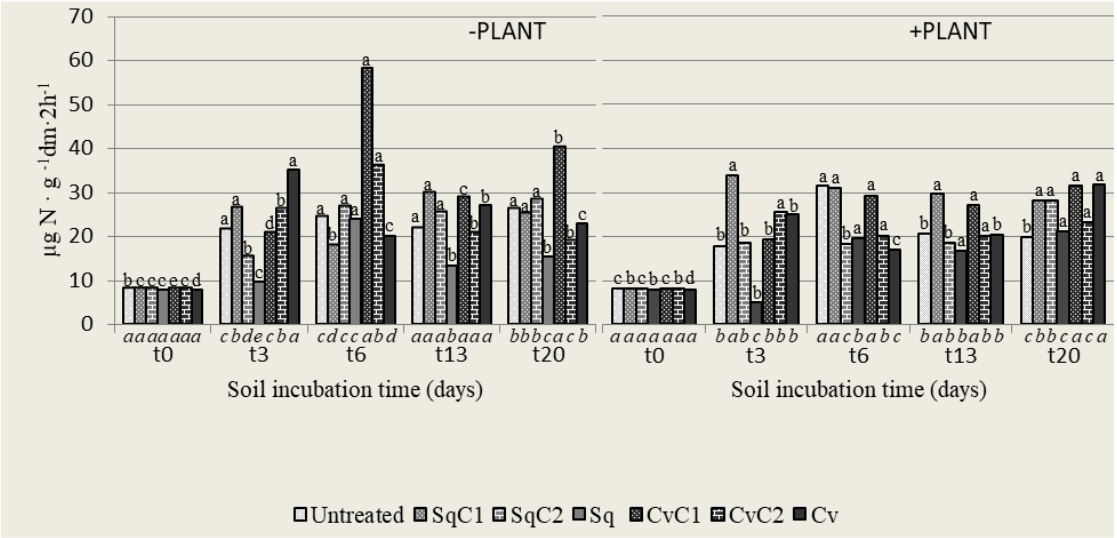


Figure 5 Urease activity (µg N per g of dry matter in 2 h) in soils without (left) and with tomato plants (right) treated with C. vulagaris and S. quadricauda extracts and live cells. The values are means of data from three replications. Values of the same treatments, in block letters, followed by different letters are significantly different ($P< 0.05$). Values of the same sampling period, in italic, followed by different letters are significantly different ($P< 0.05$).

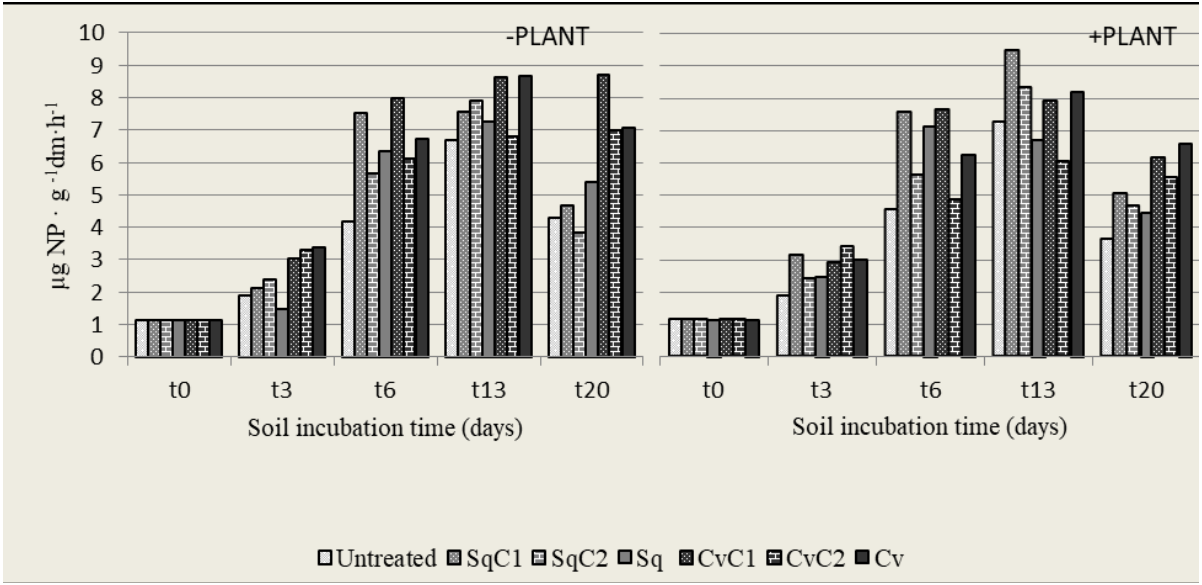


Figure 6 Biochemical index of potential soil fertility (Mw), in soils without (left) and with tomato plants (right) treated with *C. vulagaris* and *S. quadricauda* extracts and live cells. The values were calculated using the following formula: Mw= (ACP + ALP + DHA + URE $\times 10^{-1}$) × %C.

3.5 Potential biochemical index of soil fertility (Mw)

Soil is a very complex, living, dynamic system containing many free enzymes, which play a critical role in catalyzing reactions leading to the decomposition of organic matter and which serve as bioindicators of biochemical and microbial soil activity. The addition of biostimulant substances to the soil may affect soil enzymatic activity and thus the potential plant growth. In order to evaluate soil fertility, a potential biochemical index (Mw) was calculated, according to Kalembasa and Symanowicz (2012). Mw was calculated throughout the experimental period for all treatments. This index is very useful for evaluating the fertility of soil as it takes into account all the enzymatic activities calculated in the present study, in order to establish the best treatment in terms of soil fertility.

Figure 6 illustrates the values of the biochemical index of potential soil fertility (Mw). The data show that Mw values interestingly, were different in soil with or without tomato plants. These results are in accordance with the evidence that crops influence soil fertility. In naked soils the highest increments in Mw with respect to the control were achieved at t6; at this time all treatments resulted in a higher level of soil fertility index than the control, even if with some differences. In particular, at t6 the lower concentrations of the extracts from both microalgal species (*C. vulgaris* and *S. quadricauda*) proved to be the best treatments. However the treatments that proved to be most efficient at the end of the experimental period (t20) were CvC1, CvC2 and *C. vulgaris* cells.

Similarly, in soils with tomato plants at t6 the overall trend of Mw seems to be comparable to that observed in naked soils. Interestingly, the lower concentration of *S.*

quadricauda extract produced the greater levels of fertility increase with respect to the control at t13. Finally, at the end of the treatments (t20) all the values of Mw were lower (also the control); however, in this latter case (t20) the most efficient soil fertility inducers proved once again to be *C. vulgaris* cells and extracts, as observed in naked soils. Probably the presence of tomato plants interferes in some way with the effect of SqC1 on the Mw index, whereas *C. vulgaris* and its extracts are less sensitive to the action of the plants, although the effect of these treatments (especially for CvC1) in naked soils showed a greater positive action on the fertility index.

3.6 Physiological parameters in tomato plants

It has been documented that *Chlorella vulgaris*, contains high amounts of macro and micronutrients, as constituents or metabolites, like carbohydrates and proteins (Wake et al., 1992), as well as growth promoting factors, such as cytokinins (Stirk et al., 2002; Ördög et al., 2004). Furthermore, a consortium of *Stenotrophomonas maltophilia* and *C. vulgaris* used as an amendment in the soil, was shown to improve the root and leaf area of meadow clover plantlets, having a positive influence on the growth of the plants (Raposo et al. 2011). Furthermore, Elhafiz et al. (2015) found that living cells of *C. vulgaris* appear to be a promising sustainable biofertilizer, both in terms of dry weight of plant cells and chlorophyll content, for growing rice, lettuce, cucumber and eggplant. In this case the microalgae were distributed in the irrigation water.

A similar response with a greater number of leaves having a larger surface area was observed in soybean seedlings

irrigated with *Chlorella pyrenoidosa* (Dubey and Dubey, 2010).

In order to evaluate the growth of tomato plants subjected to treatment with *C. vulgaris* or *S. quadricauda* and their extracts, the dry weight and SPAD index were determined (**Table 2**).

Table 2 Leaf dry weight and SPAD index in leaves of tomato plants grown in soil after 18 days. Values followed by different letters are significantly different (P< 0.05).

	Leaf Dry weight (g)		**SPAD index**	
Untreated	1.87 ± 0.14	***b***	35.1± 2.14	***b***
Sq C1	2.34 ± 0.21	***a***	42.3± 3.73	***a***
Sq C2	2.29 ± 0.11	***a***	40.2± 2.51	***a***
Sq	2.19 ± 0.17	***a***	39.1± 1.82	***a***
Cv C1	2.48 ± 0.23	***a***	40.6± 3.27	***a***
Cv C2	2.37 ± 0.15	***a***	39.4± 2.84	***a***
Cv	2.22 ± 0.17	***a***	41.2± 3.14	***a***

It is interesting to note that the results showed that all the treatments enhanced leaf dry weight. In particular, the best biostimulant effect can be attributed to both the extracts and living cells of *C. vulgaris* (+33%, 27% and 19% for CvC1, CvC2 and Cv, respectively). Moreover, a considerable increment in dry weight was also obtained using both the extracts and living cells of *S. quadricauda* (25%, 22% and 19% for SqC1, SqC2 and Sq, respectively). These results seem to be very interesting, since applying living cells of microalgae species or their extracts (in particular, *C. vulgaris*) may result simultaneously in a better biochemical fertility in soil, as well as increased plant growth.

In accordance with leaf dry weight, the chlorophyll index content (in relative SPAD units) increased in each treatment compared to control. Overall, the same SPAD gain was observed in both *C. vulgaris* and *S. quadricauda* living cells as well as their extracts at both concentrations.

4. Conclusion

To the best of knowledge, this is the first report on the application of living *C. vulgaris* and *S. quadricauda* cells and their metabolites (under form of extracts) directly into the soils in order to evaluate simultaneously both their effect on the fertility of the soil and the biostimulant effect on the tomato plant.

The results highlighted that metabolites of *C. vulgaris* and *S. quadricauda* may induce a higher microbial activity in the soil and simultaneously increase plant growth. The soil biochemical response after adding *C. vulgaris* and *S. quadricauda* extracts or living cells, seems to be higher in the soils without vegetation, suggesting that some of the substances enhancing soil fertility may be intercepted by plants which may use them as biostimulant molecules.

In conclusion, although the extracts of microalgae seem to have a greater influence both on the fertility of the soil and plant growth, the direct use of living cells of microalgae in the soil may be a more easy-to-handle and cheaper method of treatment in order to obtain the same beneficial effects.

CHAPTER IV

Optimization of production: co-cultivation of *Chlorella vulgaris* or *Scenedesmus quadricauda* and tomato plants

1. Introduction

The previous chapters wanted to underline the multifunctionality role of microalgae.

They were initially used for the decontamination of organic and inorganic pollutants from agriculture wastewater. Subsequently, microalgal biomasses were used to extract potential substances owning a biostimulant activity on the germination and growth of *Beta vulgaris* as well as the growth of *Zea mays*. These same extracts have also proven to be able to improve soil fertility by stimulating its fundamental enzymatic activities.

Nevertheless, the biostimulation mechanism induced by microalgae extracts is a very complex mechanism and difficult to handle. For example, it could be determined by the interaction of several molecules and not necessarily by only one of them.

A clear classification of the extract obtained from these two microalgal species would therefore be necessary. Until then, there is no different way to analyze them than to evaluate indirectly the effects produced on target organisms.

In recently research, Zhang et al., (2017) observed that it was possible to cultivate hydroponically crop in association with microalgae, only using the inputs for crop. Authors attributed the microalgal biomass increments to the crop root respiration and exudation, that are sources of carbon, while algae photosynthesis enhanced the crop biomass.

As known, microalgae produce a large number of biologically active molecules. These are produced not only by microalgae but also by bacteria, coral, fungi and certain plants. These active molecules are generally secondary metabolites and are known as allelochemicals (Bacellar Mendes and Vermelho, 2013). It is very important highlight that allelopathy could be positive or negative, when

allelochemicals have beneficial or detrimental effect on the target organisms, respectively. In 1984, deep investigations convinced Rice that most organic compounds that are inhibitors at certain concentrations may be stimulating for the same processes at low concentration (Rice, 1984).

Such evidence suggests that the strong competition with the young growing tomato roots could induce microalgae to produce allelochemicals substances, in order to inhibit the growth of the competitors. However, according to Rice (1984), the low concentration of these substances does not play a inhibitory function, but stimulatory.

In recent years, numerous studies have been performed which aimed on one hand to increase the production of biomass and on the other to improve the yield and/or the quality of chemical substances that could be useful for the different purposes envisaged. Czerpak et al., (2003) demonstrated that diamines (agmatine and putrescine) and polyamines (spermine and spermidine) stimulate mitosis and increase protein, chlorophyll and monosaccharide content in *C. vulgaris*. Moreover, some growth stimulants such as phytohormones, vegetable extracts and polyamides are potentially useful for improving the productivity of microalgae (Czerpak et al., 2003). Hunt et al., (2010), employed *C. sorokiniana* to evaluate the influence of 12 biochemical stimulants on its growth and chlorophyll, protein and lipid contents. The increased biomass yield (about 0.145 g $L^{-1}d^{-1}$) was obtained by adding a combination of 1-naphthaleneacetic acid (5 ppm), gibberellic acid (10 ppm) and zeatin (1 ppm) to the culture medium. Interesting results were also obtained by adding humic acid (20 ppm) which induced a significant increase in the biomass (about 0.030 $gL^{-1}d^{-1}$) and in the chlorophyll content (0.5 $gL^{-1}d^{-1}$)

compared to the control (about 0.018 $gL^{-1}d^{-1}$ and 0.18 $gL^{-1}d^{-1}$ respectively).
Simultaneously, researches demonstrated that when humo-like substances (HLs) extracted from agro-industrial residues coming from *Brassica napus*, *Ricinus communis*, *Linum usitatissimum* and from a digestate were used to treat corn seedlings, they proved effective in increasing both protein content and the activity of those enzymes connected to the assimilation of nitrogen and photosynthesis (Ertani et al., 2013). Similarly, it has been shown that extracts from tomato plant residues obtained by alkaline hydrolysis produce a biostimulating effect on bean plants leading mainly to an increase in the quantity of nitrogen assimilated (Baglieri et al., 2014).

Aim and scope

Based on the results obtained in the previous chapters, an hypothesis may be that the increase of crop biomass could partly be attributed to the presence of biostimulating substances slowly released by microalgae in the nutrient solution.
The double goal of this chapter was to evaluate the possible biostimulating effects of living microalgae in an hydroponic cultivation of a typical regional plant such as tomato and simultaneously evaluate if an increase of the microalgal biomass and hence the biostimulant substances may exert a beneficial effect for the plants. For this purpose was firstly evaluate the possible biostimulating effects of HLs obtained by alkaline hydrolysis from different sources, among which a digestate of agro-livestock residues (D-HL), exhausted *Brassica napus* biomass residues after oil extraction (B-HL) and tomato cultivation residues (T-HL) on *C. vulgaris* and *S. quadricauda*. The alkaline extracts were added to the

microalgae growth medium to evaluate their effect on the quantity of biomass produced, hypothesizing that growth stimulation results in a better production of allelochemicals. Finally, in order to have an indirect confirmation of this hypothesis, seedling tomato plants were cultivate hydroponically in association with living microalgal species. Furthermore, the best extract, showing to act as the best biostimulant of microalgae growth, was added to the nutrient solution to evaluate its effect in the co-cultivation system.

2. Materials and Methods

2.1 Humic-like substances (HLs)

The humic-like substances (HLs) were extracted from agroindustrial wastes including: digestate from wastes of an agro-livestock farm (D-HL), oil extraction residues from rape *Brassica napus* L. (B-HL) and tomato residues from agroindustrial wastes (T-HL). Alcaline hydrolized extracts were obtained as described in Ertani et al., (2013). Briefly, 200 g of finely ground waste was treated with aqueous 0.1 mol L^{-1} KOH at a 1:5 w/v (waste/solution ratio).

The suspension was shaken under N_2 for 20 h, allowed to settle overnight and centrifuged at 3000 rpm for 20 min. In order to solubilize and totally remove the HLs, three treatments with 0.1 mol L^{-1} KOH were carried out on solid residue. The supernatant collected was freeze dried and used for further analysis. These fractions were previously characterized in already published works (Ertani et al., 2013; Baglieri et al., 2014).

2.2 Microalgae culture and experimental conditions

The experiments were conducted in triplicate by using 250 mL flask containing 150 mL of BG11 culture medium

(Baglieri et al., 2016). The culture medium was added of 50 mL of algal suspension, containing 75 mg of cells, and with two different amount of HLs (1: 100 mgL^{-1} and 2: 200 mgL^{-1}). The HLs used in all the experiments were: B-HL1, B-HL2, D-HL1, D-HL2, T-HL1 and T-HL2. BG11 without HLs was used as control. The flasks were incubated on mechanical shaker at 25–30 °C, illuminated by a 3500-lx, average photon flux (PPF) 100-µmol m−2 s−1 light source (PHILIPS SON-T AGRO 400) with a 12-h photoperiod.
Total algal biomass was determined in cultures maintained for 46 days in all the experimental conditions. Microalgae cultures were centrifuged at 2500 rpm for 10 min and the pellet was oven-dried at 70 °C until constant weight was reached, then it was weighed.

2.3 Chlorophyll a and b content determination

The determination of chlorophyll *a* and *b* was performed on the algal cultures grown for 46 days, by extraction in ethanol. After 12, 28 e 46 days, 10 mL of algal suspension were sampled to determine the chlorophyll content.
The extracts were analysed by spectrophotometry (Jasco V-530 UV-vis spectrophotometer) at a wavelength of 665 nm for chlorophyll *a* and 649 nm for chlorophyll *b* (Jeffrey and Humphrey, 1975; Licthenthaler, 1987). Chlorophyll *a* and *b* concentrations were calculated according to Wellburn and Lichtenthaler (1984).

2.4 Co-cultivation of microalgae and tomato plants

Tests were carried out in laboratory conditions. The experimental apparatus included a transparent container (40×20×10 cm), covered with wire net (mesh size 0,5 cm), used for crop fixation and 4 L of nutrient solution. This was prepared according to the modified Hoagland solution:

$Ca(NO_3)_2.4H_20$, 1250 mg dm^{-3}; KNO_3, 410 mg dm^{-3}; $NH_4H_2PO_4$, 280 mg dm^{-3}; $MgCl_2.6H_20$, 624 mg dm^{-3}; $FeSO_4.7H_20$, 60 mg dm^{-3}; EDTA-Na_2, 80 mg dm^{-3}; H_3BO_3, 6 mg dm^{-3}; $MnCl_2.4H_20$, 4 mg dm^{-3}; $ZnSO_4.7H_20$, 0,04 mg dm^{-3} and $CuSO_4.5H_20$, 0,04 mg dm^{-3} (Zhang et al., 2017). Nutrient solution was aerated by bubbling with air through an aeration pump. After 3 times washes with distilled water, microalgae were inoculated by adding 50 mL of the algal solution (OD_{680}=1.0). Successively, some plots were treated with a mix of microalgae and DHL, which gaves the best result based on the previous assay (100 mg L^{-1}). Corresponding controls received 50 mL of distilled water.
Treatments were schematize as follows:

1. **Untreated**: without microalgae and DHL;
2. **Cv**: with 50 mL of *C. vulgaris* suspension;
3. **Sq**: with 50 mL of *S. quadricauda* suspension;
4. **Cv+DHL**: with 50 mL of *C. vulgaris* suspension and DHL (100mgL^{-1});
5. **Sq+DHL**: with 50 mL of *S. quadricauda* suspension and DHL (100mgL^{-1});
6. **DHL**: with DHL (100mg/L), but without microalgae.

After germination in a vermiculite-peat substrate, five seedlings of tomato (*Solanum lycopersicum* cultivar Missouri) were transplanted at the at the appearance of the first leaf.
A completely random design with four replications was used. Distilled water was added to nutrient solution to maintain 4 L volumes, every time it was needed.
The experiment was conducted in a growth chamber at 25–30 °C, illuminated by a 3500-lx, average photon flux (PPF) 100-µmol m^{-2} s^{-1} light source (PHILIPS SON-T AGRO 400) with a 12-h photoperiod.

Plants and microalgae were harvested 46 days after transplant.
Microalgae cultures were centrifuged at 2500 rpm for 10 min and the pellet was oven-dried at 70 °C until constant weight was reached, then it was weighed.
The measurement of the SPAD index was performed using the SPAD-502 Leaf Chlorophyll Meter (Minolta Camera Co., Ltd., Osaka, Japan) on the last expanded leaf of tomato plants. The determination was carried out on 5 measurements per leaf from 10 plants per experimental condition. For dry weight measurement, plants were divided into roots and leaves, and weighed separately. The samples were placed in a drying oven for 2 d at 70 °C and allowed to cool for 2 h inside a closed bell jar, then the dry weight was measured per plant.

2.5 Statistical analysis
Data were analyzed by one-way ANOVA ($P < 0.05$) followed by the Tukey's test for multiple comparison procedures.

3. Results and discussion

3.1 Microalgal biomass and chlorophyll determination using HLs
In **Table 1** the total quantity of cells obtained at the end of the experimental period is shown, in order to evaluate the effect of the treatments on the production of biomass from the extracts **(Table 1)**. A significant increase in the biomass produced was found in both species as compared to the control following treatment with D-HL1 (41% and 31% for *C. vulgaris* and *S. quadricauda* respectively) and treatment with T-HL1 29% and 21% for *C. vulgaris* and *S.*

quadricauda, respectively). In all other cases the differences were not worthy of note.

As shown in **Table 2**, there was an increase in the chlorophyll *a* and *b* concentrations in both the microalgae after 46 days of growth with the two B-HL doses.

Our results showed that the extracts obtained by alkaline hydrolysis from a variety of food industry waste can effectively have a biostimulant effect on *C. vulgaris* and *S. quadricauda*. This biostimulant effect seems to depend on the type of extract used for cultivating the microalgae. These results can be easily justified by the fact that D-HL and B-HL contain different quantities of indolacetic acid, total phenolic acids and flavonoids as reported by Ertani et al., (2013). Moreover, it has been proved that their auxin-like behaviour effectively leads to an increase in the protein content, the activity of enzymes involved in nitrogen assimilation and photosynthesis in maize seedlings (Ertani et al., 2013).

Additionally, the results suggest that the HLs under examination can also influence the growth and development of microalgae, with variable physiological responses according to algal species and type of extract used. Furthermore, the results demonstrated that there is a greater effect on biomass production when the extract concentrations are lower. Different behavior observed at high concentrations could be due either to an increased turbidity of the medium that affected light penetration or the presence of molecules in the extract which in some way inhibited the hormone-like activity, as observed by Hunt et al., (2010).

Considering that, D-HL determined the greater increase in algal biomass **(Table 1)**, it could be used to induce

microalgae growth in the experimental condition of tomato plants in hydroponic culture.

Table 1 Biomass of microalgae grown in the absence or in the presence of different humic-like substances (HLs: D= digestate; B= Brassica; T= tomato; HL= humic-like substance; 1: 100 mg L^{-1}; 2: 200 mg L^{-1}) for 46 days. The values are means of data from three replications. Values of the same microalgae species followed by different letters are significantly different ($P< 0.05$).

Microalgae	Experimental condition	Biomass (g m^{-3} d^{-1})
C. vulgaris	Control	77.83 ***d***
	D-HL 1	109.35 ***a***
	D-HL 2	63.04 ***f***
	B-HL 1	83.48 ***c***
	B-HL 2	72.83 ***e***
	T-HL 1	100.43 ***b***
	T-HL 2	77.83 ***d***
S. quadricauda	Control	72.39 ***e***
	D-HL 1	94.57 ***a***
	D-HL 2	72.17 ***e***
	B-HL 1	64.13 ***f***
	B-HL 2	80.87 ***c***
	T-HL 1	87.83 ***b***
	T-HL 2	75.65 ***d***

3.2 Co-cultivation of microalgae and tomato plants

In order to evaluate the possible biostimulating effects of living microalgae in an hydroponic cultivation of tomato, seedling of tomato plants were cultivate hydroponically in association with living microalgal species as well as in the presence of D-HL since it resulted to be the best biostimulant of microalgal growth. To establish the stimulatory effect of the different treatments, chlorophyll content, root length and dry weight were evaluated.

As shown in **Figure 1**, chlorophyll content (as SPAD index) slightly increased in all treatments when compared to the control, nevertheless these increments do not result significantly different.

Table 2 Chlorophylls composition of microalgae grown in the absence or in the presence of different humic-like substances (HLs: D= digestate; B= Brassica; T= tomato; HL= humic-like substance; 1: 100 mg L^{-1}; 2: 200 mg L^{-1}) for 46 days. The values are means of data from three replications. Values in the same column of the same microalgae species followed by different letters are significantly different ($P< 0.05$).

Microalgae	Experimentalc ondition	Chlorophyll a (mgL^{-1})	Chlorophyll b (mgL^{-1})
C. vulgaris	Control	1.25 b	0.86 b
	D-HL 1	0.97 c	0.70 b
	D-HL 2	0.45 e	0.53 c
	B-HL 1	1.73 a	1.12 a
	B-HL 2	1.35 b	1.13 a
	T-HL 1	0.44 e	0.13 d
	T-HL 2	0.62 d	0.46 c
S.quadricauda	Control	0.75 c	0.57 c
	D-HL 1	0.48 d	0.48 d
	D-HL 2	0.35 e	0.31 e
	B-HL 1	0.92 a	0.81 a
	B-HL 2	0.86 b	0.73 b
	T-HL 1	0.40 e	0.42 d
	T-HL 2	0.51 d	0.54 c

Particularly, similar increments were obtained when the two microalgal species or the DHL were added to the nutrient solution (+10%, +10% and +11% for *C. vulgaris*, *S. quadricauda* and DHL, respectively).

The maximum SPAD index value were reached with the mixed treatment microalgae/DHL (+21% in Cv+DHL and +17% in Sq+DHL). According to these results,14 days old maize plants showed an increase of 21% in SPAD index

after a treatment for 48h with DHL using the same concentration applied in our experiments (Ertani et al., 2013).

In **Figure 2** are summarized the root length values measured at the end of the experimental periods. Interestingly, microalgal treatments increased root length by 158% and 116% for *C. vulgaris* and *S. quadricauda*, respectively. DHL augmented root length by 71%, while this increment was lower in Cv+DHL treatment (+18%) and higher in Sq+DHL treatment (+119%), when compared to the control.

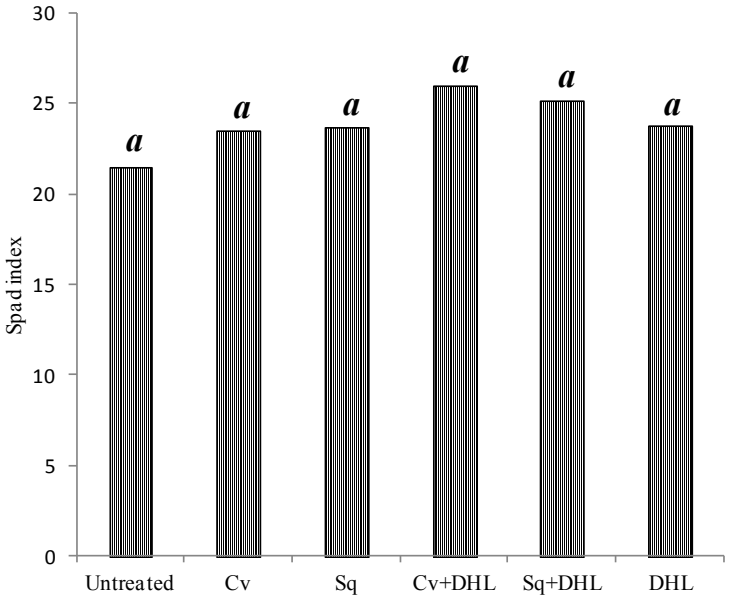


Figure 1. SPAD index in leaves of tomato plants grown in co-cultivation with microalgae after 46 days (Cv= *Chlorella vulgaris*; Sq= *Scenedesmus quadricauda;* DHL: D= digestate humic-like substance). The values are means of data from three replications. Values followed by different letters are significantly different ($P< 0.05$).

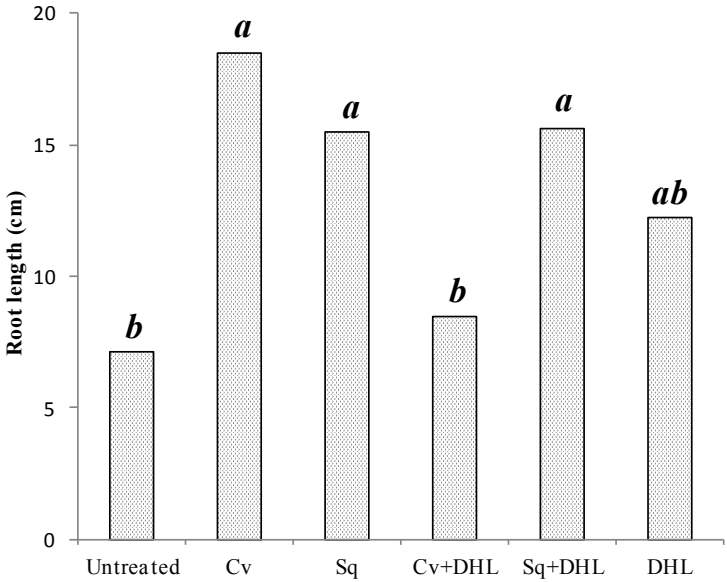


Figure 2 Length (cm) of root of tomato plants grown in co-cultivation with microalgae after 46 days (Cv= *Chlorella vulgaris*; Sq= *Scenedesmus quadricauda;* DHL: D= digestate humic-like substance). The values are means of data from three replications. Values followed by different letters are significantly different (P< 0.05).

Dry leaves weight **(Figure 3)** increased significantly in all treatment, except that Cv+DHL, which was comparable to control. The maximum level of increase was observed in plants grown in the presence of *C. vulgaris* (139%). Noteworthy, treatments with *S. quadricauda* with and without DHL increased dry leaves weight in a similar way (+77% and +70%, respectively). Conversely, a slight increase was achieved in leaves of plants treated with DHL (+45%).

Similarly, it was found that 14 days old maize plants treated with the same concentration of DHL (100 mg L^{-1})

augmented their dry weight by 14% in leaves with respect to the control (Ertani et al., 2013).

As shown in **Figure 4**, dry roots weight increased similarly in seedlings grown with *C. vulgaris* and *S.quadricauda* (+87% and 85%, respectively). Comparable effect were found in dry weight of root treated with Sq+DHL and DHL (+25% and +24%, respectively). Conversely, Ertani et al., (2013) observed that after DHL treatment of maize plants, root dry weight decreased (-18%) with respect to the control. Probably, at this concentration, the DHL biostimulating effect is higher in tomato than in maize plants, with regard of root dry weight. The different increase could also be attributed to a different duration of the DHL treatment. As well as for leaves dry weight, Cv+DHL treatment produced a tiny decrease (not significantly) in root dry weight. Therefore, *C. vulgaris* biostimulatig effect was not improved by DHL presence.

Interestingly, the DHL biostimulating action would seem to have a positive effect only on final microalgal biomass (*C. vulgaris*) with any benefit in the crop dry weight (**Table 3**).

In fact, final *C. vulgaris* biomass increased by 32% in Cv+DHL when compared with only *C. vulgaris*. This increment was smaller than the preliminary test of growth in the presence of HLs substances (+41%, **Table 1**), but in that case *C. vulgaris* was growing in an ideal nutrient solution (BG11).

Similarly, in *S. quadricauda* the biomass increment caused by DHL was lesser in hydroponic cultivation (+25%) respect to that obtained with the same concentration of DHL, when the microalgae were cultivated in BG11 (+31%).

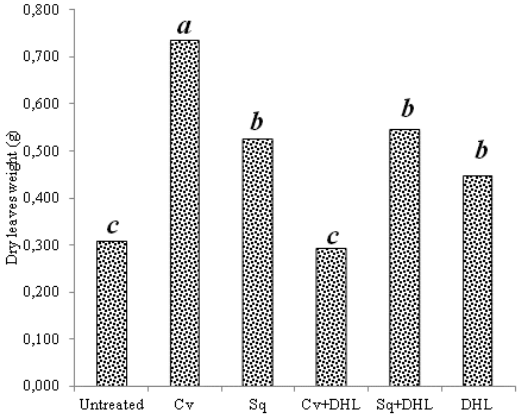


Figure 3 Dry leaves weight (g) of tomato plants grown in co-cultivation with microalgae after 46 days (Cv= *Chlorella vulgaris*; Sq= *Scenedesmus quadricauda;* DHL: D= digestate humic-like substance). The values are means of data from three replications. Values followed by different letters are significantly different (P< 0.05).

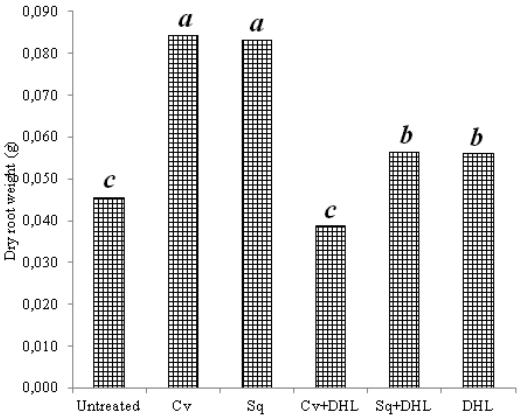


Figure 4 Dry root weight (g) of tomato plants grown in co-cultivation with microalgae after 46 days (Cv= *Chlorella vulgaris*; Sq= *Scenedesmus quadricauda;* DHL: D= digestate humic-like substance). The values are means of data from three replications. Values followed by different letters are significantly different (P< 0.05).

Table 3 Biomass of microalgae grown in co-cultivation with tomato plants after 46 days (Cv= *Chlorella vulgaris*; Sq= *Scenedesmus quadricauda;* DHL: D= digestate humic-like substance). The values are means of data from three replications. Values of the same microalgae species followed by different letters are significantly different (P< 0.05).

Treatment	Biomass (g m^{-3} d^{-1})
Cv	16.92 b
Cv+DHL	22.34 a
Sq	22.24 b
Sq+DHL	28.96 a

4. **Conclusion**

This chapter focused on two important aspects of applicative interest: by one side the increase in biomass production of microalgae and by another the use of microalgae as a biostimulant effector on tomato plants. Considering the first objective, this study showed that digestate HLs incresead *C. vulgaris* and *S. quadricauda* biomass production, that might reduce the production costs and expand the application of microalgae.

Furthermore, under this point of view, a costs reduction may be also obtained using a hydroponic co-cultivation of microalgae and crops together. Indeed, this system allows to obtain a double output using inputs only for crops.

In particular, by adding D-HL to the hydroponic system, microalgal biomass increased, along with a good increase in growth of tomato plants (Sq+DHL). All the physiological parameters of tomato plants (Cv+DHL) resulted enough similar to the values measured in the control. Therefore, the co-cultivation system in the presence of DHL may be an useful and cheap method in order to produce simultaneously microalgae as well as tomato plants.

On the other side, focusing on the plant production it is important to underline that in tomato the inoculum with *C. vulgaris* and *S. quadricauda* in nutrient solution of hydroponic is a strongly recommended strategy for a better, cheaper and ecofriendly production of tomato plants. Nevertheless, the preliminary co-cultivation in the presence of D-HL may produce firstly a great content of microalgal biomasses. Later, the biomasses obtained may be re-used in association with hydroponic cultivation of tomato plants, reaching in this way the highest performance for plants growth.

General considerations

Microalgae are photosynthetic organisms requiring very simple conditions for a high growth rate and can produce variable quantities of lipids, carbohydrates and proteins depending on the species and growing conditions. As a result, they are a very interesting option for use in various applications such as agricultural, biofuel, feed and food production.

In the first chapter of this thesis the aim of the work was to evaluate the ability of *Chlorella vulgaris* and *Scenedesmus quadricauda*, to remove organic and inorganic pollutants of agricultural origin. The microalgae were then grown in wastewater from a hydroponic greenhouse cultivation in order to evaluate the degree of removal of the main inorganic compounds. Furthermore, their ability to degrade five different active ingredients commonly used in agriculture for phytoiatric treatments was evaluated. Interestingly, the results presented in this chapter suggest that it is possible to use both *C. vulgaris* and *S. quadricauda* cultivation systems to purify wastewater from farming which contains inorganic compounds and pesticides. Furthermore, among the numerous methods for cultivating microalgae, the one proposed in this chapter could be a simple, inexpensive, easy-to-use solution coupled with the beneficial effect of wastewater purification.

In the second chapter, in order to reuse the microalgal biomass resulting from the above process (Chapter I), the final goal was to investigate the potential agricultural applications due to the presence of biostimulant substances in microalgal extracts. Successive results suggested that the extracts prepared from *C.vulgaris* and *S.quadricauda* were promising as biostimulants, both in the promotion of germination and in the early stages of plant growth in sugar

beet. These data were also confirmed by the overexpression of root traits and genes related to nutrient acquisition in sugar beet.
Simultaneously, these microalgal extracts were shown to have a positive effect on dry weight and SPAD, as well as stimulating carbohydrate metabolism in maize plants, increasing both amylase and invertase activities.
Hence, this simple, eco-friendly practice may be recommended to farmers for attaining better performance in the growth of both beet and maize plants.
In the third chapter the final objective was to determine the soil biochemical response after addition of *C. vulgaris* and *S. quadricauda* extracts or living cells. Moreover, the effects were also evaluated in soil cultivated with tomato plants.
To the best of knowledge, this is the first report on the application of living *C. vulgaris* and *S. quadricauda* cells and their metabolites (under form of extracts) directly into the soils in order to evaluate simultaneously both their effect on the fertility of the soil and the biostimulant effect on the plants. These results highlighted that metabolites of *C. vulgaris* and *S. quadricauda* may induce a higher soil biochemical fertility and simultaneously increased plant growth. The soil biochemical response after adding *C. vulgaris* and *S. quadricauda* extracts or living cells, seems to be higher in the soils without vegetation, suggesting that some of the substances enhancing soil fertility may be intercepted by plants which may use them as biostimulant molecules.
Finally the last chapter focused on two further important aspects of applicative interest:
1) to increase microalgal biomass by evaluating the effect of different natural products;

2) the simultaneous cultivation of microalgae and tomato plants in a hydroponic system.

The first objective was completely reached, in fact it was determined that alkaline extracts by digestate increased *C. vulgaris* and *S. quadricauda* biomass production. The high yield of algal biomass using residual material may reduce the production costs and expand the application of microalgae.

A further cost reduction may also be obtained using living microalgae directly in a hydroponic co-cultivation of tomato plants.

In particular, results suggested that the associated cultures of microalgae and tomato plants, in a hydroponic system to which alkaline extracted from digestate has been added, may be a useful and cheap way to produce simultaneously a higher microalgal biomass as well as promoting tomato plant growth. In fact, all the physiological parameters of the tomato plants were similar enough to the values observed in the control.

Focusing instead on the tomato plant growth, the inoculum with only *C. vulgaris* and *S. quadricauda* in a hydroponic nutrient solution is a strongly recommended strategy for a better, cheaper and eco-friendly growth of tomato plants. In fact, all the physiological parameters of the tomato plants in the test showed an increase in their values with respect to the control.

In conclusion, the extracts of microalgae seem to have a great influence on the growth of both beet and maize in hydroponic conditions, on tomato plants in soil as well as positively affecting the fertility of the soil. At the same time, the direct use of living microalgae cells increased the growth of tomato plants both in hydroponic conditions and in the soil as well as positively affecting the fertility of the

soil. Thus, based on the results obtained, a rational use of microalgae in agriculture could be envisaged. In this regard, it is possible to hypothesize that the direct use of living cells of *C. vulgaris* and *S. quadricauda,* both in hydroponic and soil cultures, may be easier to handle and a cheaper method of treatment in order to obtain the same beneficial effects. Therefore, a direct application of microalgal biomasses to the soil, obtained when possible from the purification of wastewater, may be a useful application for farmers to obtain better growth performances, at least as far as concerns sugar beet, maize and tomato. Obviously, further experimental studies are necessary to support this model of application.

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