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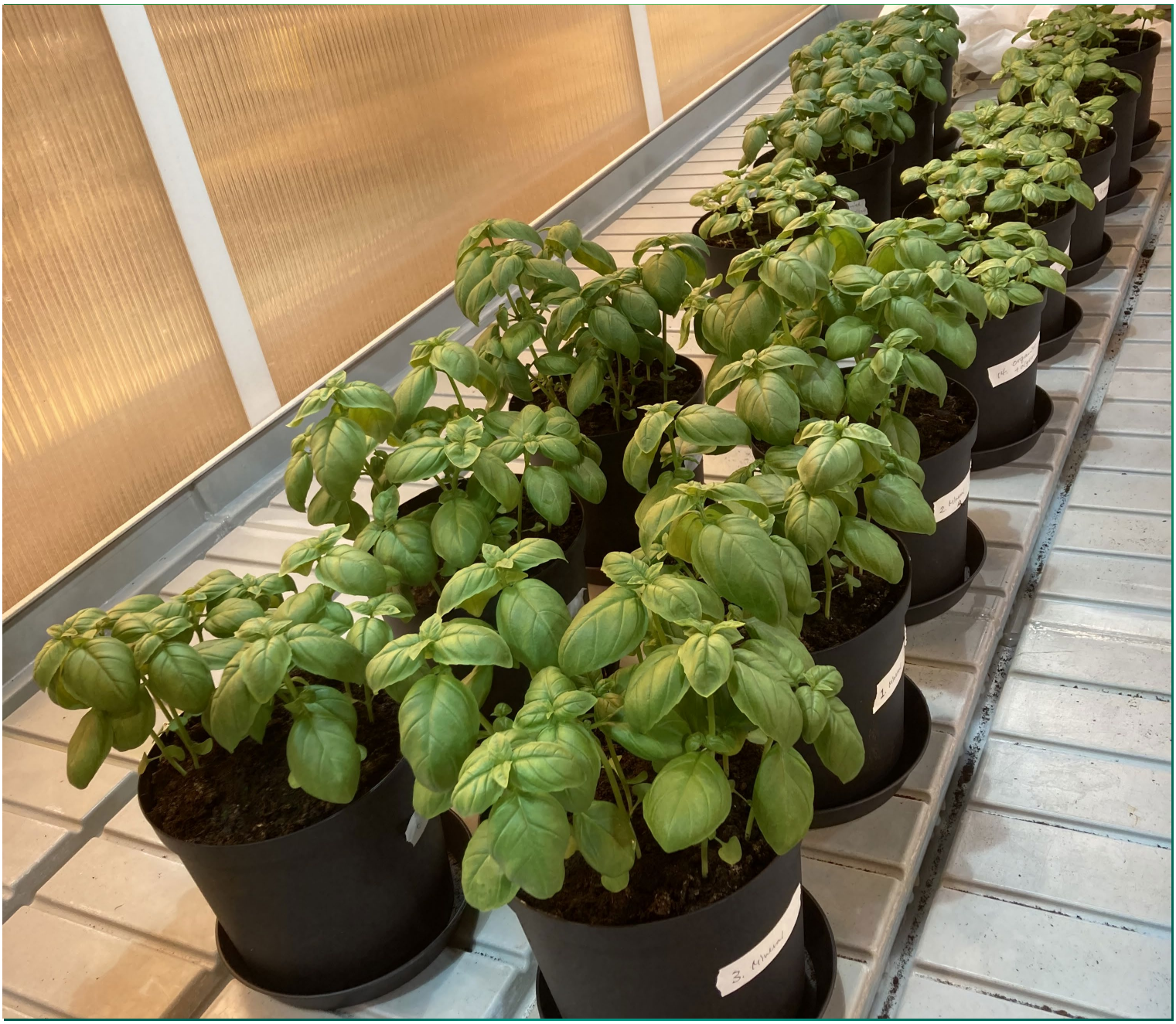

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TITTEL/TITLE

Test of biostimulant effect of microalgae (*Phaeodactylum tricornutum*) on basil (*Ocimum basilicum)*

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SAMMENDRAG/SUMMARY:

Denne rapporten viser resultater fra et forsøk hvor det ble undersøkt om et pulver av frysetørkede mikroalger (*Phaeodactylum tricornutum*) hadde en biostimulerende effekt på vekst og innhold av næringsstoffer og antioksidanter i basilikum (*Ocimum basilicum*). Effekten av mikroalgepulveret ble testet som tilskudd til enten mineralgjødsel eller en kommersiell organisk gjødsel.

Vi fant ingen signifikant effekt på avling av tilsatt mikroalgepulver, men det var en tendens til høyere avling med tilført mikroalgepulver for behandlingen med organisk gjødsel som kan skyldes ekstra nitrogentilførsel med mikroalgepulveret. Med mineralgjødsel var det motsatt tendens, høyest avling uten mikroalgepulver.

Den eneste statistisk signifikante effekten av mikroalgepulveret var en signifikant økning i konsentrasjonen av bor for forsøksleddet med organisk gjødsel. Dette var sannsynligvis en effekt av en betydelig ekstra tilførsel av bor med mikroalgebiomassen. Det var en tendens til økt konsentrasjon av kobber med tilførsel av mikroalgepulver ved både mineralsk og organisk gjødsel, selv om ekstra kobbertilførsel med mikroalgepulveret var liten. Med organisk gjødsel var det i tillegg en tendens til økte fosfor- og kaliumkonsentrasjoner med tilførsel av mikroalgepulver. Dette kan være en biostimulerende effekt da den ekstra fosfor- og kaliumtilførselen med mikroalgepulveret var liten, men effekten var som nevnt ikke statistisk signifikant. Vi fant ingen signifikante forskjeller mellom behandlingene for totalt antioksidantinnhold.

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English summary:

This report shows results from an experiment where it was investigated whether a powder of freeze-dried microalgae (*Phaeodactylum tricornutum*) had a biostimulating effect on the growth and content of nutrients and antioxidants in basil (*Ocimum basilicum*). The effect of the microalgae powder was tested as a supplement to either mineral fertilizer or a commercial organic fertilizer. We found no significant effect on the yield of applied microalgae powder, but there was a tendency for a higher yield with added microalgae powder for the treatment with organic fertiliser. This may be due to additional nitrogen supply with the microalgae powder. With mineral fertiliser, there was the opposite tendency, highest yield without microalgae powder.
The only statistically significant effect of the microalgae powder was an increase in the concentration of boron for the treatment with organic fertiliser. This was probably an effect of a significant additional supply of boron with the microalgae biomass. There was a tendency for an increased concentration of copper with the addition of microalgae powder with both mineral and organic fertiliser, although the additional copper supply with the microalgae powder was small. With organic fertiliser, there was also a tendency towards increased phosphorus and potassium concentrations with the addition of microalgae powder. This could be a biostimulating effect as the additional phosphorus and potassium supply with the microalgae powder was small, but as mentioned, the effect was not statistically significant. We found no significant differences between the treatments for total antioxidant content.

GODKJENT /APPROVED	PROSJEKTLEDER /PROJECT LEADER
Roald Sørheim	Ikumi Umetani
NAVN/NAME	NAVN/NAME

Preface

The study reported here is a part of the project “Recycling of rest raw materials from bio-based industry by production of low trophic Crustacenas (Gammaridae) for new marine ingredients (BioCycles). BioCycles is a Researcher Project financed by Research Council of Norway (NFR 295063). The project is a cooperation between SINTEF Ocean, NIBIO, RISE PFI, and CSIC (Spain). One of the subaimes of the project is to establish a zero-waste concept for co-cultivation and use of side streams of the Gammaridae production. Microalgae have been chosen as a candidate group of organisms to fulfil the concept, especially for the nutrient recycling and generation of biomass, which will further be exploited as renewable resources. As a search for a suitable bi-product, we have looked at the potential of microalgae for biostimulant product development.

Ås, 30.05.23

Ikumi Umetani

Content

1 Introduction

Biostimulants are products that stimulate plant growth, improve plant health or product quality without being a fertiliser. The biostimulants may increase plant uptake of nutrients, increase resistance towards diseases or increase tolerance towards abiotic stress like drought (Ricci *et al.* 2019). There are many kinds of biostimulants on market. It can be beneficial microbes, extracts of microalgae or seaweed, humic substances, or other components. The knowledge about the mechanism behind the eventual positive effect is often lacking or insufficient.

Recently, production of biostimulants from microalgae has gained an increasing attention (Behera, *et al.*, 2021). Microalgae are a morphologically and physiologically diverse group of organisms. They are known to contain various bioactive compounds that can be exploited as human resources. Therefore, microalgae have long been of commercial interests for pharmaceuticals, food supplements, functional food and animal feeds (Show 2022). However, their markets have been limited to only a handful number of high-value products because of high production costs and a strict safety regulation for selection of microalgal species for direct human consumption (Wells *et al.* 2017). In the frame of a circular bioeconomy, an alternative approach is to develop biofertilizers and/or biostiumulants using a combined practice of microalgae cultivation and wastewater treatment. It leads not only to a reduction in the use of synthetic fertilisers, but also to the recovery and recycling of nutrients in wastewater from e.g. aquaculture for microalgae production. In addition, an improvement of wastewater management will be achieved by the employment of an alternative or additional biological treatment of wastewater to minimize chemical treatments. This approach will support green technology for microalgae production, sustainable development of agriculture/aquaculture, and ultimately, the concept of circular economy.

Microalgae cell compounds that have biostimulant potential include phytohormones (auxins, cytokinins, gibberellins etc.), amino acids, polysaccharides, fatty acids and antioxidants (Kapoore *et al.* 2021). Despite the promising potential, the usage of microalgae as biostimulant has not yet been fully demonstrated. Several studies have shown effects of a limited microalgal species range (belonging mostly to genus *Chlorella* and *Scenedesmus*) on various plants, focusing on root development, productivity enhancement and crop quality improvement (Elalami *et al.* 2021). However, it is still challenging to distinguish between a biostimulant effect and the fertiliser effect of the nutrients applied with the microalgae biomass or its extract on plant responses.

In the experiment we report here, we tested the biostimulant effect of dried powder of a microalga (*Phaeodactylum tricornutum*) on the growth, nutrient uptake and total antioxidant content (TAC) of basil (*Ocimum basilicum*). In the growth experiment, the microalgae powder was added to treatments with either mineral fertiliser in optimal amounts or organic fertiliser with and without application of additional nutrients.

2 Material and methods

2.1 Microalgae biomass

P. tricornutum UTEX 646 was obtained from UTEX Culture Collection of Microalgae, and the stock culture was maintained in the laboratory at NIBIO. For the preparation of the experiment, the microalga was grown in 10 L Erlenmeyer flask under a LED light (BX90 NS1, Valoya, Helsinki, Finland) providing 70 µmol photons m^{-2} s^{-1} irradiation with a 12/12-hour light/dark cycle. The growth medium used for the cultivation was L1 (pH 8) (Guillard and Hargraves 1993) prepared using 30 g L^{-I} synthetic sea water (Instant Ocean, Aquariuum Systems, Cedex, France). The cultures were placed on a bench in a laboratory, where the temperature was maintained at 22 °C. The cultures received aeration with filtered (pore size of 0.2 µm) ambient air. The cultivation was conducted as a 12-day batch operation for five times. The cells were harvested by centrifugation at 4500 rpm for 10 min at 4 °C and the centrifuged cells were rinsed to minimize the salt. The pelletized cells were freeze-dried and stored in the freezer at –20 °C until the start of the experiment. The nutrient content of *P. tricornutum* biomass is listed in Table 1.

Table 1. Average elemental composition of *Phaeodactylum tricornutum* UTEX646 (dry weight basis, n=2).

Element	Concentration	Element	Concentration
C (%)	43,7	**Fe (mg/kg)**	3 159,0
N (%)	9,4	**Mo (mg/kg)**	0,5
P (%)	0,6	**Mn (mg/kg)**	367,0
K (%)	0,2	**Cu (mg/kg)**	6,6
Mg (%)	4,9	**B (mg/kg)**	874,1
S (%)	1,0	**Zn (mg/kg)**	474,0
Ca (%)	0,6		

2.2 Plant growth experiment

A pot experiment was conducted with limed sphagnum peat as growth media in 2 L pots. The sphagnum peat was limed with 12 g $CaCO_3$ per pot three days before start of the experiment. At start of the experiment, pH in the growth media was 5,2.

The experiment was performed with five treatments (Table 2) and three replicates.

Table 2. Treatments in the pot experiment.

Treatment no.	Treatment
1	Mineral fertiliser (Min.)
2	Mineral fertiliser + 400 mg microalgae biomass (Min. + microalgae)
3	Organic fertiliser (Org.)
4	Organic fertiliser + mineral K, Mg and micronutrients (Org. + min.)
5	Organic fertiliser + 400 mg microalgae biomass (Org. + microalgae)

Mineral fertiliser was applied in solutions. The organic fertiliser was a commercial product (Hageland naturgjødsel) consisting of dry pellets with a mixture of poultry manure, meat and bone meal and vinasse. The pellets were crushed before use and was added with a rate of 11 g per pot. Applied amount of nutrients to the different treatments are shown in Table 3. The applied amounts of nutrients in the treatments with mineral fertiliser are in accordance with the recommendations for commercial production of basil (Frerichs *et al.* 2019). For the treatments with organic fertiliser, it was chosen to apply approximately the double amount of N compared to the treatments with mineral fertiliser, because of the expected lower N fertilisation effect of organic N. Analyses of the organic fertiliser

revealed low concentration of some nutrients. Therefore, a treatment with additional mineral nutrients (K, S, Mg and micronutrients) was included. Microalgae biomass was added with a rate of 0,4 g per pot. Despite a small amount of applied microalgae biomass, the treatments with applied microalgae biomass received significant more N, Mg and B than the corresponding treatment without microalgae biomass (Table 3). For the macronutrients P, K and S, the additional application from the microalgae biomass was small.

Table 3. Applied nutrients (mg/pot) in the different treatments.

Nutrient	Mineral fertiliser (Treat. 1 and 2)	Organic fertiliser (Treat. 3 and 5)	Organic fertiliser + minerals (Treat. 4)	Microalgae biomass (Treat. 2 and 5)
N	415	914	914	38
P	70	482	482	2,6
K	165	113	113 + 65	0,6
S	106	74	74 + 106	4,1
Mg	20,4	33,3	33,3 + 20,4	19,5
Fe	7,7	8,0	8,0 + 7,7	1,3
Mo	0,19	0,009	0,009 + 0,19	0,0002
Mn	4,3	0,9	0,9 + 4,3	0,15
Cu	5,7	0,15	0,15 + 5,7	0,003
B	0,20	0,09	0,09 + 0,20	0,35
Zn	2,4	2,0	2,0 + 2,4	0,19

Both fertilisers and microalgae biomass were mixed into the total volume of the pot.

The pots were seeded with basil (*Ocimum basilicum* var. Marian) and thinned out to 10 plants after germination. Artificial light (white light from halogen lamps) was used to provide a photoperiod of 16 h day^{-1}. Light intensity was 200 µmol m^{-2} s^{-1}. Temperature was 24°C during day (16 h) and 19°C during night (8 h). Relative humidity (RH) was 60%. The plants were watered four times a week. Amount of water was controlled by weighing. Places of the pots were switched twice a week to ensure similar light conditions for the single pots.

Five weeks after seeding the biomass above the ground was harvested by cutting the plants with scissors approximately 1 cm above the soil surface. Before cutting, the hight of the highest plant per pot was measured. Fresh biomass in each pot was recorded. Five plants per pot were dried at 60°C for dry weight (DW) determination and element analyses. From the other five plants in the pot, two leaves were picked from each plant for antioxidant determination. The fifth and sixth leave counted from the top of the plant were picked from the plants from treatment 1, 2 and 4, and from treatments 3 and 5 the third and fourth leaves from the top were picked because of smaller plants. The leaves were stored frozen until analysis.

2.3 Chemical analyses

pH in the growth media was measured in deionised water in a solid:solution ratio of 1:25 v/v.

Nitrogen concentration in the organic fertiliser, microalgae biomass and plant tissue was determined using a CN analyser and concentration of other elements was determined by ICP-MS after digestion with concentrated HNO_3 in a microwave. Element uptake (mg pot^{-1}) was computed by multiplying dry matter production by plant tissue concentrations.

2.4 Antioxidant content analysis

Frozen leaves were homogenized in LN with mortar and pestle. 100 mg fresh weight (FW) of homogenized leaf samples was vortexed with 1000 µL of ice-cold phosphate-buffered saline (1 × PBS)

using Vortex Genie 2 (Scientific Industries) for one minute, sonicated in ice-cold water for two minutes and centrifugated for five minutes at 17000 g and 4°C. Samples were diluted 20-100 times inside of 96 well plates (Eppendorf, Germany) with distilled water. Antioxidant content was measured using spectrophotometer (Multiscan Go, Thermo Scientific, USA) at 570 nm as total antioxidant capacity in Trolox equivalents using Total Antioxidant Capacity Assay Kit (MAK187, Merck, Germany) according to the manufacturer's recommendations. Values were normalized to tissue fresh or dry weight.

2.5 Data analysis

Analysis of variance (ANOVA) was performed to identify significant treatment effects. In addition to performing analysis for individual treatment effects, two-way ANOVA for groups with and without algae was performed. For multiple comparisons, Tukey's honestly significant difference multiple comparison test was used ($\alpha = 0{,}05$).

3 Results and discussion

3.1 Yields and nutrient concentrations

The average values for yield and hight of the basil plants were highest for the treatments with mineral fertiliser, but the yield was not significantly higher than the yield for the treatment with organic fertiliser + minerals (Table 4). Organic fertiliser without addition of K, Mg and micronutrients showed very poor growth and had leaves with necroses (Fig. 1). This was probably caused by Mg, Mn or Cu deficiency, since concentrations of these elements were considerably lower in the plants with only organic fertiliser compared to the other treatments (Table 5).

The treatments with organic fertiliser showed higher plant concentrations of N, P, K, S and Fe compared with the treatments with mineral fertiliser or organic fertiliser + extra minerals. This is probably due to low yields with organic fertiliser and thereby less "dilution" of the applied nutrients.

There was no significant differences between corresponding treatments with or without applied microalgae biomass, but there was a tendency of higher yield with applied microalgae biomass to the treatment with organic fertiliser. There was the opposite tendency with mineral fertiliser: highest yield without microalgae biomass.

The lacking effect on plant growth of application of microalgal biomass together with mineral fertilser is conflicting with earlier reported results where microalgal biostumulants had positive effect on plant growth (reviewed in Elalami *et al.,* 2021). The reason can be that maximal growth in defined climatic conditions for basil plants was already reached in treatment 1. Effect of biostumlants is often detected when they can help to overcome plant stress or improve nutrient uptake. With the organic fertiliser, application of some essential nutrients was lower than the plant requirement, and thereby stressful for the plants. Therefore, we expected higher biostimulant effect in the treatments with organic fertiliser. However, the tendency of higher yield with applied microalgae biomass to the treatment with organic fertiliser in our study could be an effect of the additional N application with the microalgae biomass.

Table 4. Average plant hight of the highest plant in the pot at harvest, dry weight yield (DW) and dry matter % (n=3). Different letters indicate significant differences between individual treatments (p<0,05).

Treatment	Hight, cm	Yield, g DW	Dry matter, %
1. Min.	36,5 a	7,31 a	9,3 a
2. Min.+ microalgae	34,3 a	7,05 a	9,0 a
3. Org.	9,2 c	1,21 b	13,6 a
4. Org.+ min.	24,2 b	5,48 a	10,2 a
5. Org.+ microalgae	11,3 c	1,47 b	13,0 a

The only significant effect of applied microalgae biomass on nutrient concentrations in the basil plants was a significant increase in B concentration when fertilised with organic fertiliser (Table 5). Without the effect being statistically significant on individual treatment level, it was also a tendency of increased B concentrations (>10% increase) with mineral fertiliser and increased Cu concentration with both mineral and organic fertiliser. Plants which received microalgae biomass (when all types of fertilisation combined, i.e., 2+5) had 82% higher B concentration than plants (treatments 1+3+4) which did not get it (35.4 mg/kg and 19,4 mg/kg, respectively, p = 0,001). Additionaly, there was a tendency of increased P and K concentrations with applied microalgae biomass to the treatment organic fertiliser. The increased B concentration in the treatments with applied microalgae biomass is probably due to combination of high B application with the microalgae biomass (Table 2) and predominantly passive transport of B to plant roots (Pereira *et al.* 2021). The tendency of increased P and K concentration with applied microalgae biomass to the treatment with organic fertiliser could be a biostimulant effect as the additional P and K application with the microalgae biomass were small.

Table 5. Average nutrient concentrations in the harvested plants (dry weight basis, n=3). Different letters indicate significant differences between individual treatments (p<0,05).

	Min.	Min.+ microalgae	Org.	Org.+ min.	Org.+ microalgae
N (%)	4,84 b	5,25 b	7,78 a	5,52 b	8,23 a
P (%)	0,60 b	0,63 b	2,38 a	0,91 b	2,66 a
K (%)	2,12 bc	2,11 bc	3,07 ab	1,95 c	3,57 a
Mg (%)	1,00 a	1,04 a	0,36 b	0,47 b	0,29 b
S (%)	0,36 c	0,37 c	0,65 a	0,54 b	0,68 a
Ca (%)	3,15 a	3,09 a	2,84 ab	1,94 c	2,62 b
Fe (mg/kg)	104 b	105 b	210 a	92 b	204 a
Mo (mg/kg)	<0,4	<0,4	<0,4	<0,4	<0,4
Mn (mg/kg)	231 a	237 a	112 b	161 b	121 b
Cu (mg/kg)	6,3	9,1	3,0*	7,8	3,4
B (mg/kg)	17 b	27 b	23 b	19 b	44 a
Zn (mg/kg)	89 a	95 a	106 a	64 b	96 a

*Average of two replicates because of an extreme value in the 3. replicate (43,3 mg/kg).

3.2 Nutrient uptake

For nutrient uptake, the average values showed higher uptake of N, P and K with organic fertiliser + microalgae biomass compared to organic fertiliser without microalgae biomass, but the differences were not statistically significant (Table 6). With mineral fertiliser, there was a tendency (not statistically significant) of higher N uptake in the treatment with microalgae biomass. The tendency of increased N uptake by application of microalgae biomass could be a result of the somewhat higher N application.

Table 6. Average total uptake in above ground biomass of N, P, K, Mg and S (n=3). Different letters indicate significant differences between treatments (p<0,05).

Treatment	N, mg pot-1	P, mg pot-1	K, mg pot-1	Mg, mg pot-1	S, mg pot-1
1. Min.	352 ab	44 ab	154 a	73 a	26 a
2. Min.+ microalgae	366 a	43 ab	146 a	73 a	25 a
3. Org.	95 c	29 b	38 c	4,3 c	8 b
4. Org.+ min.	302 b	50 a	106 b	26 b	30 a
5. Org.+ microalgae	121 c	39 ab	53 c	4,2 c	10 b

Figure 1. Plants from the five treatments at the day of harvest.

3.3 Antioxidant content

Presence of antioxidants is often hypothesised as one of biostumulating mechanisms of microalgal biomass (Kapoore *et al.* 2021). Some authors report that application of microalgae increases level of small molecule antioxidants (phenols, flavonoids) and enzymatic antioxidants (ascorbate peroxidase, catalase, etc.) (Kusvuran, 2021). Little is known about the level of antioxidants in basil in response to addition of microalgal biostimulants. Antioxidant content (both small molecule and enzymatic antioxidants) was measured as TAC and calculated as Trolox equivalents per mg FW or DW. No significant differences between treatments were found (Table 7). The highest TAC per mg FW was measured with mineral fertiliser without microalgae biomass, the lowest for mineral fertiliser + microalgae biomass. Addition of microalagal biomass when organic fertiliser was used did not increase TAC of basil leaves.

Table 7. Total antioxidant capacity (TAC) of basil leaves in Trolox equivalents. Different letters indicate significant differences between treatments (p<0,05).

Treatment	TAC (nmol Trolox eq. mg-1 FW)	TAC (nmol Trolox eq. mg-1 DW)
1. Min.	49 ± 31 a	522 ± 330 a
2. Min.+ microalgae	33 ± 18 a	360 ± 153 a
3. Org.	39 ± 6 a	303 ± 92 a
4. Org.+ min.	34 ± 11 a	338 ± 120 a
5. Org.+ microalgae	37 ± 3 a	294 ± 57 a

4 Conclusion

The use of microalgae for plant biostimulant has attracted considerable interest. Microalgae contain various compounds that have potential stimulatory effect for plant growth. However, the knowledge about the mechanism behind the eventual positive effect is often lacking or insufficient. Especially, it is challenging to distinguish between a biostimulant effect and the fertiliser effect, of the nutrients applied with the microalgae biomass, on plant responses.

This study aimed to evaluate biostimulant effect of freeze-dried microalgae (*Phaeodactylum tricornutum*) biomass for the growth of basil (*Ocimum basilicum).* The effect of the microalgae powder was tested as a supplement to a mineral fertiliser in optimal nutritional amounts for the basil growth, and to a commercial organic fertiliser. We tested the effect on yield, nutrient concentrations, and total antioxidant content (TAC) in basil leaves.

This study could not confirm a biostimulant effect of the microalgae biomass. A tendency of higher yield with applied microalgae biomass could be an effect of the additional N application with the microalgae biomass. With organic fertiliser, it was also a tendency of increased P and K concentrations with application of microalgae biomass. This could be a biostimulant effect as the additional P and K application with the microalgae biomass were small, but care must be taken as the effect was not statistically significant. No significant differences between treatments were found for total antioxidant content.

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NIBIO - Norwegian Institute of Bioeconomy Research was established July 1 2015 as a merger between the Norwegian Institute for Agricultural and Environmental Research, the Norwegian Agricultural Economics Research Institute and Norwegian Forest and Landscape Institute.

The basis of bioeconomics is the utilisation and management of fresh photosynthesis, rather than a fossile economy based on preserved photosynthesis (oil). NIBIO is to become the leading national centre for development of knowledge in bioeconomics. The goal of the Institute is to contribute to food security, sustainable resource management, innovation and value creation through research and knowledge production within food, forestry and other biobased industries. The Institute will deliver research, managerial support and knowledge for use in national preparedness, as well as for businesses and the society at large.
NIBIO is owned by the Ministry of Agriculture and Food as an administrative agency with special authorization and its own board. The main office is located at Ås. The Institute has several regional divisions and a branch office in Oslo.

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