PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

Report Form for PhD student annual evaluation (XXXVII and XXXVIII cycles)

Name of PhD student: **Lorenzo Mollo**
Title of PhD research: Development of algae-based biostimulants according to the principles of circular economy
Name of PhD supervisors: Alessandra Norici (DISVA), Nicola Rovelli (Enereco S.p.A.)
Research lab name: Laboratory of Algal and Plant Physiology
Cycle:
[ ] XXXVI
[X] XXXVII

PhD Curriculum::
[ ] Marine biology and ecology
[ ] Biomolecular Sciences
[X] Civil and environmental protection

DISVA instrumentation labs/infrastructure eventually involved in the project:
[ ] Actea Mobile Laboratory
[X] Advanced Instrumentation lab
[ ] Aquarium
[ ] MassSpec lab
[ ] MaSBiC
[ ] Simulation/informatics lab
[ ] Other. Please, indicate:

ABSTRACT:
Circular economy is a model of production and consumption where inputs are reduced as far as possible through the creation of a closed-loop system. In this perspective, phycoremediation of wastewaters and production of high-value algal biomass is a way to reduce inputs, to create added value and to reduce environmental pollution.
One sector which could definitely benefit from the circular economy is agriculture. Currently, conventional agriculture is a high-intensive process with higher levels of input and output per unit of agricultural land area. A way to make agriculture more sustainable may consist in creating new values from the anaerobic digestion of crops/food waste, remediating the by-products (digestate) using microalgae and apply these algae as biostimulants on plants.
The goals of this work were: 1) optimisation of digestate remediation using a microalgal consortium composed of three chlorophytes which were previously selected on the base of their growth in synthetic digestate; 2) evaluating the potential use of the algal extracts as plant biostimulants. So far, the consortium displayed higher growth performances and resilience compared to single species behaviour while biostimulant activity was observed both for consortium and monospecific samples. Results are promising and they bring us closer to a sustainable circular process.

Part 1. Scientific case of the PhD Research (2 to 3 pages, including figures)

BACKGROUND

Digestate is an ammonium-rich wastewater produced during the anaerobic digestion (AD) of biomass. As a secondary product of the process, it must be properly treated to reduce its nutrient and organic loads. Several research found that digestate can efficiently be used as a growth medium for microalgae, but its chemical composition, mainly depending on the anaerobic digestor input, can make the remediation process quite challenging. The digestate here used comes from the AD of olive mill wastewaters, silages and chicken manure and its resulting composition is a mixture of nutrients such as ammonium and phosphates, and phenols. The three of them when present at high concentrations, may cause toxicity as already demonstrated during the 1st year of PhD and in other research studies (Mollo et al., 2023, Q.Li et al., 2018, Ariz et al., 2011).

A microalgal consortium could be a way to reduce the effects caused by the digestate. It was proved that higher resilience and performance can be achieved using co-cultures of algae or algae-bacteria due to their cooperation and mutualism among single species resembling what occurs in a natural environment (Subashchandrabose et al., 2011). In a consortium remediation of pollutants is improved due to the synergy among algal metabolisms. Moreover, from ongoing studies, it was observed an allelopathic effect between species which led to an increased biomass production.

Biostimulation using macroalgae is a well-known phenomenon and tons of literature explored the benefits and potential of this approach; however, little is known about microalgae application on crops and even less about the application of a microalgal consortium. Moreover, it's a common use to apply only a fraction of the algal extract while discharging the remaining one. Our research tried to fill the gap by investigating the potential of a crude extract from a microalgal consortium as biostimulant.

SCIENTIFIC AIMS

The PhD project aims to develop a circular process where resource inputs (i.e. water, nutrients) are reduced and high-value outputs (i.e. algal biomass, clean water) are produced and maximized according to the principles of the circular economy. The algal biomass produced during the remediation process will be used to biostimulate crops; as a result further inputs to achieve high harvest yield will be reduced.

Based on 1st year results, a three-species algal consortium was chosen for the circular process. The 2nd year of PhD project focused on extending knowledge of the consortium, evaluating its potential for the remediation of a real digestate, and testing its biostimulant activity.

WORKPLAN AND RESEARCH ACTIVITIES

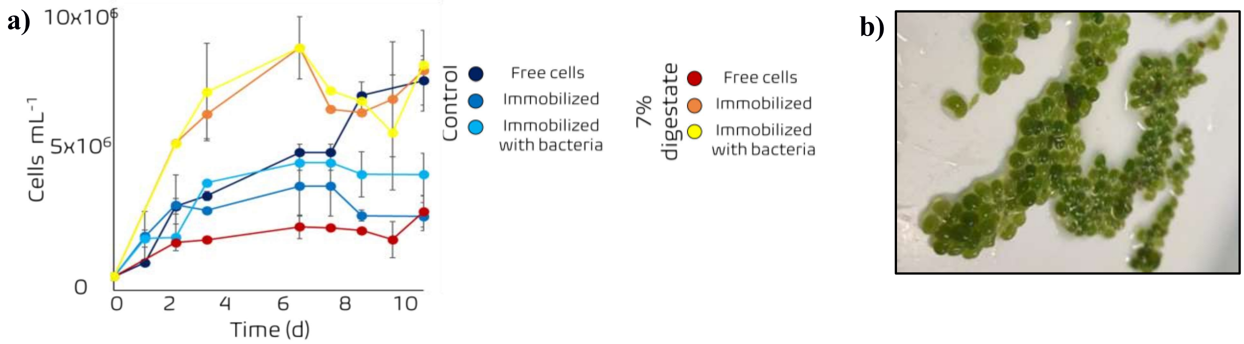
WP 1. Phycoremediation of digestate.

Methods. The consortium was kept and maintained for several generations in a standard growth medium (BG11) to reach a composition stability among species (*Tetradesmus obliquus, Chlamydomonas reinhardtii, Auxenochlorella protothecoides*). Growth curves were then carried out in increasing digestate concentrations (diluted in BG11) to find the best compromise between digestate dilution and algal growth. Once the proper concentration was chosen, growth was assessed by immobilising algae onto alginate beads with or without a probiotic bacterium (*Azospirillum brasilense*). Growth was daily followed and once the stationary phase was reached samples were observed using a cytofluorimeter to evaluate differences in shape and species-proportion.

Obtained Results. The maximum density was highly affected by digestate concentration. At the lowest concentration, 2%, the consortium had a higher density than the control. Even at 4%, the results were comparable to those in the control. Contrarily, concentrations higher than 10% were found to be deleterious to the consortium. Digestate concentration of 7% was found to be the highest concentration which still allowed an algal growth and was then chosen for further experiments.

The consortium was then acclimated to the growth medium modified with 7% digestate. Once cells were acclimated, growth was assessed as free cells or using the immobilisation approach (Fig. 1b) with or without probiotic bacteria (De-Bashan et al., 2004). Compared to the free cells condition, immobilised algae grew much better and reached values comparable to the control condition (Fig. 1a). The presence of the bacteria did not seem to significantly improve the growth performance of algae. Moreover, proportion variation among species was not affected by the growing condition as observed using the cytofluorimeter. The consortium then was stable and grew efficiently in the 7% digestate when immobilised onto alginate beads.

Figure 1: a) growth of consortium in standard growth medium (Control) and in 7% digestate. b) algae immobilized on alginate beads

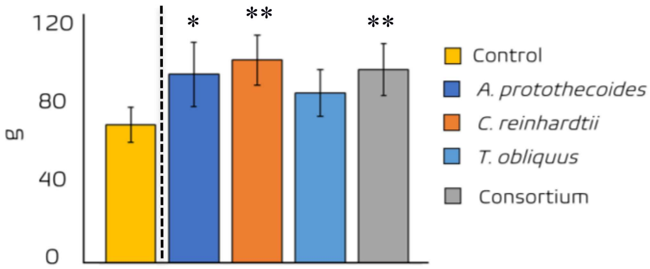


WP 2. Evaluation of microalgal biostimulation potential.

Methods. Crude extracts of the microalgal consortium and the single species were tested as biostimulants on *Diplotaxis tenuifolia* L. plants grown in a greenhouse under controlled environmental conditions. Crude extracts were made using a high-pressure disruptive cell system. The crude extracts were then kept at 4°C until plant application. A leaf treatment was carried out on plants at the full-expanded leaves stage: one treatment every 7 days for a total of 3 treatments. Physiological parameters related to chlorophyll *a* fluorescence, pigment, antioxidant, sugar and nitrate contents were evaluated after 48 and 72h from the treatment.

Obtained Results. Sample and data analysis are ongoing, but previous results showed us that each treated plant had a significative higher value of Fv/Fm (photosynthetic efficiency) than the control condition. Fresh weight of treated plants was significantly higher than control plants (*T. obliquus* as the only exception) (Fig. 2). In addition, plants were in better shape, and they had considerably bigger leaves.

Figure 2: Fresh weight of *Diplotaxis tenuifolia* L. after three weeks from the first algal application.

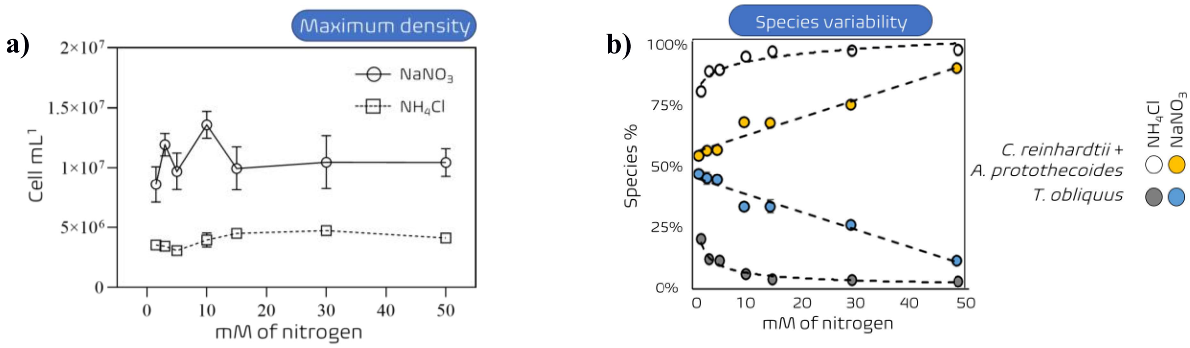


WP 3. Assessment of ammonium tolerance.

Methods. Based on Ariz et al. 2011 methodology, we assessed the growth, pigment composition, species proportion and isotope fractionation of the consortium with increasing concentrations of ammonium. Nitrate was used as N-source for the control condition and concentrations ranged from 1.5 to 50 mM. Isotope fractionation was assessed using a CHN/IRMS to evaluate a correlation between toxicity and $\delta^{15}N$.

Obtained Results. The N chemical form and concentration affected both growth rate and maximum density. At the lowest concentration, ammonium-supplied cultures had a higher growth rate than nitrate ones, however, with increasing N concentration the difference was less marked. On the contrary maximum density was always lower in the cultures supplied with ammonium compared to the density in control ones, proving that even at the lowest concentration (1.5 mM) ammonium was toxic (Fig. 3a). Also the chlorophyll content was highly affected by ammonium. Nonetheless, an interesting result was that both maximum density and chlorophyll content were not affected by ammonium concentration (in the experimental range). Such results suggest that ammonium exploited its effects even at small concentrations, but consortium proved high resilience maintaining a constant cell density. The high resilience of the consortium could be also shown by a variation in the proportion of the species: with increasing ammonium concentrations, the percentage of *T. obliquus* declined while *A. protothecoides* and *C. reinhardtii* increased (Fig. 3b). The adaptation of the consortium in different environmental conditions is the key to an effective consortium and it is the advantage compared to cultures of single species.

Figure 3: a) maximum density of the consortium. b) percentage of species in the consortium as observed with the cytofluorimeter.

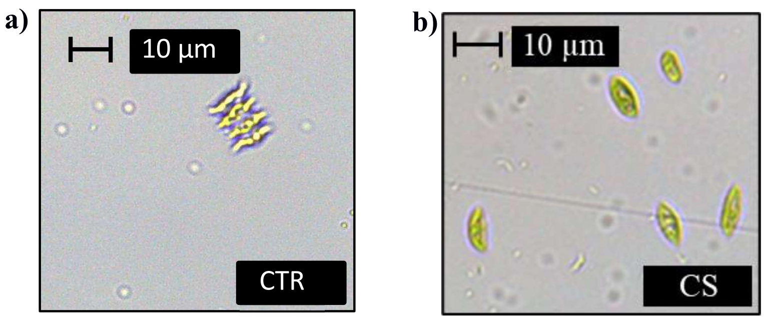


WP 4. Evaluation of allelopathic effect within consortium algal species (Ongoing).

Methods. The allelopathic effect of molecules released by algae was assessed using 0.2 µm filtered spent medium. The spent medium was obtained as the supernatant of algal cultures after centrifuge. Each species (and consortium) was grown in tubes with half volume of the medium being the spent medium and the remaining half volume a standard growth medium. Growth was daily followed in terms of cell density and biomass.

Partial Results. So far, the effect of allelopathic substances has been tested only on *T. obliquus*. It was observed that spent medium coming from *C. reinhardtii*, *A. protothecoides* and consortium cultures enhanced both the cell number and the biomass of the tested species. When *T. obliquus* was grown in its spent medium there was a significantly higher growth enhancement than using the other spent media as compared to the growth in control condition (*T. obliquus* grown in standard medium). A quite interesting effect was observed on the morphology of *T. obliquus* colonies: this species formed colonies of 4-8 cells in the control condition (Fig. 4a) and when supplied with *T. obliquus* spent medium; when other spent media were used as growth media no more colonies were visible (Fig. 4b). Similar effects have already been observed in other algae even if the context was different (prey-predator interactions) (Rigby, K., & Selander, E., 2021). More literature analysis should be carried out, nonetheless, it was proved that *T. obliquus* benefited from the co-cultivation with the other species of the consortium.

Figure 4: a) *T. obliquus* cultures in the control condition. b) *T. obliquus* cultures supplied with consortium spent medium



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Subashchandrabose, S. R., Ramakrishnan, B., Megharaj, M., Venkateswarlu, K., & Naidu, R. (2011). Consortia of cyanobacteria/microalgae and bacteria: biotechnological potential. Biotechnology advances, 29(6), 896-907.

Part 2. PhD student information on the overall year activity (courses/seminars/schools, mobility periods, participation to conferences)

List of attended courses/seminars/schools

1. Rischio climatico – Prof. Falco

2. Microbial-mediated processes in aquatic ecosystems: from basic to applied research toward solving environmental problems – Prof. Dell'Anno

3. FTIR and Raman Imaging – Prof- Gioacchini, Prof. Giorgini

List of conferences/workshops attended and of contributions eventually presented

1. Riunione annuale Algologia della Società Botanica Italiana. Ora presentation

2. III Workshop Aisam giovani, Poster presentation

3. EUBCE 2023: 31th European Biomass Conference & Exhibition. Oral presentation and student award in my symposium

4. EPC 2023: European Phycological Congress. Poster presentation

Part 3. PhD student information on publications

List of publications on international journals

J1. 2022 - Garofalo, C.; Norici, A.; Mollo, L.; Osimani, A.; Aquilanti, L. Fermentation of Microalgal Biomass for Innovative Food Production. Microorganisms 2022, 10, 2069. https://doi.org/10.3390/microorganisms10102069

J2. 2023 - Mollo, Lorenzo, Filippo Drigo, Matteo Moglie, Alessandra Norici. Screening for tolerance to natural phenols of different algal species: Toward the phycoremediation of olive mill wastewater. Algal Research 75, 2023. https://doi.org/10.1016/j.algal.2023.103256.

J3. 2023 - Mollo Lorenzo, Alessandra Petrucciani, Alessandra Norici. Growth and physiological analysis of microalgae in an artificial digestate with the perspective of sustainable phycoremediation (Ready for submission).

11/10/2023

Student signature

Supervisor signature