PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

Report Form for PhD student annual evaluation (XXXVI and XXXVII 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 supervisor: 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
[ ] Advanced Instrumentation lab
[X] Aquarium
[ ] MassSpec lab
[ ] MaSBiC
[ ] Simulation/informatics lab
[ ] Other. Please, indicate: .....

ABSTRACT
Many initiatives can be implemented in the fight against climate change: one of these is the exploitation of microalgae, microscopic and versatile organisms that can act as natural carbon sinkers and produce several innovative bio-products in alternative to products derived from fossil fuels, that can remediate wastewaters by removing organic carbon and mineral elements from the growth medium.
The main goal of the PhD project is to realise a circular process where microalgae will be used to remediate the liquid fraction of the digestate (a wastewater) coming from the anaerobic digestion of organic waste and the CO_2 extracted from biogas during the upgrade to biomethane [1,2]. The algal biomass produced during the remediation will be exploited as biostimulants to enhance crops performances [3]. So far, performance of several microalgae has been assessed in terms of growth potential in a synthetic digestate medium. The best species will be further grown as an algae-bacteria consortium in a pre-treated and diluted digestate.

Part 1. Scientific case of the PhD Research

SCIENTIFIC AIMS

The final aim of the PhD will be to develop a circular process where inputs (i.e. water, nutrients) will be reduced and high value outputs (i.e. algal biomass, clean water) will be maximized according to the principles of the circular economy. An LCA analysis will be further carried out to assess the environmental sustainability of the project.

The aim of the PhD first year was to identify algal species in monospecific cultures or co-cultures that were able to: 1) remediate effluents (e.g. digestate) deriving from the anaerobic digestion of agro-food waste products, 2) bio-stimulate growth and abiotic/biotic resistance of crops. The most suitable consortium was hypothesized according to scientific literature and then it was tested in simplified synthetic digestate. Optimization of algal growth and remediation yield was also preliminary assessed by modifying growth conditions.

WORKPLAN AND RESEARCH ACTIVITIES

WP 1. Literature analysis and choice of the proper algal species

Methods.

Main search engines such as *pubmed* and *google scholar* were used to obtain data and info about anaerobic digestion, remediation of digestate, biostimulant activity and CO2 tolerance by algae and algal growth at high CO_2 concentration. Several searches were made in order to choose 5 to 10 algal species with the following features:

1) **Freshwater species**: the wastewater produced during the anaerobic digestion does not contain high salt concentrations which could allow a proper growth of marine species; furthermore, enriching algal growth medium with salts would represent an additional cost and compromise the sustainability of the process.
2) **Mixotrophic species**; suitable algae must have a recognized ability to remediate wastewaters and their components: phosphorous, nitrogen, and organic carbon. Indeed, the digestate is a medium rich in minerals and simple organic molecules to be removed before it is released into the environment.
3) **Tolerance to high CO_2 concentration**; to upgrade the biogas produced during the anaerobic digestion to biomethane, the extra CO_2 will be used as additional carbon source for the algal growth. Microalgae should tolerate and fix CO_2 at higher concentrations than the atmospheric one.
4) **Biostimulant activity**: experimental algae should be already tested as biostimulants. Biostimulation should improve plant growth by enhancing plant resistance against abiotic and biotic stresses.

Obtained Results.

Based on the literature research, 8 species were chosen and each of them had the desidered features. The algal species were: *Auxenochlorella protothecoides, Chlamydomonas reinhardtii, Tetradesmus obliquus, Chlorella vulgaris, Euglena gracilis, Arthrospira platensis, Anabaena sp., Synechocystis sp.*.

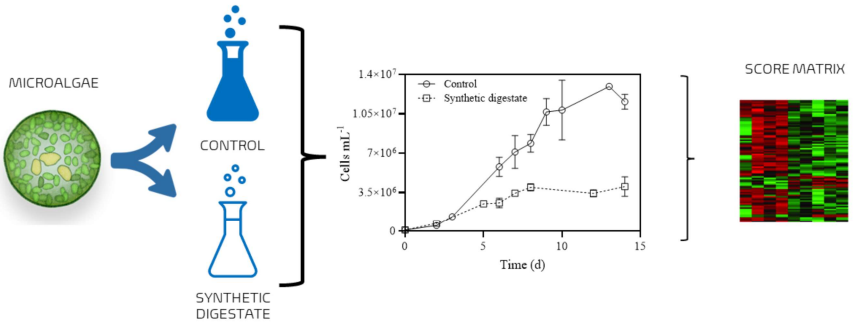
The species were acquired from CCAP algal collection or they were already present in the research lab.

WP 2. Experimental selection of microalgae through a synthetic media

Methods.

Algal species selected from the literature as already described, were maintained in standard growth medium until the beginning of the experiment. Experimental design is graphically reported below and, as a first step, it required algal cultivation in a synthetic medium mimicking a simplified digestate. The synthetic medium contained half of the average digestate concentrations of ammonia, phosphates and organic carbon (e.g. sodium acetate) [4]. Algae were grown in three independent replicas for a total of 15 days at 20°C, 100 µmol photons m^{-2} s^{-1}, continuous light; growth in standard growth medium was used as control condition. To assess algal growth performance and remediation yield, several parameters were measured during growth: cells density, [PO_4], [NH_4], [proteins], [pigments], [trace elements], C/N ratio. A score matrix was used to select the species whose growth and mineral remediation were the highest.

Obtained Results.



Data regarding mineral remediation and algal growth in the synthetic digestate are reported in the following table. Except for *Anabaena sp.*, all species were able to survive the change in external medium and increase in cell density even if growth rates were considerably lower than the one in control cultures. Cyanobacteria were more affected by the synthetic digestate than the other strains: nutrients removal and growth rate were quite low for *Arthrospira platensis* and *Synechocystis*. Nitrogen was highly removed from the medium even if ammonium/ammonia at high concentrations is toxic for most of the algal organisms. Contrary, phosphate remediation was lower than we expected and only *Euglena gracilis* reached a remediation yield higher than 50%.

Through the elaboration of the data achieved during the experiments a score matrix was made and the following rankingwas obtained: 1) *A. protothecoides,* 2) *C. reinhardtii*, 3) *T. obliquus*, 4) *E. gracilis*, 5) *C. vulgaris*, 6) *A. platensis*, 7) *Synechocystis sp.,* 8) *Anabaena sp.* Due to the high scores obtained by the first four species it was decided to use these species for future experiments.

Growth parameters and remediation yield - *Values*

Species	µ (d^{-1})	Productivity (mg mL^{-1} d^{-1})	Biomass at stationary phase (mg mL^{-1})	Nitrogen removal (%)	Phosporous removal (%)
Auxenochlorella protothecoides	0,530 ± 0,025	0,247 ± 0,042	1,578 ± 0,208	89% ± 0,4%	57% ± 3%
Tetradesmus obliquus	0,627 ± 0,014	0,110 ± 0,013	0,794 ± 0,038	94% ± 0,2%	42% ± 13%
Chlamydomonas reinhardtii	0,510 ± 0,009	0,298 ± 0,018	1,625 ± 0,049	90% ± 0,1%	30% ± 3%
Euglena gracilis	0,243 ± 0,005	0,143 ± 0,017	0,926 ± 0,182	91% ± 0,2%	61% ± 1%
Chlorella vulgaris	0,550 ± 0,015	0,081 ± 0,014	0,644 ± 0,183	92% ± 0,1%	36% ± 10%
Synechocystis sp	0,075 ± 0,034	0,014 ± 0,010	0,415 ± 0,046	83% ± 0,1%	32% ± 4%
Anabaena sp	-0,531 ± 0,077	-0,028 ± 0,000	0,000 ± 0,000	32% ± 1,9%	12% ± 97%
Arthrospira platensis	0,365 ± 0,042	0,168 ± 0,068	0,856 ± 0,128	66% ± 7,0%	21% ± 91%

WP 3. Optimization of the remediation and algal consortia analysis

Methods.

The four species selected were further studied to optimize their remediation and biomass values. Based on the previous results, it was decided to assess two additional growing conditions:

1) *Growth in potassium enriched synthetic digestate.* The NH_4^+ ion can be uptaken by algal cells in several ways, one of these is through the K^+ ion channel becoming a competitor of this element [5,6]. According to our hypothesis, the addition of potassium to the external medium would result in a lower ammonia toxicity for the algae by reducing its flux inside the cell and re-establishing intracellular K^+ quota. The N:K^+ proportion present in the standard medium was tested.
2) *Enhanced phosphorus removal by phosphorous starved algae in synthetic digestate.* Phosphorous acquisition by algal cells is governed by a luxury uptake mechanism [7,8]. The mechanism allows cells to uptake a larger amount of phosphorous than immediately needed during the first stages of growth and store it as polyphosphate granules. This reservoir is then used during time to provide phosphorus to daughter cells. The hypothesis was to enhance phosphorous uptake by using starved cells [9], thus by stimulating the luxury uptake mechanism. Cells were kept in standard media depleted with phosphorous for about ten days; after the starvation step growth and P removal in synthetic digestate were assessed.

Obtained Results.

Data analysis is still ongoing; however, some considerations may be done on the growth performance of the species in the two conditions. The phosphorous starvation contributes to the lowest growth and biomass values across the different conditions, hence excluding this treatment for further application. The addition of potassium affected algal growth in different ways depending on the species: while *T. obliquus* was highly favoured by the supplementation of K^+ and reached biomass values comparable to those of the control, *A. protothecoides* and *C. reinhardtii* were negatively affected, their growth (μ_{max} and maximal density) was lower as compared to the growth in standard medium and in synthetic digestate without K^+ addition.

From our study, P-starvation did not contribute to a higher phosphorous removal rate and higher biomass values. On the contrary, the addition of K^+ could lead to a growth comparable to the one in control conditions while maintaining a high remediation yield.

WP 4. First insight into the biostimulant activity of *Chlamydomonas reinhardtii*

Methods.

Chlamydomonas reinhardtii, as one of the species selected from the growth in the synthetic digestate, was tested for a primary experiment of biostimulation on rucola (*Eruca vesicaria ssp. sativa*). The experiments were carried out in collaboration with the Agricultural department of University of Milano. *C. reinhardtii* was grown in standard growth medium for 15 days at 20°C, 100 µmol photons m^{-2} s^{-1}, 12:12 light photoperiods in a 50 L closed vertical photobioreactor present in the aquarium facility of UNIVPM. Algae in stationary phase were harvested by centrifugation and concentrated at about 30 g/L. A cell disruption method using high pressure was carried out and the algal extract was finally stored at 4°C until the biostimulation experiments. The algal extract was then diluted at several desired concentrations (1-5 mg mL^{-1}) and sprayed on the leaves of the plants. Growth performance and responses were assessed based on standard parameters: chlorophylls content, Fv/Fm and photosynthesis performance, sugars content, leaves surface of the plants .

Obtained Results.

Preliminary results suggest that *C. reinhardtii* has a high biostimulant activity even at low concentrations. Further studies are required since different concentrations of the algal extract may enhance or even repress the growth of *Eruca vesicaria*

REFERENCES

1. Bahr, M.; Díaz, I.; Dominguez, A.; González Sánchez, A.; Muñoz, R. Microalgal-Biotechnology As a Platform for an Integral Biogas Upgrading and Nutrient Removal from Anaerobic Effluents. *Environ. Sci. Technol.* **2014**, *48*, 573–581, doi:10.1021/es403596m.
2. Koutra, E.; Grammatikopoulos, G.; Kornaros, M. Selection of Microalgae Intended for Valorization of Digestate from Agro-Waste Mixtures. *Waste Management* **2018**, *73*, 123–129, doi:10.1016/j.wasman.2017.12.030.
3. Colla, G.; Rouphael, Y. Microalgae: New Source of Plant Biostimulants. *Agronomy* **2020**, *10*, 1240, doi:10.3390/agronomy10091240.
4. Vasseur, C.; Bougaran, G.; Garnier, M.; Hamelin, J.; Leboulanger, C.; Chevanton, M.L.; Mostajir, B.; Sialve, B.; Steyer, J.-P.; Fouilland, E. Carbon Conversion Efficiency and Population Dynamics of a Marine Algae–Bacteria Consortium Growing on Simplified Synthetic Digestate: First Step in a Bioprocess Coupling Algal Production and Anaerobic Digestion. *Bioresource Technology* **2012**, *119*, 79–87, doi:10.1016/j.biortech.2012.05.128.
5. Andrews, M.; Raven, J.A.; Lea, P.J. Do Plants Need Nitrate? The Mechanisms by Which Nitrogen Form Affects Plants: Do Plants Need Nitrate? *Ann Appl Biol* **2013**, *163*, 174–199, doi:10.1111/aab.12045.
6. Szczerba, M.W.; Britto, D.T.; Kronzucker, H.J. K+ Transport in Plants: Physiology and Molecular Biology. *Journal of Plant Physiology* **2009**, *166*, 447–466, doi:10.1016/j.jplph.2008.12.009.
7. Brown, N.; Shilton, A. Luxury Uptake of Phosphorus by Microalgae in Waste Stabilisation Ponds: Current Understanding and Future Direction. *Rev Environ Sci Biotechnol* **2014**, *13*, 321–328, doi:10.1007/s11157-014-9337-3.
8. Portielje, R.; Lijklema, L. Kinetics of Luxury Uptake of Phosphate by Algae-Dominated Benthic Communities. 10.
9. Solovchenko, A.; Khozin-Goldberg, I.; Selyakh, I.; Semenova, L.; Ismagulova, T.; Lukyanov, A.; Mamedov, I.; Vinogradova, E.; Karpova, O.; Konyukhov, I.; et al. Phosphorus Starvation and Luxury Uptake in Green Microalgae Revisited. *Algal Research* **2019**, *43*, 101651, doi:10.1016/j.algal.2019.101651.

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. Design of research – Prof. Paone

2. Technology transfer and innovation – Prof. Iacobucci

3. Getting started with R – Dr. D'Errico

4. Environmental sustainability: the life cycle assessment, LCA – Dr. Amato

5. Analisi di regressione mediante Microsoft Excel – Prof. Beolchini

6. APRE 2021: Horizon Europe: European Partnership

7. CCAP 2022: Algaculture for biotechnology

List of conferences/workshops attended and of contributions eventually presented

1. CEWS 2021: Circular Economy for the Sustainable Bio-based Products: from waste to soil

2. CIRCC 2022: XVIII PhD Day → contribute with the following presentation: "Phytoremediation of wastewaters using microalgae"

3. EUBCE 2022: 30th European Biomass Conference & Exhibition

4. La domesticazione delle Alghe: Cibo del Futuro? → 21 Febbraio 2022, conferenza organizzata da collettivo Gulliver. Conferenza organizzata assieme alla docente di Algae in Human nutrition Alessandra Norici

Part 3. PhD student information on publications

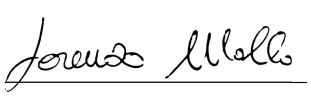
List of publications on international journals

J1. Submitted and under minor revision → Garofalo C., Norici A., **Mollo L.**, Osimani A., Aquilanti L. "Microalgae fermentation for innovative food production", Microorganisms

J2. In preparation → **Mollo L.**, Drigo. F., Norici A. "Toxicity of three Olive Mill WasteWaters phenols on several microalgae strains: a comparison"

[Date] 14/10/2022

Student signature



Supervisor signature