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Communication

First application of different *Chlorella* sp. microalgal strains for the treatment of vegetation waters derived from unconventional oil extractions enriched with citrus byproducts

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Abstract: The Mediterranean diet has among its cornerstones the use of olive oil for its nutraceutical and organoleptic properties. Despite the numerous merits, olive-oil mill wastewater (OMWW), which is generated by the olive-oil extraction process, is one of the most serious environmental pollutants in the Mediterranean countries. The polluting potential of OMWW is due to its high content of tannins, polyphenols, polyalcohols, pectins and lipids. In this experiment, we tested the ability of five microalgae of the *Chlorella* group (SEC_LI_ChL_1, CL-Sc, CL-Ch, FB and Idr) in lowering the percentage of total phenolic compounds in vegetation water. In order to close the recovery cycle of a fortified citrus olive oils previously developed, we tested the vegetation water obtained with three different extraction processes (conventional, lemon and orange peels) at three concentrations each (10%, 25% and 50%). Results showed that strains Idr, FB and CL-Sc from the Lake Massaciuccoli can tolerate vegetation water from conventional and lemon peels extraction up to 25%; these strains can also reduce the phenolic compounds within the tests. The results demonstrate that the application of microalgae for OMWW treatment represent an interesting opportunity, and an eco-friendly low-cost solution to be developed within the companies as a full-scale approach.

Keywords: olive-oil mill wastewater; wastewater; microalgae; *Chlorella* sp.; phenolic compounds

1. Introduction

One of the biggest problems in olive oil production is the enormous amount of solid and liquid waste produced by the extraction process, called olive oil mill wastewater (OMWW). The high phenolic nature of OMWW and its organic contents make it highly resistant to biodegradation. Their composition is variable depending on a wide range of influences such as climate, cultivation and the particular milling method used in oil extraction [1, 2].

According to literature, chemical oxygen demand (COD) of OMWW samples can range from 35 to 200 g/L, biochemical oxygen demand (BOD) from 15 to 135 g/L, suspended solids (SS) from 6 to 69 g/L, total phenols from 2 to 15 g/L, while pH ranges from 4.5 to 5.8

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[3]. This is one of the highest organic loads of all known concentrated effluents and it is 100–200 times higher than domestic wastewater.
A number of processes have been previously used for OMWW treatment including lagooning, physio-chemical treatment, electro coagulation, Fenton and electro Fenton processes [1]. Chemical treatment is the most common for OMWW with membrane separation [4]. However, reports indicate significant disadvantages in these treatment methods and showed that no single technology can treat OMWW effectively as a stand-alone process [1].
In the last decades, the increasing need in finding new alternative, eco-friendly and economically sustainable solutions to the common water treatments brought to the development of biological strategies; these last are mainly based on the use of microbial biomasses that help providing for almost of the total removal of nitrogen [5], sulfur [6], phosphate [7], as well as BOD and COD (Biological/Chemical Oxygen Demand) decrease. Microbial biomasses commonly used in wastewater (WW) treatment are mainly composed by bacteria, but a central role is also played by fungal biomasses [8], protists [9], and microalgae [10]. It has been widely demonstrated that microalgae can find an optimum growth medium both in domestic [11] and in industrial WW [12], because they require high amounts of nitrogen and phosphorous for their growth, as well as organic matter and carbon sources, that are all present in large quantities in WWs [13]. Microalgae are able to significantly decrease the amount of nutrients, and so, to reduce BOD [14]. In this context, the application of microalgae could represent a useful alternative in processing methods for OMWW treatment, and represents a possible solution to develop a new agro-food chains derived by a food waste.
In the perspective of the food supply chains sustainability, in a previous work [15] the waste from the citrus fruit supply chain (orange and lemon peels) were used in order to obtain, through a process of cryomaceration and subsequent co-extraction of citrus peels with olives, fortified citrus olive oils, with enhanced nutritional properties. However, the produced oils, despite the inestimable qualities and virtues conferred by the bioactive compounds of citrus fruits, originate production waste that is difficult to dispose of, as they are particularly rich in bioactive compounds. In this context, the first purpose of the following work was therefore to close the recovery cycle of the citrus flavored olive oils, promoting the removal of the originated OMWW; the second, was to test whether microalgae could represent putative candidates for the treatment of vegetation water, and if they were able in reducing the level of phenolic compounds from the matrix. The vegetation water obtained with three different extraction processes [15] were evaluated (conventional, lemon and orange peels) at three concentrations each (10%, 25% and 50%). Five environmental microalgal strains belonging to the *Chlorella* group were tested in a 7-days experiment, in presence of the different crude vegetation waters. The quantification of photosynthetic pigments was considered as growth parameter for the microalgal strains exposed to the different treatments [16], compared to the control test (the growth in absence of vegetation water, indicated as 0%). At the end of the experiment, the reduction of the phenolic compounds was evaluated.
To the best of our knowledge no data are available about the use of these specific *Chlorella* strains in treatment of OMWW nor citrus OMWW.

2. Materials and Methods

2.1 Microalgal strains and growth conditions

The five microalgal strains selected for this experiment were of environmental origin. Strain SEC_Li_ChL_1 [17] was sampled in an artificial lake at the "Rosignano Energia Ambiente. S.p.A." location (now called "Scapigliato Energia s.r.l. Company") in Rosignano Marittimo (LI), Italy (43° 27′ 45.34′′ N, 10° 28′ 24.42′′ E). The strain was characterized as belonging to the *Chlorella*-Micractinium clade. Strains Idr, CL-Sc, CL-Ch and FB were sampled from the Lake Massaciuccoli, in different sites of the Lake area; they were all characterized as belonging to the *Chlorella sorokiniana* group [18]. All five strains were

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grown in Tris-Ammonium Phosphate (TAP) medium [19] and maintained in the laboratory collection of the Dept. of Agriculture Food and Environment under the growth conditions described in Chiellini et al., [16].

2.2 Citrus Olive Oil Extraction and wastewater sample

Orange and lemon citrus peels (came from Massa) were cryomacerated with solid carbon dioxide (1:1 in weight) overnight and then directly added (22% in weight) to olives before milling. The extraction was carried out using a micro oil mill (Oliomio Baby®, by "Toscana Enologica Mori", Tavernelle Val Di Pesa, Florence, Italy) able to mill 20–30 kg of olives. The technical characteristics of the micro oil mill and the working conditions used followed the method previously described (Flori et al., 2020).
After the olive oil extraction, the wastewater was separated by the olive pomace with a laboratory centrifugation treatment (4000 rpm x 5 minutes). All the samples obtained were stored at 4°C under Nitrogen for 24 h before the microalgae treatment.

2.3 Experimental setup

Two distinct experiments were carried out, both monitored for one week, according to Chiellini et al [16], and under a light condition of 16/08 h day-night cycle with PPFD of 120 mmol photons m-1 s-1 from cool-white light lamps (Gavita Lep 330 Plasma fixtures, Gavita Holland Light Emitting Plasma, Netherlands), and a constant 22/24°C temperature. Both experiments were conducted in sterile glass tubes, in a total volume of 20 mL. In both experiments, for each kind of vegetation water three dilutions (in TAP medium) were tested in triplicate: 10%, 25% and 50%; a triplicate control test (100% TAP medium) was included in the experiments, hereinafter indicated as "Ctrl_0%" test. In the first experiment, all the five strains (SEC_Li_ChL_1, Idr, CL-Sc, CL-Ch and FB) were tested against the crude vegetation water without any preliminary treatment. Three different vegetation waters were tested: i) conventional, ii) lemon peels and iii) orange peels olive oil extraction.
In the second experiment, a subset composed of 3 strains was selected, including those that survived in vegetation water in the first screening (Idr, CL-Sc and FB). The vegetation water obtained from orange peels was removed from the experimentation since results from the previous test revealed that no strains were surviving in its presence, and those who survived were not able to significantly reduce the total phenolic compounds. Finally, in this second experiment, a pre-treatment to vegetation water, consisting in a 5 minutes centrifuge at 800 g was carried out, in order to simulate a decantation process that might occur naturally within a treatment plant. The supernatant obtained after centrifuge was used for the test.

2.4 Growth parameters and total phenolic compound reduction measure

Microalgal growth parameters measured at the end of the experiment were chlorophyll a, chlorophyll b and total chlorophyll content. The photosynthetic pigment extraction was performed in 100% acetone, using the procedure described in Chiellini et al [16]. Briefly, 1 ml of each sample-test was centrifuged (1500 rpm, 5 min at 4°C) and the pellet was resuspended in 1 ml 100% acetone (Sigma Aldrich, MI, United States) and submitted to 10 min sonication (Branson 1210, Bransonic Ultrasonic Cleaner, United States). Samples were then kept in the dark at 4°C overnight, and subsequently centrifuged (12,000 rpm, 5 min). All the centrifuges were performed in a Speedmaster 14R, Euro Clone, Milano (Italy). Finally, the absorbance of the supernatant was spectrophotometrically analyzed (UV-1800 Spectrophotometer, Shimadzu, Japan) with regards to the blank at 661.6 and 644.8 nm, according to the equations indicated in Lichtenthaler [20]. The carotenoid content was not measured since the emission spectra of the three different vegetation waters were interfering with the wavelength of the carotenoids (470 nm, data not shown). According to Chiellini et al [16], once the pigment concentrations of the three replicates were calculated,

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the average value was calculated; data were then elaborated and expressed as % compared to the pigment content of T0, representing the beginning of the experimentation.

2.5 Analysis of the Phenolic Content

Phenolic substances were determined by Folin-Ciocoltau method as previously described by Flori et al., 2020. The determination of the total phenols content was expressed in g/L of gallic acid. The phenolic reduction was calculated following the equation (1):

$$\%\ \boldsymbol{reduction} = \left(\frac{C_0 - C_f}{C_0}\right) \times \mathbf{100} \qquad (1)$$

where C_0 and C_f are respectively the concentration of phenols at the beginning and at the end of the experiment.

2.6 Statistical analysis

The results are the means ± CI (Confidence Interval) values of three independent experiments. The significance of differences among means was determined by one-way ANOVA (CoStat, Cohort 6 software). Comparisons among means were performed by Tukey's test ($p < 0.05$).

3. Results and discussion

In both the experiments all the microalgal strains were able to grow in the experimental conditions in absence of vegetation water for the whole experimental duration: accordingly, an increment in total chlorophyll content was measured for all five microalgal strains after 1-week (i.e. T0 values vs. Ctrl_0%, Figure. 1).

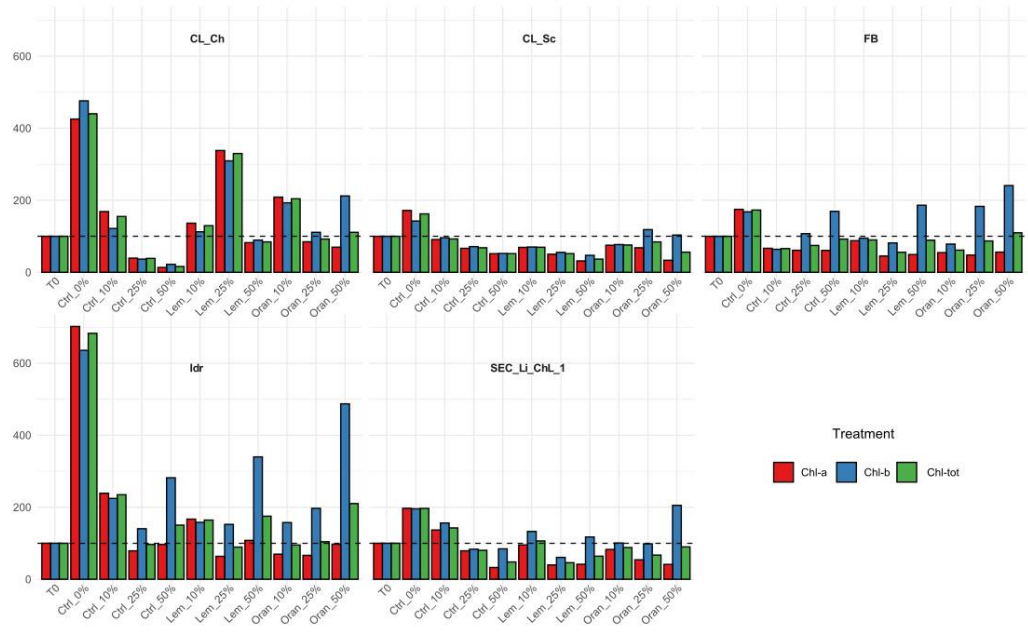

Figure 1. Pigment contents of the first screening experiment expressed as % compared to the pigment content of T0 (the microalgal culture at the beginning of the experiment). The 100% pigment content of T0 is represented by the dashed line. "Ctrl" samples: vegetation water obtained with traditional method; "Lem" samples: vegetation water obtained with lemon peels extraction; "Oran" samples: vegetation water obtained with orange peels extraction.

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This result confirms that any observed growth variation, might depend on the presence of the treatment with vegetation water. The 7-days experimental duration was chosen on the base of previous conducted experiments with the same algal species [16, 18], and also on the base of similar experiments testing microalgae in presence of OMWW (i.e. 10 days duration) [21].

The five treated microalgae replied very differently to the vegetation water samples, especially when different dilutions were used. In fact, some microalgae have shown a greater aptitude to adapt to higher concentrations of OMWW and others to a lower one. This observation is in line with previous data on the same microalgal species exposed to different environmental contaminants [16, 18]. According to the measure of photosynthetic pigments content, in the first screening experiment (Figure. 1) a % increment in chlorophylls content in presence of vegetation waters was observed mainly in CL-Ch and Idr strains under the treatments Ctrl_10%, Lemon 10% and 25% and Orange 10%; in CL-Sc and FB treated with Ctrl_10%, and in SEC_Li_ChL_1 exposed to Ctrl_10% vegetation water. Overall, the only strain that seemed to not tolerate the treatments (about 20-35% photosynthetic pigment content respect to T0) was strain CL-Ch exposed to Ctrl 25% and 50% vegetation water.

Despite the fact that strain CL-Ch is the one showing the greatest percentage increment in photosynthetic pigment content, especially in presence of Lemon 25% treatment, the values of the reduction of phenolic compounds of this strain were not the most promising, accounting for 10.4% (table 1).

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Table 1. Total phenols at the starting time and their percentage reduction after 1-week treatment in the preliminary screening performed on all the five microalgal strains (only best data are reported).

	OMWW sample	Total phenols at the starting time (g/L gallic acid)	% Reduction $[((C_0 - C_f)/C_0) \times 100]$
SEC_LI_ChL_1	10% CONTROL	0.39±0.05	5.3±1.1
	10% ORANGE	0.58±0.02	1.3±1.0
	10% LEMON	0.35±0.01	2.5±1.3
	25% CONTROL	0.82±0.05	21.0±1.1
CL-SC	10% CONTROL	0.39±0.04	18.7±1.0
	10% ORANGE	0.58±0.02	9.9±1.0
	10% LEMON	0.35±0.01	24.0±1.3
	25% CONTROL	0.82±0.06	27.0±1.0
	25% LEMON	0.73±0.03	28.2±1.0
	50% CONTROL	1.54±0.05	30.3±1.2
CL-CH	10% CONTROL	0.39±0.03	13.9±1.0
	10% ORANGE	0.58±0.02	21.8±1.1
	10% LEMON	0.35±0.06	26.5±1.1
	25% ORANGE	1.31±0.03	11.8±1.0
	25% LEMON	0.73±0.02	10.4±1.0
	50% LEMON	1.36±0.04	4.0±1.2
IDR	10% CONTROL	0.39±0.02	45.8±1.0
	10% LEMON	0.58±0.01	53.9±1.3
FB	10% CONTROL	0.39±0.06	32.4±1.0
	10% ORANGE	0.58±0.03	16.5±1.3
	10% LEMON	0.35±0.02	33.5±1.2
	25% CONTROL	0.82±0.01	17.5±1.0
	25% LEMON	0.73±0.04	18.4±1.1

Values are the mean of three technical replicates +/CI

According to phenols reduction, this microalgal strain seems to prefer OMWW obtained from pressing with citrus fruits, orange and lemon at lower concentrations (10%), as shown in table 1 by the reduction values of 21.8 and 26.5, respectively.
Interestingly, despite strain SEC_LI_ChL_1 exposed to Ctrl_25% treatment did not highlight an increment in pigment content respect to the T0 (Figure. 1), it showed a significant reduction of phenolic compounds (21%), unlike the other samples whose reduction appears negligible.
Strain CL-Sc is apparently more predisposed to adaptation and this translates into a more effective phenolic reduction. In particular, some tendency can be seen for OMWW at higher concentrations (25 and 50%) in both the Lem and Ctrl treatments. Analogously to strain SEC_LI_ChL_1, also in this case the data related to the growth increment (i.e. increase in pigment content respect to the T0, Figure. 1) does not reflect the tendency of CL-Sc in reducing phenolic compounds. On the light of such data, we can hypothesize that

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despite a reduced growth of this strain in presence of 25% and 50% Lemon and Control treatments, the microalgal strain is much more effective in reducing phenols.
According to phenolic compounds reduction, strain Idr seems to be the best microalgae tested, with a greater adaptation to low treatments concentrations (10%) and especially in presence of lemon treatment, recording a phenolic reduction of 53.9%, the highest ever measured. As previously stated, this phenols reduction reflects an increment in pigment content respect to the beginning of the experiment (T0), suggesting not only a decontamination activity of such strain, but also a growth in terms of biomass. The peculiar behavior of this strain compared to the other ones isolated from Lake Massaciuccoli, somehow reflects its "geographical" and "phylogenetic" isolation, as previously discussed [16].
Finally, FB has demonstrated excellent potential for use in phenol reduction, with better results at low concentrations (10%) with both Lem and Ctrl treatments.

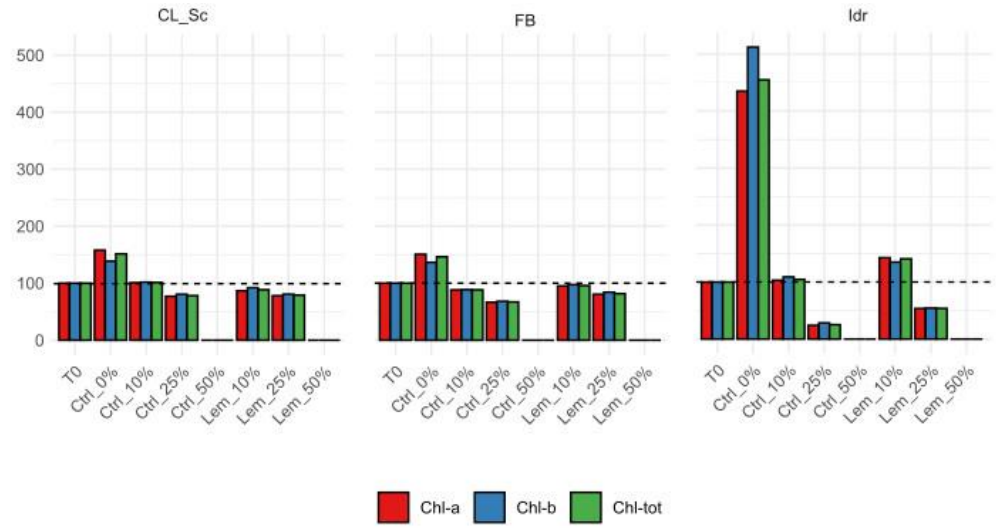

Figure 2. Pigment contents of the second experiment expressed as % compared to the pigment content of T0 (the microalgal culture at the beginning of the experiment). The 100% pigment content of T0 is represented by the dashed line. "Ctrl" samples: vegetation water obtained with traditional method; "Lem" samples: vegetation water obtained with lemon peels extraction; "Oran" samples: vegetation water obtained with orange peels extraction.

According to the results of the first experiment, the second experiment was performed on 3 microalgal strains (Idr, CL-Sc and FB), and on the vegetation water from conventional extraction method and from extraction with lemon. Results related to the microalgal growth (Figure.2), were in line with the preliminary experiment showing that all the three strains were able to survive in presence of vegetation water from conventional (Ctrl) and from lemon (Lem) method, up to a concentration of 25%.

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Table 2. Total phenols at the starting time and their percentage reduction after 1-week experiment in the second experiment performed on the best microalgae strains (only best data are reported).

	OMWW sample	Total phenols at the starting time (g/L gallic acid)	% Reduction $[((C_0 - C_f)/C_0) \times 100]$
CL-SC	10% CONTROL	0.39±0.03	24.4±1.0
	10% LEMON	0.35±0.04	35.3±1.1
	25% CONTROL	0.82±0.01	32.0±1.1
	25% LEMON	0.73±0.05	34.9±1.0
	50% CONTROL	1.54±0.06	52.9±1.0
IDR	10% CONTROL	0.39±0.02	45.7±1.3
	10% LEMON	0.35±0.04	44.8±1.0
FB	10% CONTROL	0.39±0.03	26.7±1.0
	10% LEMON	0.35±0.02	34.0±1.3
	25% CONTROL	0.82±0.03	45.1±1.2
	25% LEMON	0.73±0.05	50.3±1.0

Values are the mean of three technical replicates +/CI

Analyzing the percentage of reduction of phenolic compounds (Table 2), it is possible to observe that strain CL-Sc, as previously shown, adapts very well to high concentrations of OMWW, showing a reduction of phenolic compounds of 52.9% with the traditional vegetation water treatment. Strain Idr records a reduction of approximately 45% in both tested samples (control and lemon), with a better yield at low concentrations (Table 2). Finally, strain FB works very well at intermediate OMWW concentrations in both tested samples (Ctrl and Lem) even if the best results are obtained with the lemon OMWW sample, with a reduction of 50.3%.

Despite the use of microalgal strain for the treatment of olive mill wastewater is a still almost unexplored field, some experiments demonstrated the ability of two commercial strains, namely "Scenedesmus quadricauda, no. 76 of the Algal Collection at the University of Texas at Austin (UTEX), and Ankistrodesmus braunii, no. 202.7a of the Culture Collection of Algae and Protozoa, Cumbria, UK", in the reduction of phenol contents in olive oil wastewater [22]. Interestingly, the two strains were efficient in removal in dark conditions, and thus under a heterotrophic metabolism. In both the experiments here described, the microalgae were exposed to light for the whole experimental duration (16/08 h day-night cycle with PPFD of 120 mmol photons m-1 s-1). Anyway, if we consider that the vegetation water has a purple dark color at all the tested concentrations, we are not completely aware by the possibility that our strains, might have performed heterotrophic metabolism as well, since it is already documented that *Chlorella*-like strains from environmental origin are able to growth and survive in dark conditions [17].

4. Conclusions

In conclusion, strains CL_Sc, Idr and FB from the Lake Massaciuccoli, all related to the *Chlorella sorokiniana* clade, showed a high potential to reduce total phenolic compounds from the OMWW, opening new perspectives for the biological treatment of such wastewater.

In other hand, the OMWW represent three different culture medium, with different potential for adaptation; in particular, the orange OMWW was the least effective, on the contrary, the lemon and control OMWW represent excellent candidates for an industrial application.

This experiment, tested on five different microalgal strains of environmental origin, allowed us to verify the possibility of adaptation to OMWW, brought about good results for a possible use at an industrial level, with the aim of reducing pollution by the olive oil supply chain and with the intend to generate by-products that can be used in different chains for the production of cosmetics and food supplements.

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References

[1] Kiril Mert, B., Yonar, T., Yalili Kiliç, M., Kestioğlu, K. Pre-treatment studies on olive oil mill effluent using physicochemical, Fenton and Fenton-like oxidations processes. J Hazard Mater 2010, 174 (1–3), 122–128. DOI: 10.1016/j.jhazmat.2009.09.025

[2] Venturi, F., Sanmartin, C., Taglieri, I., Nari, A., Andrich, G., Terzuoli, E., Donnini, S, Nicolella, C., Zinnai, A. Development of Phenol-Enriched Olive Oil with Phenolic Compounds Extracted from Wastewater Produced by Physical Refining. Nutrients 2017, 9, 916. DOI: 10.3390/nu9080916

[3] Bettazzi, E., Morelli, M., Caffaz, S., Caretti, C., Azzari, E., Lubello, C. Olive mill wastewater treatment: an experimental study. *Water Sci Technol* **2006**, 54 (8) 17–25. DOI: 10.2166/wst.2006.781

[4] Rahmanian, N., Jafari, S., Galanakis, C. Recovery and Removal of Phenolic Compounds from Olive Mill Wastewater. *Journal of Oil & Fat Industries*, **2014**, 91, 1-18. DOI: https://doi.org/10.1007/s11746-013-2350-9

[5] Chiellini, C., Munz, G., Petroni, G., Lubello, C., Mori, G., Verni, F., Vannini, C. Characterization and comparison of bacterial communities selected in conventional activated sludge and membrane bioreactor pilot plants: a focus on Nitrospira and Planctomycetes bacterial phyla. *Curr Microbiol* **2013**, 67(1), 77-90. DOI: https://doi.org/10.1007/s00284-013-0333-6

[6] Vannini, C., Munz, G., Mori, G., Lubello, C., Verni, F. Petroni, G. Sulphide oxidation to elemental sulphur in a membrane bioreactor: Performance and characterization of the selected microbial sulphur-oxidizing community. *Syst Appl Microbiol* **2008**, 31(6-8), 461-473. DOI: https://doi.org/10.1016/j.syapm.2008.07.003

[7] Rout, P.R., Bhunia, P. Dash, R.R. Simultaneous removal of nitrogen and phosphorous from domestic wastewater using Bacillus cereus GS-5 strain exhibiting heterotrophic nitrification, aerobic denitrification and denitrifying phosphorous removal. *Biores Technol* **2017**, 244, 484-495. DOI: https://doi.org/10.1016/j.biortech.2017.07.186

[8] Siracusa, G., Yuan, Q., Chicca, I., Bardi, A., Spennati, F., Becarelli, S., Levin, D.B., Munz, G., Petroni, G. Di Gregorio, S. Mycoremediation of Old and Intermediate Landfill Leachates with an Ascomycete Fungal Isolate, Lambertella sp. *Water* **2020**, 12(3), 800. DOI: https://doi.org/10.3390/w12030800

[9] Foissner, W. Protists as bioindicators in activated sludge: Identification, ecology and future needs. *Eur J Protistol* **2016**, 55, 75-94. DOI: 10.1016/j.ejop.2016.02.004

[10] Lutzu, G.A., Ciurli, A., Chiellini, C., Di Caprio, F., Concas, A. Dunford, N.T. Latest developments in wastewater treatment and biopolymer production by microalgae. *J. Environ. Chem. Eng* **2020**, 104926. DOI: https://doi.org/10.1016/j.jece.2020.104926

[11] Rawat, I., Kumar, R.R., Mutanda, T. Bux, F. Dual role of microalgae: phytoremediation of domestic wastewater and biomass production for sustainable biofuels production. *Appl energ* **2011**, 88(10), 3411-3424. DOI: 10.1016/j.apenergy.2010.11.025

[12] Ajayan, K.V., Selvaraju, M., Unnikannan, P., Sruthi, P. Phycoremediation of tannery wastewater using microalgae Scenedesmus species. Int J Phytoremediat 2015, 17(10), 907-916. DOI: 10.1080/15226514.2014.989313

[13] Barsanti, L., Gualtieri, P. Algae: Anatomy, Biochemistry, and Biotechnology. **2014,** CRC Press.

[14] Emparan, Q., Harun, R., Danquah, M.K. Role of phycoremediation for nutrient removal from wastewaters: A review. *Appl Ecol Env Res* **2019**, 17, 889–915. DOI: http://dx.doi.org/10.15666/aeer/1701_889915

[15] Flori, L., Macaluso, M., Taglieri, I., Sanmartin, C., Sgherri, C., De Leo, M., Ciccone, V., Donnini, S., Venturi, F., Pistelli, L., Martelli, A., Calderone, V., Testai, L., & Zinnai, A. Development of Fortified Citrus Olive Oils: From Their Production to Their Nutraceutical Properties on the Cardiovascular System. *Nutrients* **2020**, 12, 1557. DOI: 10.3390/nu12061557

[16] Chiellini C., Guglielminetti L., Pistelli L., Ciurli A. Screening of Trace Metal Elements for Pollution Tolerance of Freshwater and Marine Microalgal Strains: Overview and Perspectives. *Algal Res* **2020,** 45, 101751. DOI: https://doi.org/10.1016/j.algal.2019.101751

[17] Ciurli A., Modeo L., Pardossi A., Chiellini C. Multidisciplinary integrated characterization of a native Chlorella-like microalgal strain isolated from a municipal landfill leachate. *Algal Res* **2021**, 54, 102202. https://doi.org/10.1016/j.algal.2021.102202.

[18] Chiellini C., Guglielminetti L., Sarrocco S., Ciurli A. Isolation of four microalgal strains from the Lake Massaciuccoli: screening of common pollutants tolerance pattern and perspectives for their use in biotechnological applications. *Front Plant Sci* **2020**, 11, 1954. DOI: https://doi.org/10.3389/fpls.2020.607651.

[19] Gorman, D.S., Levine, R.P. Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of Chlamydomonas reinhardi. *P Natl Acad Sci USA* **1965**, 54, 1665–1669. DOI: 10.1073/pnas.54.6.1665.

[20] Lichtenthaler, H.K. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes, in *Methods in Enzymology*, eds Abelson, J., Simon, M., Verdine, G., Pyle A. (Cambridge: Academic Press), 1987, 350–382. DOI: 10.1016/0076-6879(87)48036-1.

[21] Pinto, G., Pollio, A., Previtera, L. Temussi, F. Biodegradation of phenols by microalgae. *Biotechnol Lett* **2002**, 24(24), 2047-2051. DOI: https://doi.org/10.1023/A:1021367304315

[22] Pinto, G., Pollio, A., Previtera, L., Stanzione, M. Temussi, F. Removal of low molecular weight phenols from olive oil mill wastewater using microalgae. *Biotechnol Lett* **2003**, 25(19), 1657-1659. DOI: https://doi.org/10.1023/A:1025667429222