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TITOLO: LIPIDS PRODUCTION FROM ULVA RIGIDA, PRODUZIONE DI LIPIDI DA ULVA RIGIDA

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LIPIDS PRODUCTION FROM *ULVA RIGIDA*

PRODUZIONE DI LIPIDI DA ULVA RIGIDA

***Abstract** – This preliminary study was performed to assess* Ulva rigida *ability to produce and store lipids. In 72 hours lipid content increased 4 fold from environmental scores (2.3%) in nitrogen depleted, stressful conditions in the dark, reaching values of 8.8%. Peak values of 15% were measured in dark/light cycles. The role of stress in lipid production was briefly evaluated.*

***Key-words:** lipid, Ulva rigida, production, stress, depleted.*

Introduction - In the last few decades fossil fuels contributed to improve human living standards in many countries and, following globalization, the rising need for energy resources and fuels have become a growing issue. Moreover the recent Paris agreements on the reduction of CO_2 emissions encouraged the development of alternative processes and technologies in resource and energy provision. The first attempt to replace fossil fuels with biofuels from renewable sources led to production of first and second generation biofuels: produced from edible and non-edible plants and oils, respectively. The search for further more sustainable developments, where not even arable land is needed to yield crops, led the focus of researchers on microalgae production. Microalgae grow 100 times faster than terrestrial plants and accumulate large quantities of lipids inside cells (with common oil levels of 20-50%; Christi 2007). The structures for microalgae cultivation can be set up on "brown fields" or non cultivable lands, fostering local economies, but requires an investment of knowledge and money superior to common cultivation practices. The yields of these implants largely exceed the most intensive productions of terrestrial plants for oil provision, such as oil palm or *Jatropa*, however, few doubts have been raised about the sustainability of microalgae production. The analysis of some life cycles examined the energy efficiency ratios (EER; energy output/energy input) of different oil bearing crops revealing commonly higher values for terrestrial plants in comparison to microalgae. The latter often show values <1, highlighting that high productivity in microalgae can be propelled by a negative energy balance, consuming energy instead of farming it (Lam et Lee, 2012). The main critical factors affecting the sustainability of this crop were assessed to be: fertilization and harvesting. With this in mind, we decided to focus our attention on other fast-growing organisms that could minimize these critical issues: the choice fell on macroalgae. The historical choice of the cultivation of microalgae is perhaps linked to their higher growth rates and their usually higher lipid content. However, macroalgae like *Ulva rigida* C. Agardh are a very common occurrence in many transition environments and can be a harbinger of many problems if not properly managed producing green-tides and summer anoxic crises, followed by fish death and spot ecological degradation (Bastianini, 2013). The possibility of harvesting biomass grown in coastal environments, by absorbing nutrient loads coming from agricultural leaching, would solve the critical issues related to the use of fertilizers, reclaiming eutrophicated areas. Moreover the harvesting operations of these macroscopic organisms would be less energetically and technologically intensive, avoiding the by product pollution related to chemical flocculants or highly energy demanding processes for filtration. Despite the high growth rates up to 10% d^{-1} reported for *U. rigida* (Sfriso et Sfriso, 2017) the usually low lipid content (<3% dw; Sfriso et al. 1994) makes this resource unattractive for the production of oils. This led our research in the development of techniques to increase lipid production in *U. rigida* "post harvesting", following the knowledge acquired on microalgae cultivation. The optimal conditions for lipid production in microalgae were reported to be in the dark, at salinities higher than 35 psu, in presence of stressful conditions and in a nitrogen depleted environment (Ma et al. 2016). The results here depicted represent the very first reported trials on this topic.

Materials e methods - Small young thalli and old thalli of the macroalga *U. rigida* were collected from the artificial rocky shores of the Lido island in Venice. All the glassware was washed with "Contrad", HNO_3 1% and $NaHCO_3$ 1% buffer solution. Artificial seawater was prepared (in one liter of Milli-Q water: NaCl 24.6g, KCl 0.67g, $CaCl_2$ 1.36g, $MgSO_4x7H_2O$ 6.29g, MgCl 4.66g, $HNaCO_3$ 0.18g) and 100 ml were poured into flasks with 500mg of seaweed. Seaweeds were stressed in the dark in a nitrogen/phosphorus depleted environment with silver nanoparticles as stressor (AgNP; synthesized by the citrate reducing method) at three concentrations: 0.05ppm, 0.5ppm and 5ppm. Lipid production was monitored at 24h, 48h and 72h by epifluorescence microscopy with Nile red dye. The experiment was carried out at 17°C. Total lipids (LPD) were extracted from dried seaweeds by hexane/isopropanol mixture and measured spectrophotometrically by the charring assay of Marsh et Weinstein (1966). A CHNS analyzer (Vario-micro, Elementar) was employed to measure the raw composition changes of the seaweeds. The percentages here reported all refers to dry weight (dw). The oxidative stress was measured as malondyaldeide by the lipid peroxidation assay and expressed ad µmol g^{-1} fresh weight (fw). The experimental replicates were done in double and all the analyses in triplicate.

Results - Samples of young and old *Ulva* were collected to investigate differences in the lipid production behavior of seaweeds at exponential and stationary growth phase. The total lipid content of *U. rigida* collected *in situ* was very low accounting for 2.3% in young short thalli and 1.6% in old floating gibbous thalli, respectively. The oxidative stress measured at harvesting time (T_0) was higher for old *Ulva* (165±10µmol g^{-1}) in comparison to young *Ulva* (21±1.6µmol g^{-1}). Differences were also highlighted by CHNS analysis. Old thalli (N: 3.8%; C: 32%; H: 5.3%; S: 19%) displayed an higher sulfur content for lower values of nitrogen, carbon and hydrogen in comparison to young thalli (N: 5.4%; C: 37%; H: 5.7%; S: 15%), highlighting a starting low nitrogen content in old *Ulva*. All these differences reflected in the lipid production experiment of **Fig. 1a/b**.

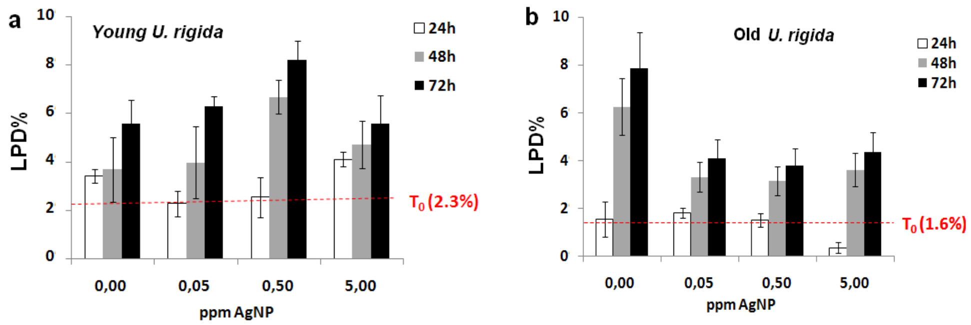

Fig.1: Total lipid dw percentages in increasing AgNP concentrations at 24h, 48h and 72h. Red dotted line is the T_0 value. The error bars represent the experimental standard deviation. **a**) Young *Ulva*; **b**) Old *Ulva - Lipidi totali, percentuali dw a concentrazioni crescenti di AgNP a 24h, 48h, 72h. La linea rossa tratteggiata rappresenta il T_0. Le barre d'errore rappresentano la deviazione standard. a) Ulva giovane; b) Ulva vecchia.*

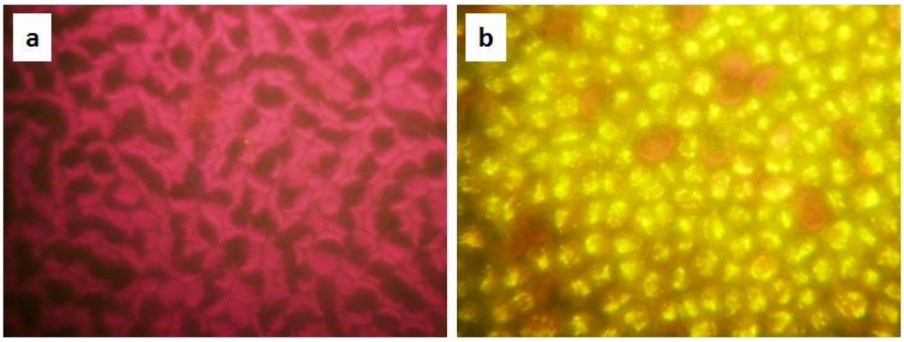

Fig. 2: a) Control *U. rigida* (60X), chlorophyll-*a* autofluorescence; b) *U. rigida* (40X) in 0.5ppm AgNP, neutral lipid yellow vescicles. – a) Controllo *U. rigida* (60X) – autofluorescenza della clorofilla-*a*; b) *U. rigida* (40X) in 0.5ppm AgNP, vescicole di lipidi neutri visibili in giallo.

Under these conditions, the lipid content in young *U. rigida* reached 8.8% at 0.5ppm AgNP. Stressful conditions too high (5ppm AgNP) resulted in a moderate lipid production, highlighting that the stress should be measured and/or "dosed" to induce the best lipid yield. This was highlighted also by **Fig.1b** showing that old *Ulva*, already stressed when collected, produced up to 7.9% of lipids at 72h in the control without further stress (AgNP) addition. The values reached by *Ulva* were still low compared to those reported for microalgae but replicates performed in a dark/light cycle of 12h reached lipid values up to 15% already at 24h in a 5ppm AgNP solution. This increase in lipid content is related to an increase of the neutral lipid fraction as can be assessed by epifluorescence microscopy (**Fig. 2a/b**). Only the red autofluorescence of chlorophyll-*a* is visible in the control but many yellow vesicles appears at 0.5ppm AgNP due to neutral lipid reaction to Nile red.

Conclusions – Further tests shall be performed to find out better lipid producing conditions for *Ulva rigida*, inducing stress by less toxic substances or by physical treatments. The lipids scores obtained are still lower than microalgae but lipid content should further increase in *Ulva* up to values exceeding 15-20%. Therefore, *Ulva* could become a valuable resource for oil production, surely competitive with oil bearing terrestrial plants that are nowadays still the major global producers of lipids (**Tab.1**). This despite the much higher areal productivity of microalgae whose production is still limited. Microalgae are still further but macroalgae will slowly catch up.

Tab.1: Lipid productivity in terrestrial plants, *U. rigida* and microalgae. - Produttività di lipidi in piante terrestri, *U. rigida* e microalghe. (Chisti, 2007; FAOSTAT, 2014; Rio et al., 1996; Rodolfi et al., 2009; Sfriso et al., 1994; Sfriso et al.,2017; www.valori-alimenti.com).

species	biomass productivity		dw/fw	average LIPID %dw	**LPD Productivity g m^{-2} year^{-1}**
soybean (soia)	0.18	Kg fw m^{-2} year^{-1}	31%	22%	**10**
mais	0.53	Kg fw m^{-2} year^{-1}	90%	5%	**20**
safflower (catramo)	0.09	Kg fw m^{-2} year^{-1}	92%	15-34%	**10-30**
sesame seed (semi di sesamo)	0.08	Kg fw m^{-2} year^{-1}	96%	54%	**40**
cotton seed (semi di cotone)	0.17	Kg fw m^{-2} year^{-1}	96-90%	27-36%	**40-50**
olives	0.28	Kg fw m^{-2} year^{-1}	25-32%	75-91%	**50-90**
sunflower seeds (semi girasole)	0.18	Kg fw m^{-2} year^{-1}	86-94%	50%	**80**
rapeseed (colza)	0.22	Kg fw m^{-2} year^{-1}	85-90%	44%	**80-90**
coconut - (noce di cocco)	0.52	Kg fw m^{-2} year^{-1}	47%	64%	**160**
oil palm fruit	1.12	Kg fw m^{-2} year^{-1}	76%	43-58%	**370-490**
***Ulva rigida* in situ**	15-25	Kg fw m^{-2} year^{-1}	20%	1-9-15%	**30-450-750**
***Ulva rigida* in tank**	40	g dw m^{-2} d^{-1}	-	1-9-15%	**150-1300-2200**
unspecified μalgae-raceway ponds	0.01-0.050	Kg dw m^{-2} day^{-1}	-	15-75%	**500-14000**
Chlorella vulgaris	20	mg L^{-1} h^{-1}	-	27%	**11000**
Nannochloropsis sp.	0.30-0.36	g L^{-1}day^{-1}	-	32-60%	**11000-19000**

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