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Potential Biodiesel Production from Mixed Phytoplankton Cultures Collected off the Lebanese Coast

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Abstract—Seawater samples were collected from the coastal area along the Lebanese coast. The samples were fertilized using Guillards F/2 medium and cultured in 2 L flasks. Out of five promising blooms, three were selected and cultured in 70 L cylinders and in 300 L raceway ponds under greenhouse conditions. A culture of Chlorella "marine" was used as reference. The growth dynamics were determined and dry matter yield, lipid content, fatty acid profile and potential biodiesel yield were determined. Dry matter production varied among blooms and ranged between 455 and 641 mg/L/harvest, with the cylinders giving a 23-33% greater productivity in terms of mg dry matter/L/day. The lipid content in blooms varied between 16 and 18% DM and reached about 19% in the Chlorella isolate. In race way ponds as well as in the cylinders, two of the natural blooms yielded biodiesel in proportions similar to the Chlorella reference isolate. The Saida bloom gave a biodiesel production of 5.2 mg/L/day in cylinders and the Nahr ElKalb (NK2) bloom 4.3 mg/L/day in raceway ponds.

Index Terms—Renewable energy, Microalgae, Mixed natural blooms, Lipid content, Fatty acid profile, Biofuel.

I. INTRODUCTION

Algae are a diverse collection of chlorophyll *a* containing organisms with a thallus not differentiated into roots, stems and leaves [1]. Algae are responsible for about 40 to 50% of the photosynthesis that occurs on earth despite the fact that their biomass represents only around 0.2% of that on land [2]. They comprise macro and microalgae. Microalgae, prokaryotic or eukaryotic photosynthetic microorganisms, include unicellular and simple multi-cellular microorganisms. The most common habitat of many of the microalgae is open waters, and thus are known as phytoplankton [3]. Microalgae are autotrophs that have the capacity to grow rapidly due to their unicellular or simple multicellular structure and can produce all year long, leading to continuous supply of lipids, proteins, carbohydrates, vitamins and antioxidants [4]. Oil content of microalgae can vary between 14 and 50% of dry weight but has been recorded as little as 4 % in some strains and as high as 80% in others [5, 6, 7]. Manipulating the composition of the culture media and environmental conditions can alter the lipid productivity of microalgae [8]. The annual productivity and oil content of microalgae is far greater than seed crops since microalgae are capable of year round production [9].

Several studies speculate that at the current rates of fossil oil consumption, world reserves could be exhausted in less than 50 years. Furthermore, combustion of fossil fuel is considered a major source of greenhouse gases involved in global warming. Among other ecologically friendly alternative sources for energy production, renewable CO_2 neutral biofuels are urgently needed as alternatives to fossil fuels. Biodiesel is a mixture of monoalkyl esters of long chain fatty acids (FAME) derived from a renewable lipid feedstock such as algal oil [10] which can be used directly in conventional diesel engines. After the extraction processes (see Section II, F), the resulting product algal oil can be converted into biodiesel through a process called transesterification. Transesterification is a chemical reaction between triglycerides and alcohol in the presence of a catalyst to produce mono-esters that are termed as biodiesel [11].

Microalgae have long been recognized as potentially good sources for biofuel production because of their high oil content and rapid biomass production. They were initially examined as a potential replacement fuel source for fossil fuels in the 1970s [12]. In recent years, use of microalgae as an alternative biodiesel feedstock has gained renewed interest particularly because most of the lipid content of microalgae is in the form of triacyglycerols (TAG) which is the form needed to produce biodiesel [13]. Therefore, microalgae are currently considered as third generation feedstock for biofuel production because of unique properties in terms of efficiency of production and sustainability. Production of microalgae in seawater or waste water does not compete with food or feed crops neither for fresh water resources nor for land suitable for agriculture production [14]. Microalgae may also

produce valuable co-products such as proteins and residual biomass after oil extraction, which may be used as a high protein food or feed supplements [15, 16], fertilizers [17], antioxidants, antimicrobial compounds [18] or fermented to produce ethanol or methane [19].

Most commercial or industrial scale microalgal production occurs in large open systems. However, only a handful of microalgal species can succeed in such conditions and such systems are prone to contamination by fungi, bacteria or other microalgae. Alternatively, closed systems which include flat, tubular and column photobioreactors overcome some of the limitations of open systems because light is distributed evenly and contamination risks are minimized, with a real chance of producing single- species of microalgae. Unfortunately, production costs are greater as compared to using open pond systems.

Commercial microalgal biodiesel production is not yet economically feasible, primarily because of high energy inputs required for culturing and harvesting [20]. The investment and operating costs should be drastically decreased to make commercial biodiesel production feasible, unless other high value products are produced simultaneously [21]. Accordingly, the success of a commercial project will depend on many factors including the selection of an adapted species and the selection of an economical and reliable cultivation system.

The present study reports preliminary data on the evaluation of three locally isolated mixed blooms of microalgae for their potential use as feedstock for biodiesel production in open and closed continuous production systems.

II. Materials and Methods

A. Phytoplankton Culture Collection

Seawater (SW) samples (2L) were collected from 20 sites along the Lebanese coast, mainly from small ports. The water was filtered on site using a 100μm filter to remove macroalgae or zooplankton. Guilliards F/2 medium was added to the seawater samples. The sample flasks were transported to the American University of Beirut and placed by an East-facing window for acclimatization. After observing slight coloration of the water samples, the flasks were transferred to a growth chamber equipped with daylight lamps (450 lux) with a photoperiod of 14/10 hrs. The blooms that showed the most dense growth were selected for further analysis.

B. Upscaling

Seawater collected from a shore well was used in all experiments. For small volumes, water was filtered through a 0.22 μm filter. For larger volumes, water was chlorinated using 5 ml of Clorox per 20 L of sea water and then de-chlorinated using sodium thiosulfate at a rate of 0.2 g per 20 L. The selected natural blooms were scaled up in 5 L gallons, then in 20L flat bioreacators, followed by growth in 70L cylindrical bioreactors (30 cm diameter) with forced aeration from the bottom, and then in 300 L raceway ponds equipped with paddlewheels. Throughout the experiments, all the seawater used was amended with Guillard f/2 medium.

C. Isolation and Identification of Algal isolates

Two methods were followed for isolation of single cultures; the plate streaking technique and the serial dilution method. Once a unialgal strain was isolated, it was transferred into 5 ml of enriched liquid media, then upscaled gradually to one liter cultures. Molecular tools based on polymerase chain reaction (PCR) and sequencing of resulting amplicons were used for identification of single isolates (Data not presented in this manuscript).

D. Growth Kinetics

The cylinders and raceway ponds were installed in a single span plastic house, equipped with a fan that circulates air when the temperature rises above 26 ˚C. A data logger was installed to monitor temperature and relative humidity. One cylinder and one raceway pond were used to grow a reference isolate (*Chlorella* marine sp.) provided by Dr. Ionanis Tzovenis (NKUA, Greece). Samples (10 ml of cultures) were collected 5 times a week and microalgae growth was estimated by measuring the optical density of the culture suspension at 560 nm. Upon reaching the stationary phase, about 90% of the culture was harvested and SW amended with f/2 medium was replenished and a new cycle of production was initiated. Reported results are based on a minimum of three cycles of production.

E. Dry Matter Content

Microalgae were harvested from 10 L of culture media using a centrifuge. The resulting paste was washed with distilled water and centrifuged again at 4000 rpm. The process was repeated two more times and the final pellet was stored at -5 ˚C and then freeze-dried overnight.

F. Lipid Content

Total lipid content was determined gravimetrically using the Folch method [22]. A methanol: dichlorormethane (DCM) (2:1) mixture was added to a known mass of freeze-dried algae. The mixture was homogenized to a volume 20 times the volume of tissue sample (1 g in 20 mL of solvent mixture). Tubes were

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placed in an ultrasonic bath for 60 minutes (at 40ºC) then vortexed. Homogenates were filtered into a clean screw capped tubes and 0.4 mL of water was added (8 ml of water for 20 ml of solvent mixture). After vortexing for few seconds, mixtures were centrifuged for 10 minutes at 4000 rpm to allow phase separation. Upper phases were discarded while the lower phases representing the DCM containing lipids were retained. Sodium sulfate was then added for moisture absorption. The mixture was decanted into a clean tube and the solvent was evaporated under reduced pressure. Dry weights of tubes were measured and lipid content estimated. Proportion of lipids in dry sample weight was calculated as follows:

% Lipid = (weight of lipid in g/ dry weight of sample in g) x 100.

G. Determination of Fatty Acid Methyl Esters and Biodiesel Production

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After the lipid extraction processes, the resulting algal lipids were converted into biodiesel *via* transesterification. Fatty acid methyl esters (FAME) were prepared according to Freedman et al [23]. GC-MS was used for an efficient separation, identification and qualification of the particular fatty acid methyl ester present in the sample. The injector of the GC was set at a temperature of 260ºC. 1µL sample volume was injected into the split injector with a dilution split ratio of 100:1. Helium was used as a carrier and was injected in the DB 23 column at a rate of 30 cm / sec (0.54 ml/min). Initial column temperature was set to 50ºC for 2 minutes, then temperature was increased to 220ºC at a rate of 4ºC / min. Temperatures of the transfer line as well as of the MS were set at 260ºC
The transformation or conversion of lipids into biodiesel (FAME) [24] was performed by base-catalyzed methylation. The algal lipids (50 mg) were added to 15 mL of 1% MeONa in methanol and refluxed for around 2 hours. After complete conversion as monitored by thin layer chromatography (TLC), the mixture was extracted with hexane (2 X 15 mL) and the combined hexane layers were washed with water until pH was neutral. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to recover the fatty acid methyl ester fraction (biodiesel).

III.   RESULTS

A. Growth Dynamics

In the preliminary tests, only five samples out of the twenty samples collected developed good blooms. One of the blooms (Jbeil) had a low lipid content (14.5% DW) and another (Damour) was not tolerant to high

temperatures prevailing in the green house. Therefore, three of these blooms were selected for evaluation in cylinders and in raceway ponds, namely Saida, Ein el Mreiseh (EM) and Nahr el Kalb (NK2) as shown in figure 1.
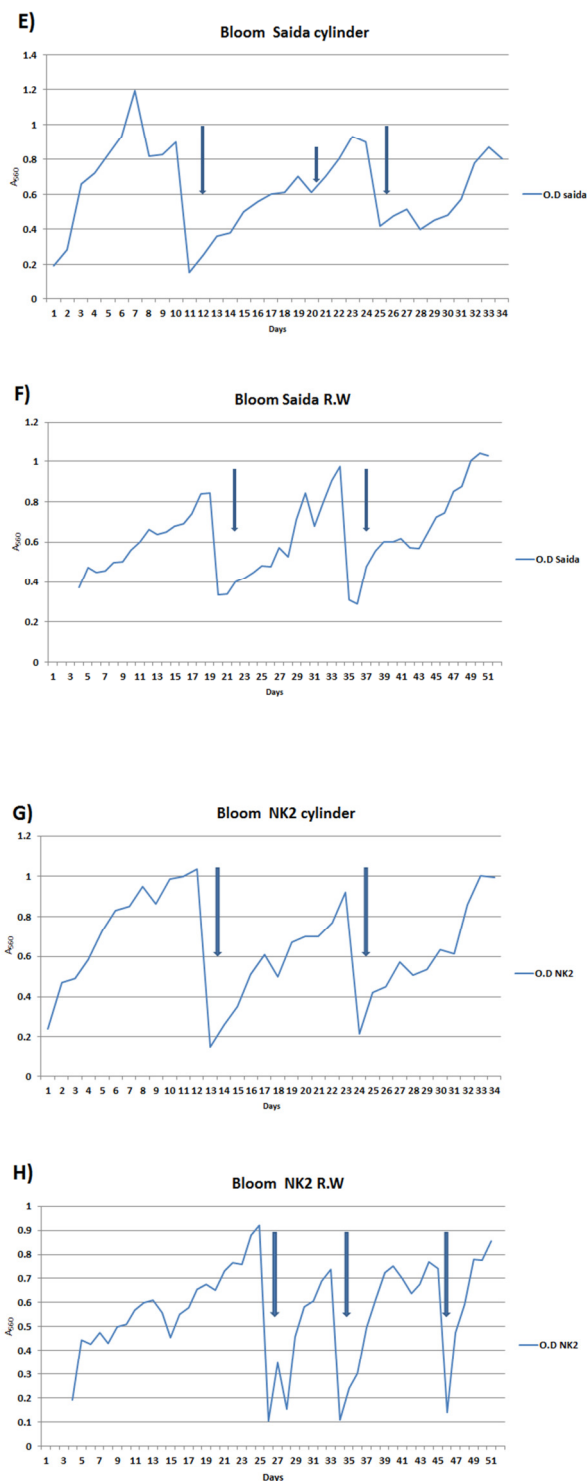

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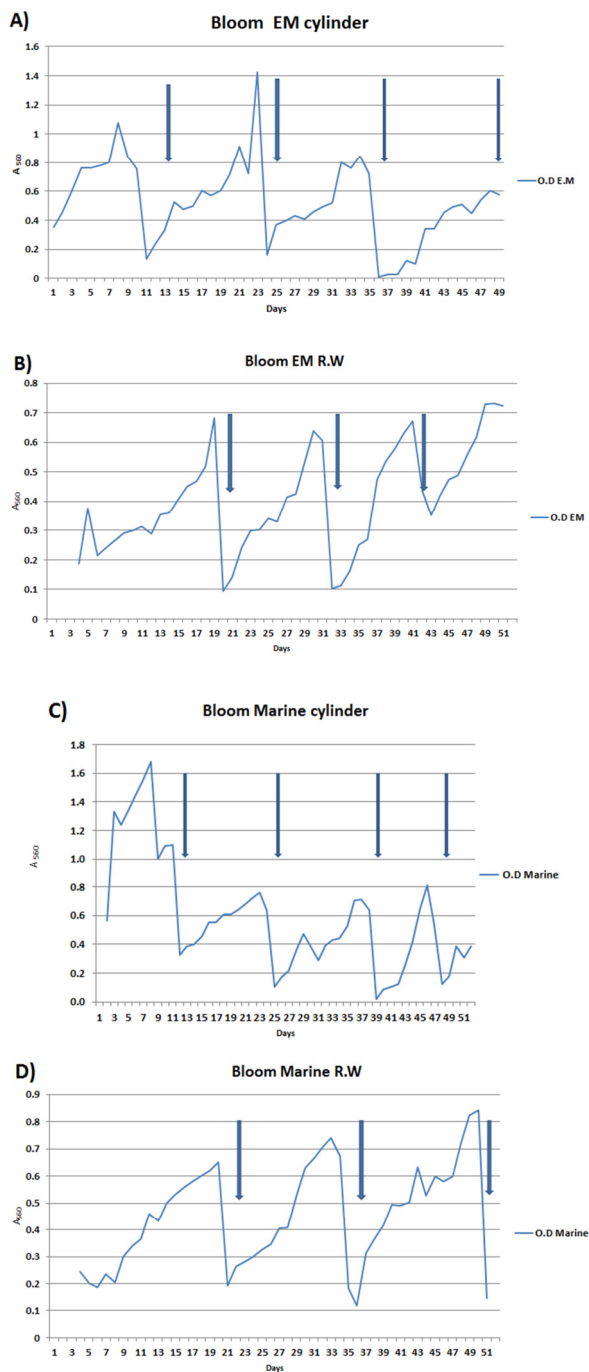
Fig. 1. Growth dynamics of natural mixed microalgal blooms (EM), (NK2), (Saida) and the reference Chlorella "marine spp. (Marine) in cylinders (A, C, E & G) and in raceway ponds (B, D, F & H) as determined by the A_{560} readings (Arrow indicates harvesting day). June 9- July, 2014

The growth dynamics of the various blooms in raceways and in cylinders are summarized in figure 1.

B. Microscopic Observations

The three blooms were composed mainly of green algae with a lower proportion of diatoms except for the Saida bloom where the percentages were equal. However, the percentage of diatoms dropped with time with some variations between the cylinders and raceway ponds. For example at the end of the trial, in Saida bloom, the raceway pond contained 70% green algae 30% diatoms, while in the cylinder it was mainly Green algae with very low proportion of diatoms. The NK2 contained mainly green algae with a predominance of *Tetraselmis* sp. In both cylinders and raceway ponds, the "Chlorella marine" got contaminated, with "Chlorella" ranging between 40-50%, *Tetraselmis* sp. 40-50% and 10% diatoms in the raceway pond and less than 1% in the cylinder .

C. Dry matter and lipid content

Under the experimental conditions in the raceway ponds, the Saida and NK2 blooms yielded between 545-549 mg dry matter/L of culture (Table 1), a yield more or less similar to that of the reference isolate (519 mg/L). The dry matter yield of EM bloom was significantly less (Table 1). In the cylinders the yield ranged between 455-641 mg dry matter/L. Saida bloom gave the highest yield, followed by EM, Marine and NK2.

Total lipid content varied between 16.1 and 18.9% of total biomass dry weight (Table 1). In the raceway ponds, the EM and NK2 blooms had the greatest total lipid content (c. 18%), followed by Saida bloom (c. 17%) which had lipid content approximately equivalent to the reference isolate. In the cylinders, the reference isolate contained the greatest lipid content (approximately 19%).

D. Fatty acid methyl ester profile

Fatty acid profiles are presented in Table 2 and 3. Total FAMEs ranged from 85% to 95% of total lipid content, with EM, NK2, and Saida presenting the highest values and Marine the lowest one. Hexadecanoic acid (palmitic acid) and methyl tetradecanoate were the most frequent saturated fatty acids, with contents varying from 15% to 27% of total fatty acid content. Total mono-unsaturated fatty acids varied in these four isolates from 30% to 50%, with 9-octadecenoic acid methyl ester (27-40%) and 9-hexadecenoic acid methyl ester (3-10%) as the most predominant. 9,12-Octadecadienoic acid methyl ester (linoleic acid methyl ester) (6-12%) and 7, 10-Hexadecenoic acid methyl ester (2 %) are the only di-

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unsaturated fatty acid present and ranged from 6 to 12 %. Total polyunsaturated fatty acid (PUFA) content is characterized by good quantities of 9,12,15-Octadecatrienoic acid methyl ester (linolenic acid methyl ester) (8-20%) and 5,8,11,14,17-Eicosapentaenoic acid, methyl ester (EPA) (3-9%). Others poly-unsaturated fatty acids were signaled, but only as traces with a values lower than 2%.

Table 1. Dry matter production and lipid content of mixed algal blooms grown in 70L cylinders or in 300L raceway ponds

Blooms	Cylinders (70L)			Raceway ponds (300 L)		
	O.D. value	dry weight mg/ L	Lipid content %	O.D. value	dry weight mg/ L	Lipid content %
EM	1.15	564	16.9	0.86	413	18.2
NK2	0.92	455	17.3	0.75	545	18.1
Saida	0.80	641	16.1	0.97	549	17.2
Marine	0.64	555	18.9	0.75	519	16.8

Table 2. Percentages of saturated and mono-unsaturated FAMEs presents in 4 isolates of microalgae. 16:0, palmitic acid, 14:0, Methyl tetradecanoate. 16:1 (ω -7), 9-octadecenoic acid, 18:1 (ω -9), 9-hexadecenoic acid.

Blooms	Saturated fatty acid		Mono-unsaturated fatty acid	
	16:0	14:0	16:1 (ω -7)	18:1 (ω -9)
EM	33.7%	0.61%	33.6%	-
NK2	27.5%	0.46%	30%	2.85
Saida	26.3%	1.71	27.2%	10
Marine	21.3%	-	39.4	-

Table 3. Percentages of di-unsaturated and poly-unsaturated FAMEs present in 4 isolates of microalgae. 18: 2 (ω-6): 9, 12 octadecadienoic acid, 16: 2 (ω-8) : 7, 10 hexadecanoic acid, 18:3 (ω-3): 9,12,15-Octadecatrienoic acid, 22:5 (ω-3).

Blooms	di-unsaturated fatty acid		poly-unsaturated fatty acid	
	18: 2 (ω -6)	16:2	18:3 (ω-3)	22:5 (ω -3)
EM	8.3%	2%	11.8%	4.4%
NK2	6.3%	1.3%	19.3%	8.5%
Saida	11.6%	2%	14.6%	2.8%
Marine	6.9%	1.6%	8.7%	2.7%

E. Potential for Biodiesel Production

Potential biodiesel production in mg biodiesel/gm of dry matter harvested or mg biodiesel/L of culture medium at the beginning of the stationary phase are summarized in figs 2 and 3. During the summer period, the Saida bloom gave similar amounts of biodiesel production as the reference isolate whether in raceway ponds or in cylinders. In the raceway ponds, the NK2 and Saida blooms could produce about 4 - 4.3 mg biodiesel/L/day, a level of production similar to that of the reference isolate used in the present study. In the cylinder, only the Saida bloom yielded similar production to the reference isolate (5.2 mg/L/Day), followed by EM and NK2 blooms.

IV. DISCUSSION

As the world gets richer and populations grow, levels of fossil fuel consumption are expected to increase and price is expected to rise. Moreover, a shortage in the supply of fossil fuels is expected within the coming few decades. Microalgae represent a good renewable resource for biofuel production. They have the potential

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to grow rapidly producing large quantities of biomass with several advantages. They need less water than terrestrial crops and can be cultivated in sea water or brackish water on non-arable land, thus reducing the load on freshwater sources [25]. Their farming uses marginal land that does not compete with traditional land usage so algae culture does not necessitate land-use changes nor traditional lifestyle upheaval of rural people. Moreover, there are very few if any environmental effects associated with algae culture [26], thus minimizing impacts on production of food, fodder or any other products derived from crops [27]. Additionally, to produce 1 kg of dry algal biomass about 1.8 kg of CO_2 are utilized, making the use of microalgae for fuel production carbon neutral and potentially reducing pollution and the effect of greenhouse gases on climate change [28]. What's more, nutrients for microalgae cultivation (especially nitrogen and phosphorus) can be obtained from wastewater thus providing cheap growth medium coupled with treatment of organic effluent from the agri-food industry [29].

Within the framework of the Med-Algae project, five locally isolated blooms were evaluated for their potential use as feedstock for biodiesel production. Most of the literature published so far focused on unialgal cultures. However, large-scale algal monocultures may be ecologically unstable due to contamination/competition by other microorganisms. The present study focused on mixed blooms with the assumption that these may be ecologically more stable, less prone to contamination and/or more resilient to environmental changes throughout the year. Even though seasonal change in the algal population occurs, careful selection of blooms may allow for good quality biodiesel year round. Effectively, during the course of this study, the "Chorella marine" bloom was rapidly contaminated in raceway ponds and this affected the lipid content of harvested biomass, reducing it to 16.9% on dry weight while in the initial isolate it ranged between 20-21%.

Under the experimental conditions, the Saida bloom showed similar potential for biodiesel production to that of the reference isolate, "Chlorella marine sp." The other two locally isolated blooms also showed promise, especially if comparison is extended over the whole year. However, the highest level of biodiesel obtained with all blooms did not exceed 5.2 mg/L/day. This may be attributed to two factors, dry matter production and lipid content. Both parameters may be affected by the growth conditions including nutrients, temperature, irradiance and culture systems [30]. Therefore, the data obtained in this study are considered as preliminary, since no optimization of any of the latter parameters was performed. The dry matter produced in the systems used in this trial was of the same order reported in several articles, i.e. about 500-600 mg/L/harvest or 33-

40 mg/ L/ day [31, 32]). However, the dry matter yields are considerably lower than those reported under optimized growth conditions in flat bioreactors (green wall photobioreactors), where the dry matter yield reached about 300 mg/L/day and the total lipids 60% under nitrogen deprivation [33].
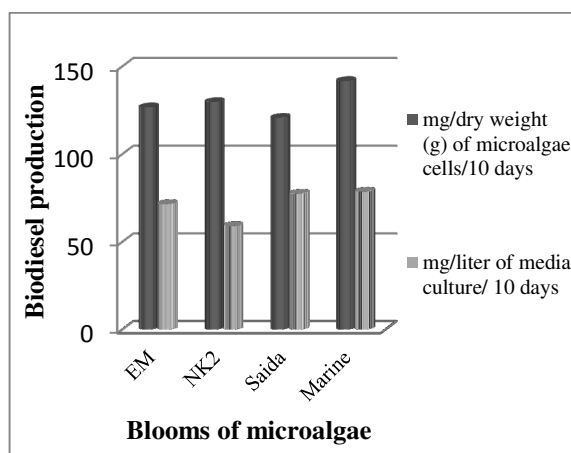

Fig. 2. Biodiesel production of 4 blooms in cylinders
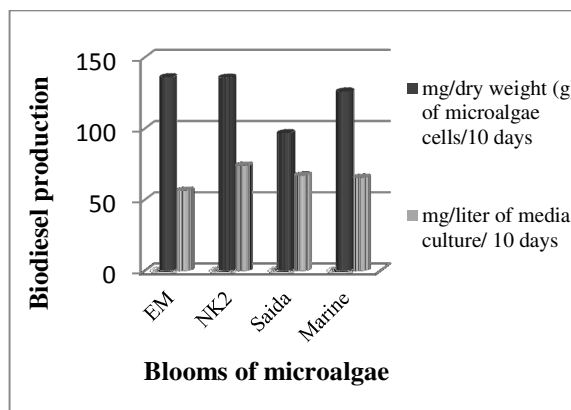

Fig. 3. Biodiesel production of 4 blooms in raceway ponds

The Fatty acid profile of the Saida and EM blooms contains over 78% of saturated + mono- and di-unsaturated FAMEs, a profile suitable for good quality biodiesel production. While the NK2 bloom contained a high percentage of omega 3 FAs (27.8% of total FAMEs), which make it suitable for consideration as feed supplement.

While the raceway ponds may represent the least expensive culture system, the use of more expensive vertical systems may be warranted in many Mediterranean countries, where land is limited and land rent is high. These closed systems confer higher productivity and lower risk of contamination than open systems. For small Mediterranean countries like Lebanon, the cost of land is high and biodiesel production may not be considered economically

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sustainable in the short to medium term. However, if other high value products such as protein supplements, antioxidants, antimicrobial compounds etc. are produced from microalgae as the primary product, while biodiesel is considered a byproduct, then economically viable projects may be expected in the short term. Effectively, this team has cultured several local microalgae isolates, and evaluations are in progress for their potential use for the production of protein supplements, omega 3 fatty acids, nutraceutical and antioxidant pigments and plant growth biostimulants.

V. ACKNOWLEDGEMENT

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