Egypt. J. Exp. Biol. (Bot.), 11(1): 77 – 88 (2015)

RESEARCH ARTICLE

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OPTIMIZATION OF MEDIUM COMPONENTS USING PLACKETT-BURMAN DESIGN FOR HIGH PRODUCTION OF PROTEIN, CARBOHYDRATES AND LIPIDS IN THE MICROALGA *TETRASELMIS CHUII*

ABSTRACT:

Microalgae are the most important plankton for nutrition of marine larval organisms. The objective of this study is to apply the Plackett-Burman statistical design to specify which nutrient factor(s) optimizes the nutritional contents (protein, carbohydrates, lipids, and omega3 fatty acids) in the marine microalga *Tetraselmis chuii*. *Tetraselmis chuii* was cultured on F/2 medium (as control) reaching the maximum growth in the 10^{th} day. By application of the design, contents of protein and carbohydrates increased at high concentrations of NO_3 and PO_4 by 43.5% and 50%, respectively. Total lipid increased by 91.5% at low concentrations of NO_3 and PO_4. Also, omega3 fatty acids, including Alpa-linolenic acid, Eicosatetraenoic, Eicosapentaenoic, and Decosahexaenoic, increased about 3 times. The enrichment of *Artemia franciscana* with *T. chuii* cultured on the optimized media showed increases in the total protein, carbohydrates and lipids by 58.9, 106.6 and 173%, respectively. The omega3 fatty acids in *A. franciscana* enriched with the optimized *T. chuii* medium constitute 61.1% of the total fatty acids versus 34.8% for that in the F/2 control medium. These acids including C18:3, C20: 5, and C22: 6 are similar to those found in *T. chuii*. Therefore, algal species rich in omega3 fatty acids may cover the requirements for the high survival, growth and quality of fish larvae.

KEY WORDS:

Marine microalga-Protein, Carbohydrates, Omega3 fatty acids, *Artemia franciscana*

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ARTICLE CODE: 10.02.15

INTRODUCTION:

Microalgae play a crucial nutritional role in the aquaculture. They supply marine organisms with the basic food as protein, carbohydrates, lipids and polyunsaturated fatty acids –PUFAs [such as omega3 (ω_3) and omega6 (ω_6)]. Due to the global shortage of fish oil and fish meal, researchers are looking increasingly into alternative sources of lipid from algal biomass. However, relatively few studies have been carried out to evaluate the microalgal lipids in feeds for farmed fish (Ganuza *et al.*, 2008).

The fatty acid composition of microalgae varies significantly depending on the taxonomic position of the algal species (Volkman, 1989). Algae can directly produce highly unsaturated fatty acids (HUFAs) such as arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The average lipid in algal cells varies between 1% and 70% but can reach 90% of dry weight under certain conditions (Metting, 1996). Both lipids and carbohydrates accumulate up to 60–65% of dry weight when the microalgal cells are cultivated under stress conditions like temperature, salinity, light intensity and nutrient starvation/limitation (Markou *et al.*, 2012; Yao *et al.*, 2013).

Some fatty acids are essential for many marine animals (Nichols, 2003) and similar requirements exist for the growth and

metamorphosis of many larvae (Becker, 2004). PUFAs contribute to the acceleration and increase of growth, reduction of mortality, improvement of fecundity, egg hatchability and the overall quality of the brood stock (Mata *et al.*, 2010). There is a great demand for EPA-enriching feed in aquaculture (Borowitzka, 1997). Fish oil is currently the major source of EPA, but it is believed that fish get EPA from microalgae instead of synthesis by themselves (Wen and Chen, 2003). Robert *et al.* (2009) estimated that the marine microalga *Pavlova salina* (Haptophyta and Pavlovophyceae) produces lipids containing approximately 50% ω_3 long-chain PUFAs including eicosapentaenoic and docosahexaenoic acids.

Izquierdo *et al.* (2001) studied the essentiality of eicosapentaenoic and docosahexaenoic acids in brood stock diets to improve reproductive performance and egg/larvae quality such as fecundity, embryo development and hatchability. Eicosapentaenoic acid is effective in preventing blood platelet aggregation and reducing blood cholesterol (Cohen, 1988). Docosahexaenoic acid is important for correct brain and eye development in infants and has been shown to support cardiovascular health in adults (Ward and Singh, 2005). PUFAs also serve as precursors for eicosanoids, an important group of paracrine hormones responsible for a whole range of physiological activities including development, immunity and reproduction (Chamberlain and Barlow, 2000).

The present work aims to develop a special new formulated media by using Plackett-Burman statistical design for optimization the production of protein, carbohydrates, lipids and fatty acids composition in the selected marine microalga (*Tetraselmis chuii*) which commonly used in aquaculture. Also, we aim to investigate changes in the protein, carbohydrates, lipids and fatty acids of brine shrimp (*Artemia franciscana* metanauplii) fed on the selected algal species. *Artemia franciscana* is used for feeding newly hatched embryo of marine fishes.

MATERIAL AND METHODS:

The microalgal species used in this study the flagellate *T. chuii* (Chlorophyceae) was obtained from National Institute of Oceanography and Fisheries, Alexandria, Egypt.

Cultivation of the microalga:

Culture medium:

The control algal culture was grown on F/2 enriched sea water medium (Guillard, 1975). One liter of the medium was poured in two liter Erlenmeyer flasks, the Erlenmeyer flasks were inoculated with 200 ml of the pre-culture organism (cell density 15 x 10^4 cell.ml^{-1}) and incubated under continuous fluorescent light of 80 µmol $m^{-2}s^{-1}$ and temperature at 26ºC ± 1ºC. The culture flasks were aerated to accelerate algal growth. Before bubbling into the culture, the mixture was allowed to pass through bacterial filter (0.2 µm diameter).

The growth of the tested alga was determined basing on number of cells counted every two days using haemocytometer. *Tetraselmis Chuii* was harvested at late exponential growth stage.

Applying Plackett-Burman experimental design:

In this part special statistical treatments by applying Plackett-Burman experimental design (1946) were conducted on the selected algal species which commonly used in marine hatchery in order to determine which nutrient factor(s) controls the optimization of the growth and synthesis of some important metabolites (protein, carbohydrates, lipid, and fatty acids content).

The design matrix used was developed for 7 variables (elements) over 8 runs (Table 1). Each independent variable was investigated at a high (+) and a low (-) level. The low level (-) of medium components was taken as a half concentration in the given media (F/2), while the high level (+) means that element is present by 150% i.e. one and half of the variable concentration in the standard F/2 medium.

Table 1. Plackett - Burman experimental design with low and high levels of selected seven elements.

Trials (n=8)	Variables (factors)						
	$NaNO_3$	NaH_2PO_4	Na-EDTA	$FeCl_3$	$CuSO_4.5H_2O$ + $ZnSO_4.7H_2O$	$CoCl_2.6H_2O$ + $MnCl_2.4H_2O$	$Na_2MoO_4.2H_2O$
	g.L^{-1} stock solution				g.100 ml^{-1} stock solution		
+ level	112.5	7.5	6.54	4.73	1.5+3.3	1.5+2.7	0.945
- level	37.5	2.5	2.18	1.58	0.5+1.1	0. 5+0.9	0.315
1	+	+	+	-	+	-	-
2	+	+	-	+	-	-	+
3	+	-	+	-	-	+	+
4	-	+	-	-	+	+	+
5	+	-	-	+	+	+	-
6	-	-	+	+	+	-	+
7	-	+	+	+	-	+	-
8	-	-	-	-	-	-	-

According to F/2 medium, table 2 illustrates an array for n=8 trials that well test independent 7 variables ($NaNO_3$, NaH_2PO_4, Na-EDTA, $FeCl_3$, $CuSO_4.5H_2O$ + $ZnSO_4.7H_2O$, $CoCl_2.6H_2O$ + $MnCl_2.4H_2O$and$Na_2MoO_4.2H_2O$), while vitamins concentrations was constant.

The main effect of each variable was determined with the following equation: E xi = (∑Mi+ - M i-) / N

Where E xi is the variable main effect, Mi+ and Mi- are the result in the trials. The independent variable (xi) was present in the high and low concentrations, respectively, and N is the number of trials divided by 2. A main effect with a positive sign indicates that the high concentration of this variable is near to optimum and a negative sign indicates that the low concentration of this variable is nearer to optimum. Standard t-values for two sample assuming unequal variances are obtained from Statistical Methods (Cochran and Snedecor, 1989) by using Microsoft Excel for determination of the variable significance.

Artemia franciscana was produced by hatching *Artemia* cysts through decapsulation technique. They were incubated in sea water to hatch as described by Lavens and Sorgloos (1996). The produced nauplii were harvested after 24 hrs, then washed with filtered sea water, and after 6 hrs from hatching time, *Artemia nauplii* enriched with *Tetraselmis chuii* grown on the F/2 and optimized media for 24 hrs. Enriched *Artemia* was harvest by plankton net (100 µm), and the nutritional value of *Artemia franciscana* (metanuplii) enriched with the tested alga grown on the two media was evaluated as total protein, carbohydrate, total lipid, and ω_3FAs.

Determination of the biochemical constituents in *T. chuii* and *Artemia franciscana*:

Total protein content was estimated using the method of Bradford (1976).

Total carbohydrate content was quantitatively determined by the method of Phenol-Sulphoric acid described by Kochert (1978).

Total lipid content was extracted following the modified Folch method (Folch *et al.*, 1957).

Fatty acids analysis:

After the lipid was extracted as described previously by (Folch *et al.*, 1957), and methylation was done according to Radwan (1978**)** in a tube weight 50 mg of lipid, add 5 ml of methanolic sulphuric acid (1 ml concentric sulphuric and 100 ml methanol) and 2 ml of benzene, close the tube well and put it in a water bath at 90°C for 1.5 hours. Cool, add 8 ml water and 5 ml petroleum ether shake strongly and separate out the ethereal layer in a dry tube. Evaporate to dryness, and by using gas liquid chromatography (HP-6890 gas–liquid chromatography), the fatty acids was estimated.

GC Conditions:

- Device Model: HP (Hewlett Packard) 6890 GC.
- Detector: FID (Flam Ionization Detector).
- Detector temperature: 250°C.
- Injector temperature: 220°C, injection volume 2µl, splitless mode.
- Column: HP-5 (5% diphenyl, 95% dimethyl polysiloxane), 30 m, 0.32 mm ID, 0.25 µm film thickness.
- Carrier gas: Nitrogen, gas flow: 1 ml/min.
- Oven program: initial temperature 150°C for 2 min.

Ramp	Rate (°C /minutes)	Temperature (°C)	Hold time (minutes)
1	10	200	-
2	5	250	9

Statistical Methodology:

Results were presented as mean ± SD (standard deviation) for three replicates. The statistical analyses were carried out using SAS program (1989-1996) version 6.12. Data obtained were analyzed statistically to determine the degree of significance between treatments using one, two and three-way analysis of variance (ANOVA) at $P \leq 0.001$ and 0.01.

RESULTS:

Algal growth on F/2 medium:

The growth of *T. chuii* on F/2 medium increased gradually from the day zero to the 10th day (stationary phase) and then decreased gradually till the 14th day; death phase (Fig.1).

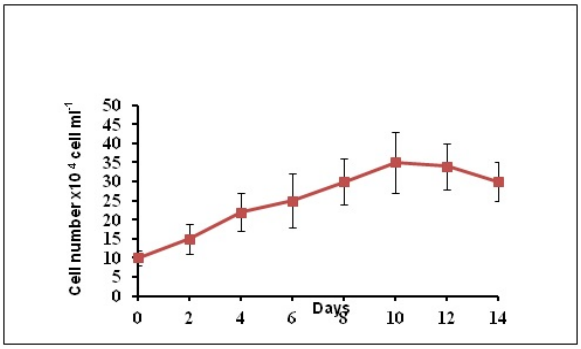


Fig. 1. Growth curve of *T. chuii* grown on F/2 medium. Each value is the mean of three readings ± standard deviation.

Nutritional factors optimizing algal growth:

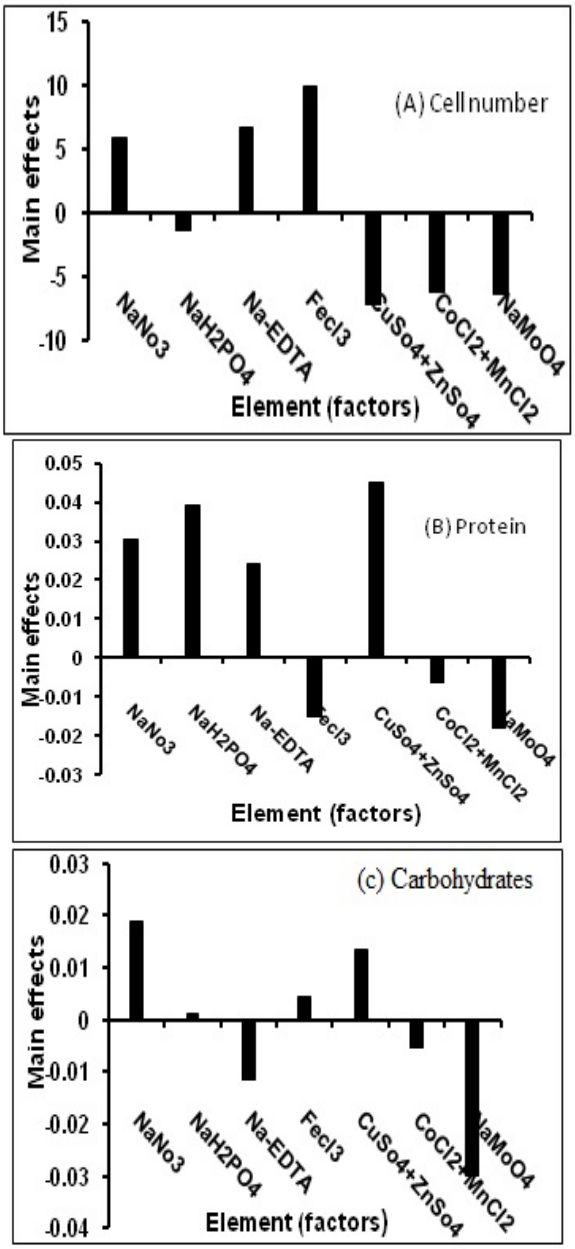
Applying Plackett-Burman experimental design showed that trial 7, where +ve concentrations of PO_4, EDTA, Fe, Co + Mn and –ve concentrations of NO_3, Cu + Zn, MoO_4, produced the highest cells number (Table 2). There were three variables (NO_3,

EDTA and Fe) had a +ve main effect on the cells number of *T. chuii* (Fig. 2A), while the other four variables had a -ve main effect. The t-value of these 7 variables was non-significant at $P \leq 0.05$.

Table 2. Cell number, protein, carbohydrates and total lipid contents (mg.ml^{-1}) in *Tetraselmis chuii* after 10 days of growth after applying Plackett -Burman design

Trials (n=8)	Element(factors)							Cell number	Protein	Carbohydrates	Total Lipid
	NO_3	PO_4	EDTA	Fe	Cu + Zn	Co + Mn	MoO_4				
1	+	+	+	-	+	-	-	62.3 ± 2.4	0.27 ± 0.02	0.052 ± 0.002	0.104 ± 0.003
2	+	+	-	+	-	-	+	66.3 ± 3.5	0.16 ± 0.004	0.022 ± 0.003	0.115 ± 0.005
3	+	-	+	-	-	+	+	58.3 ± 6.9	0.16 ± 0.003	0.002 ± 0.001	0.175 ± 0.01
4	-	+	-	-	+	+	+	37.3 ± 5.5	0.19 ± 0.003	0.014 ± 0.001	0.195 ± 0.005
5	+	-	-	+	+	+	-	60.7 ± 3.1	0.18 ± 0.004	0.063 ± 0.003	0.09 ± 0.01
6	-	-	+	+	+	-	+	61.3 ± 4.1	0.16 ± 0.005	0.012 ± 0.002	0.166 ± 0.01
7	-	+	+	+	-	+	-	67.3 ± 6.1	0.17 ± 0.005	0.012 ± 0.002	0.135 ± 0.008
8	-	-	-	-	-	-	-	58.3 ± 5.8	0.13 ± 0.003	0.023 ± 0.003	0.134 ± 0.005

Each value is the mean of three readings ± standard deviation



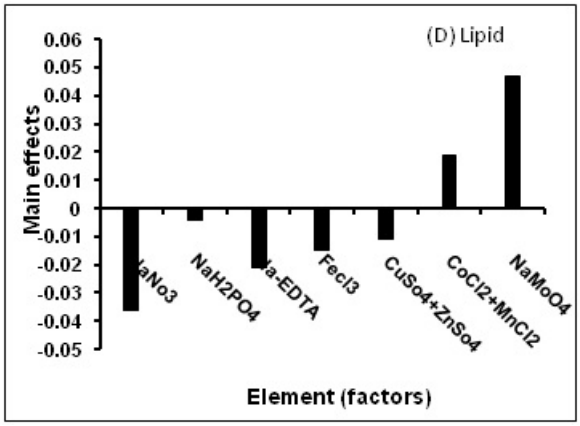


Fig. 2. The main effects of different variables on the growth and biochemical metabolites of *Tetraselmis chuii.*

In trial 1, *T. chuii* yielded the highest protein content (0.27 mg.ml^{-1}) where NO_3, PO_4, EDTA and (Cu + Zn) were present in +ve concentration and the other elements were present in –ve concentrations (Table 2). The t-value and degree of significance point to that Fe, MoO_4 and (Co + Mn) have a -ve main effect on the protein content, while the other variables have a +ve one (Fig. 2B). Statistical analysis estimated that (Cu + Zn) was the significant variable on protein content at $P \leq 0.05$.

Carbohydrate contents in *T. chuii* (Table 2) showed its maximum in trial 5 (+ve concentrations of NO_3, Fe, Cu + Zn and Co + Mn and –ve concentrations of PO_4, EDTA and MoO_4). According to levels of variables, it was appeared that (Co + Mn), EDTA and MoO_4 have -ve main effect, while the other variables exhibited a positive one (Fig. 2 C). Statistical analysis for the significance of the seven tested variables estimated that MoO_4 was the significance one at $P \leq 0.05$.

It was found that in the trial number 4, *T. chuii* produced the higher amount of lipid content (Table 2), where NO_3, EDTA and Fe were present in –ve concentrations and the

other variables were present in +ve concentrations.

MoO_4 and (Co + Mn) have the +ve main effects, while the other variables exhibited a -ve main effects on the lipid content (Fig. 2D). Statistical analysis for the significance of the seven factors estimated that MoO_4 was the significant variable at $P \leq 0.05$.

Fatty acids in *T. chuii* after the application of Plackett- Burman experimental design (Table 3) consisted mainly of saturated types including Capric, Lauric, Myristic, Palmitic, Margaric, Stearic and Arachidic (C10: 0, C12: 0, C14: 0, C16: 0, C17: 0, C18: 0, and C20: 0, respectively). The highest content of these acids (62.5 $\mu g.g^{-1}$dry wt) were recorded in trial 1. The monounsaturated fatty acids (MUFAs) included C16: 1, C17: 1, and C18: 1, recording the highest content in trial 8 (9.94 $\mu g.g^{-1}$dry wt). The polyunsaturated fatty acids (PUFAs) were linoleic acid (C18: 2), ALA (C18: 3), ETA (C20: 4), EPA (C20: 5) and DHA (C22: 6), these acids were found with highest amounts in trial 8 (48.6 $\mu g.g^{-1}$dry wt), where all variables were present in –ve concentrations.

Table 3. Fatty acids content ($\mu g.g^{-1}$dry wt) based on Plackett-Burman statistical design in *Tetraselmis chuii* after 10 days of growth

Fatty acids			Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8
Saturated	C10:0	Capric	0	0	0	0	0. 74	0	0	0
	C12:0	Lauric	6	6.8	8	4.7	0.5	5.5	0	0.47
	C14:0	Myristic	47.5	40	42.9	35.4	20.8	36	32.2	30.6
	C16:0	Palmitic	8.5	5.7	2.9	4.1	8.5	3.9	5.3	1.3
	C17:0	Margaric	0	0.85	0.7	0 .42	0.68	1.1	1.5 9	0.87
	C18:0	Stearic	0.5	0	0	0	0 .57	0	0 .37	0.5
	C20:0	Arachidic	0	0	0	0	0.85	0	0.83	0.9
Total			62.5	53.35	54.5	44.2	30.48	46.5	37.5	33.74
% to total fatty acids			57.8	58.9	51.1	51.4	39.5	52.3	41.5	36.6
Mono unsaturated	C16:1	Palmitoleic	0	0	0	0	0.47	0	0.28	0.54
	C17:1	Margaroleic	0	0	0	0	0.9	0	0	0
	C18:1	Oleic	4.5	2.5	8.6	4.6	11 .1	4.0	5.5	9.4
Total			4.5	2.5	8.6	4.6	1.37	4.0	5.78	9.94
% to total fatty acids			4.2	2.8	8.1	5.3	1.8	4.5	6.4	10.8
Poly unsaturated	C18:2	Linoleic	1.7	3 .4	4.2	3 .6	10.9	3.1	4.6	7.1
	C18:3	Alpha-linolenic (ALA)	37.8	30.3	34.2	32.7	31.6	33	38.1	38.6
	C20:4	Eicosatetraenoic (ETA)	0	0	0	0	1.8	0	2.6	2.9
	C20:5	Eicosapentaenoic (EPA)	1 .3	2.0	3.3	2	2 .8	1.7	1.7	3.9
	C22:6	Decosahexaenoic (DHA)	1.6	2.4	1.9	2.5	1.1	0.6	3. 9	4 .9
Total			41.1	34.7	43.6	37.2	45.4	38.4	47	48.6
% to total fatty acids			38.0	38.3	40.8	43.3	58.7	43.2	52.1	52.6
Total fatty acids ($\mu g.g^{-1}$dry wt)			108.1	90.55	106.7	86.0	77.25	88.9	90.28	92.28

Based on the Plackett-Burman experimental design (Table 4), the maximum omega 3 fatty acids (ω_3FAs) content in *T. chuii* was recorded in trial 8 at day 10 (49.3% of the total fatty acids), where all the variables present in –ve concentrations. PO_4 and EDTA have the +ve main effects, while other variables showed -ve main effects on the ω_3 FAs content. Statistical analysis showed that NO_3 was the most significant variable at $P \leq 0.05$.

Table 4. Main effects, t-value and degree of significance of the seven different variables affecting the total omega 3 fatty acids (ω_3FAs) ($\mu g.g^{-1}$dry wt) in *Tetraselmis chuii* after 10 days of growth

Trials (n=8)	Element (factors)							The sum of ω_3FAs	% to the total fatty acids
	NO_3	PO_4	EDTA	Fe	Cu + Zn	Co + Mn	MoO_4		
1	+	+	+	-	+	-	-	45.7	37.2
2	+	+	-	+	-	-	+	34.7	36.9
3	+	-	+	-	-	+	+	39.4	36.9
4	-	+	-	-	+	+	+	37.2	41.3
5	+	-	-	+	+	+	-	37.3	40
6	-	-	+	+	+	-	+	35.3	39.7
7	-	+	+	+	-	+	-	46.3	47.7
8	-	-	-	-	-	-	-	50.3	49.3
Main effects	-2.9*	0.4 n	1.8n	- 4.8 n	- 3.8 n	- 1.5 n	- 8.3n		
t- value	-1.4	-0.84	-1	0.62	-.022	0.66	-1.8		
p- value	0.22	0.42	0.34	0.55	0.82	0.52	0.13		

Each value is the mean of three readings ± standard deviation; *significant at $P \leq 0.05$ using t- test two sample assuming unequal variances; n non significant at $P \leq 0.05$ using t- test two sample assuming unequal variances

Effect of optimized Plackett-Burman media on growth and metabolites of *T. chuii*:

From the all previous results and based on the results of the main effects, composition of the newly formulated media for optimization the growth and some metabolites in *T. chuii* are listed in table 5. The maximum production of total lipid and omega3 fatty acids was observed with low NO_3 concentration (-ve sign). With respect to PO_4 the low concentration (-ve sign) gives higher total lipid content in *T. chuii*. EDTA was present in low concentration led to higher contents of total lipid, carbohydrate. Fe was present in +ve sign gives higher carbohydrate contents. Cu + Zn were present in +ve sign showed the higher contents of protein and carbohydrate. Co + Mn were present in +ve sign attained the maximum production of total lipid. Finally, the protein, carbohydrates and omega 3 fatty acids showed higher values with low MoO_4 concentration -ve sign. On the other hand, the total lipid showed higher values with high MoO_4 concentration +ve sign.

Table 5. Constituents of the newly formulated media for optimization of protein, carbohydrate, lipid, and omega 3 fatty acids in *Tetraselmis chuii*

Parameter	Variables (factors)							
	NO_3	PO_4	EDTA	Fe	Cu + Zn	Co + Mn	MoO_4	Vitamins $ml.L^{-1}$
	$g.L^{-1}$ stock solution			$g.100\ ml^{-1}$ stock solution				
+ level	112.5	7.5	6.54	4.73	1.5+3.3	1.5+2.7	0.945	0.5
-level	37.5	2.5	2.18	1.58	0.5+1.1	0. 5+0.9	0.315	0.5
F/2 medium	75	5	4.36	3.15	1 + 2.2	1 + 1.8	0.63	0.5
Protein	+	+	+	-	+	-	-	0.5
Carbohydrate	+	+	-	+	+	-	-	0.5
Total lipid	-	-	-	-	-	+	+	0.5
Omega3 fatty acids	-	+	+	-	-	-	-	0.5

Culturing the alga species to optimize their protein, carbohydrates, total lipids and omega3 fatty acids:

In this part the newly formulated media by applying the Plackett-Burman statistical design as compared to F/2 medium (as control) were used for culturing the algal species (*T. chuii*) after 10 days of culturing.

Protein, carbohydrates and total lipid contents increased in the tested alga cultured in the optimized medium after 10 days of culturing by 43.5%, 50%, and 91.5%, respectively as compared with F/2 medium (Table 6).

Table 6. Total protein, carbohydrate, lipid in *Tetraselmis chuii* grown on F/2 medium as compared with optimized medium after 10 days culturing

Algae	*T. chuii*	
media	F/2 medium	optimized medium
Total protein $mg.ml^{-1}$	0.46 ± 0.1	0.66 ± 0.08*
Carbohydrate $mg.ml^{-1}$	0.12 ± 0.01	0.18 ± 0.01**
Total lipid $mg.ml^{-1}$	0.14 ± 0.005	0.27 ± 0.02*

Each value is the mean of three readings ± standard deviation; **Highly significant at $P \leq 0.05$ using one way analysis of variance (ANOVA); * Significant at $P \leq 0.01$using one way analysis of variance.

The total ω_3FAs in *T. chuii* cultured on the optimized medium increased about 3 times than cultured on F/2 medium (Table 7), forming 98.5 $\mu g.g^{-1}$ dry wt, and it represented by 52.7 % of the total fatty acids. The ω_3FA fractions were ALA, ETA, EPA, and DHA.

Table 7. Omega 3 fatty acids (ω_3FAs) concentrations ($\mu g.g^{-1}$dry wt) and their percentage (%) to the total fatty acids in *T. chuii* grown on optimized medium in comparison to F/2 medium after 10 days of growth

	F/2 medium		optimized medium	
	%	Concentration ($\mu g.g^{-1}$dry wt)	%	Concentration ($\mu g.g^{-1}$dry wt)
C18:3 Alpha-linolenic (ALA)	23.8	21.6	35	65.5
C20:4 Eicosatetraenoic (ETA)	3.9	3.5	3.7	7
C20:5 Eicosapentaenoic (EPA)	3.2	2.9	7.4	13.7
C22:6 Decosahexaenoic (DHA)	6.4	5.8	6.6	12.3
Total ω_3FAs ($\mu g.g^{-1}$dry wt)	37.3	33.8	52.7	98.5
Total fatty acids ($\mu g.g^{-1}$dry wt)		90.8		186.9

***Artemia franciscana* enriched with *T. chuii*:**

The nutritional value of *A. franciscana* fed on *T. chuii* cultured on the optimized medium represented by protein, carbohydrate, total lipid, was increased about 58.9, 106.6, and 173, respectively than that the alga cultured in the control medium (F/2) (Table 8). One way analysis of variance showed that there was a significant increase in protein, carbohydrate, and total lipid in *A. franciscana* fed on *T. chuii* cultured on the optimized medium ($P \leq 0.001$).

Table 8. Comparison between protein, carbohydrate, total lipid contents in *Artemia franciscana* fed on *Tetraselmis chuii* grown on the F/2 and optimized media

Parameter	*Artemia fed on T. chuii* grown on F2 medium	*Artemia fed on T. chuii* grown on optimized medium
Total protein ($mg.g^{-1}$)	30.24 ± 4.1	48.07 ± 3.5*
Carbohydrate ($mg.g^{-1}$)	17.04 ± 2	35.2 ± 4**
Total lipid ($mg.g^{1}$)	0.3 ± 0.03	0.82 ± 0.05*

Each value is the mean of three readings ± standard deviation; * Highly significant at P ≤ 0.001 using one way analysis of variance (ANOVA); ** Highly significant at P ≤ 0.0 1 using one way analysis of variance (ANOVA).

The results showed that, ω_3 fatty acids of *A. franciscana* enriched with *T. chuii* and grown on the optimized medium, respresented 61.1% of total fatty acids compared to 34.8 % in *Artemia* fed on *T. chuii* grown F/2 medium (Table 9). These fatty acids were C18: 3, C20: 5, and C22: 6 present in *A. franciscana* fed on *T. chuii* cultured in optimized medium, while C22: 6 not present in *A. franciscana* fed on *T. chuii* grown on F/2 medium.

Table 9. Comparison between Omega3 fatty acids (ω_3FAs) and their percentage (%) to total fatty acids in *Artemia franciscana* enriched with *Tetraselmis chuii* grown on F/2 and optimized media

ω_3FAs	*A. franciscana* fed on *T. chuii* grown on F/2 medium			*A. franciscana* fed on *T. chuii* grown on optimized medium		
	% to total fatty acids	% to total ω_3FAs	Concentration ($\mu g.g^{-1}$dry wt)	% to total fatty acids	% to total ω_3FAs	Concentration ($\mu g.g^{-1}$dry wt)
C18:3 Alpha-linolenic (ALA)	26.5	76	37.12	18	29.4	24.61
C20:5 Eicosapentaenoic (EPA)	8.3	24	11.7	32	52.4	43.88
C22:6 Decosahexaenoic (DHA)				11.1	18.2	15.25
Sum ω_3FAs	34.8		48.82	61.1		83.74
Total fatty acids		140.28			137.01	

DISCUSSION:

Cultivated microalgae have long been integral to the hatchery production of many farmed finfish, shellfish and other commercially important aquaculture species. Microalgae providing those proteins, carbohydrates, vitamins, essential polyunsaturated fatty acids (PUFAs), pigments and sterols which are transferred up through the food chain (Nichols *et al.*, 1989).

As regarded to the growth of *T. chuii* on the optimized medium, it was significantly increased 3 folds over control (F/2 medium) at low trace metals concentration (0.5 of F/2) and high NO_3 concentration (1.5 of F/2). Similar results obtained by Feng *et al.* (2011) who indicated that the addition of NO_3 is one of the factors promoting cell division of the marine microalga *Isochrysis zhangjiangensis*.

The present results showed that, in *T. chuii,* the protein increased by increasing the concentration of NO_3, PO_4 by 1.5 times over that found in the cells grown in F/2 medium. Nitrogen availability can affect the synthesis and accumulation of cell constituents, such as pigments, proteins, carbohydrates, amino acids, nucleic acids and lipids. Most of the cell nitrogen is in the protein form. Thus, nitrogen consumption from the medium directly affects protein synthesis (Utting, 1985). This result is in agreement with that obtained by Vergara and Niell (1993) who concluded that total soluble protein increased when the algal cells incubated under nitrogen sufficient condition.

Phosphorus is the primary limiting nutrient for microalgae in many natural environments (Larned, 1998). Kilham *et al.* (1997) demonstrated that phosphorus starvation reduces protein content in algal cells.

Carbohydrate contents in *T. chuii* increased in the optimized medium by 50% greater than that cultured on the F/2 medium. Carbohydrate increased by increasing of NO_3, PO_4, Fe, and Cu + Zn by 1.5 times of F/2 medium and decreasing of EDTA, Co + Mn, and MoO_4. The optimization of carbohydrates synthesis usually took place when one or more factors at negative levels. Thomas *et al.* (1984) reported increase in carbohydrate content of *Phaeodactylum tricornutum* when cultured in N sufficient medium. El-Sayed (2007) reported that carbohydrate of *Nannochloropsis salina* content increased by 98% compared to basal medium at 1.5 times of NO_3 and MoO_4 that of basal medium.

Lipid production usually differs between genera, species and strains of microalgae (Barclay *et al.*, 1994). Our results showed that there was a significant increase in the total lipid by 91.48 % in *T. Chuii* using the optimized medium than F/2 medium. Lipid content increased in *T. chuii* by decreasing the concentration of NO_3 and PO_4 by 0.5 times those of the F/2 medium concentration.

A number of factors have been shown to influence the lipid content of algae, such as nitrogen deficiency (Yeesang and Cheirsilp, 2011), phosphate limitation (Gouveia and Oliveira, 2009), salt stress (Hu and Gao, 2006), and iron content of the medium also affect algal growth along with lipid content (Tang *et al.*, 2010; Yeesang and Cheirsilp, 2011).

Lipid content in *T. chuii* rose as nitrate and phosphates concentration declined in the medium. These results are in accordance with Yeesang and Cheirsilp (2011) who reported that algal cells accumulate carbon metabolites as lipids under nitrogen deficient conditions. On the other hand, Li *et al.* (2010) found that phosphorus limitation also leads to accumulation of lipids in *Scenedesmus* sp.

Stephenson *et al.* (2010) showed that in some species, removing nutrients such as nitrogen from the growth medium decreased the cell division and induced a stress behavior in which cell size increases and neutral lipid accumulates in the cytoplasm as observed in *Chlorella vulgaris*. In addition, Halling-Sørensen *et al.* (2000) concluded that the changes in algal nitrogen status cause an increase in lipid content of *Selenastrum carpricornutum*. They found also that, nitrogen starvation induced an increase in lipid content of *S. carpricornutum* from 17 to 44% of the algal dry weight.

Nitrogen limitation would cause three changes: decreasing of the cellular content of thylakoid membrane, activation of acylhydrolase and stimulation of the phospholipid hydrolysis. These changes may increase the intracellular content of fatty acid acyl-CoA. Meanwhile, nitrogen limitation could activate diacylglycerol acyl transferase, which converts acyl-CoA to triglyceride (TAG) (Takagi *et al.*, 2000). Therefore, nitrogen limitation could both increase lipid and TAG content in microalgal cells.

Total ω_3FAs in *T. chuii* cultured on the optimized medium increased about 3 folds than cultured on F/2 medium, forming 98.5 $\mu g.g^{-1}$ dry wt, and it represented by 52.7 % of the total fatty acids when PO_4 and EDTA present in positive concentration while other variables NO_3, Fe and trace element present in negative concentrations than that F/2 medium.

Abd El-Baky *et al.* (2004) reported that under nitrogen limitation, *Dunaliella salina* contained high amount of total lipid rich in ω_3 polyunsaturated fatty acids and antioxidant compounds. Carotenoids and fatty acids are two examples for non-enzymatic classes of substances which are able to protect the organism from oxidative damage (Sies and Stahl, 1995; Christaki *et al.*, 2013)

The present results found that the ω_3 fatty acids fractions in *T. chuii* were present as C18: 3, C20: 4, C20: 5, and C22: 6 (ALA, ETA, EPA and DHA) respectively. Similar results were recorded by Brown and Jeffrey (1992) as they reported huge variations in the fatty acid composition between different microalgae. This indicates that the fatty acid profile is species-specific.

The significant role of nitrogen in the production of ω_3 fatty acids has been demonstrated in some other microalgal species (Yongmanitchai and Ward, 1991). With respect to the fatty acids production, *Botryococcus braunii, Dunaliella bardawil,* and *Dunaliella salina* produced a higher percentage of ω_3 fatty acid EPA (C20: 5) under low N-levels (Ben-Amotz *et al.*, 1987). These results agree with our results concerning *T. chuii* where ω_3 fatty acids increased with decreasing nitrogen concentration. On the contrary, N and P starvation reduces the level of ω_3 fatty acids ARA, as well as EPA in *Nannochloropsis* sp (Hu and Gao, 2006). The proportion of EPA in the red microalga *Porphyridium cruentum* decreases during N starvation while the proportion of ARA increases (Cohen, 1990).

Our results confirmed the potential of *T. chuii* to produce a high amount of valuable long chain PUFAs (ω_3 fatty acids) under different nutrient concentration and Alpha-linolenic acid (C18: 3) was the dominant ω_3 PUFAs with the optimized medium and represented by (35% of the total FAs in *T. chuii*). This makes the strain a promising candidate for aquaculture and biotechnology. While the results of El-Sayed (2007) proved that Decosahexaenoic (C22: 6) was the dominant PUFAs in the optimized medium of *Dunaliela salina*, *Nannochloropsis salina, Chlorella vulgaris* and *Tetraselmis chuii* compared to basal medium.

One of the major applications of microalgae in aquaculture is the enrichment of rotifers in order to improve their nutritional content before being fed to fish larvae. Today, *Artemia* is one of the most-used live feeds for the larval rearing of marine fish and crustaceans. Additionally, there is an increasing demand from hatcheries for *Artemia* biomass to induce ovarian maturation of fish.

This work revealed significant increases in the biochemical composition of the *A. franciscana* (total protein, carbohydrate, total lipid, and omega3 fatty acids) after 24 h of enrichment with *Tetraselmis chuii* cultured on newly optimized media. This declared the importance of the biochemical composition of the food alga in modulating the biochemical composition of the filter-feeder. Good quality microalgae constitute an excellent diet for enrichment since they provide rotifers with essential fatty acids (Dhert *et al.*, 2001). The brine shrimp *Artemia* is probably the most popular live diet in aquaculture. Zaki and Saad (2010) concluded that the nutritional adequacy of zooplankton used for feeding in newly hatched larvae in marine hatcheries depend on nutritional value of microalgae used to enrich brine shrimp *Artemia salina.*

Results showed that the percentage increases in the contents of total protein (58.9%), carbohydrates (106.6%) and lipid (173%) in *A. franciscana* metanuplii enriched with *T. chuii* cultured on newly proposed

medium than those cultured in the F/2 medium for 24 h. Similar results have been obtained by Reitan *et al.* (1997), demonstrating that after 24 h enrichment with *Tetraselmis suecica* and *Isochrysis galbana*, protein and lipid contents increases between 50 and 70% with regard to the initial values. Ben-Amotz *et al.* (1987) found a close relation between both the gross biochemical composition and lipid profile of rotifers and those of their algal diet. Lipids are an important component of diet, both as energy and essential fatty acids sources needed by fish for basic functions including growth, reproductive and maintenance of healthy tissues (Sargent *et al.*, 1989).

The content of polyunsaturated fatty acids (PUFAs), in particular eicosapentaenoic acid (C20: 5, EPA) and docosahexaenoic acid (C22: 6, DHA), is of major importance in the evaluation of the nutritional composition of an algal species to be used as food for marine organisms. The present study showed that the total Omega3 fatty acids composition of *A. franciscana* enriched with *T. chuii* grown on newly proposed optimized medium had higher omega3 fatty acids content (83.74 $\mu g.g^{-1}$dry wt) than those cultured on the F/2 medium (48.82 $\mu g.g^{-1}$dry wt,). This was due to the higher omega3 fatty acids content of the algae used through enrichment process. These results were in accordance with those obtained by Reitan *et al.* (1993), reporting that the fatty acids composition of rotifers are closely related to that of the diets used, and short term feeding of rotifers with algae will shift the fatty acid composition towards that of the algal species used.

The microalgae, commonly used to produce mass quantities of rotifers, are rich in ω_3PUFAs, mainly represented by C20:5n-3 (eicosapentaenoic acid or EPA) and C22:6n-3 (docosahexaenoic acid or DHA). After having ingested these algae, the PUFA content of rotifers increases and in a few hours they become an essential food source for the early larval stages of marine fish and crustacean species (Brown *et al.*, 1997). On the other hand, the amount of C18: 3 Alpha-linolenic (ALA) present in *A. franciscana* enriched with *T. chuii* grown on newly proposed optimized medium was 24.61 $\mu g.g^{-1}$dry wt and C20: 5 Eicosapentaenoic was 43.88 $\mu g.g^{-1}$dry wt. Also C22: 6 Decosahexaenoic was 15.25 $\mu g.g^{-1}$dry wt).

The present results also demonstrated that C20: 5 Eicosapentaenoic (EPA) and C22: 6 Decosahexaenoic (DHA) were provided in algal diet with low concentration but appear in *A. franciscana* after enrichment process with high concentration, and this may due to that linoleic acid (18: 2 ω_6) and linolenic acid (18: 3 ω_3) are thus essential dietary fatty acids, which can easily be converted by fish larvae via a series of desaturation and elongation reactions to very long-chain (C20 and C22 PUFAs), for example, the principal ω_3PUFAs, eicosapentaenoic acid (EPA, 20: 5ω_3) and docosahexaenoic acid (DHA, 22: 6ω_3), and the ω_6PUFA, arachidonic acid (ARA, 20: 4ω_6) (Jobling and Bendiksen, 2003).

One of the challenges facing aquaculture of fish is the high demand for fish meal and oil as food for the farmed fishes (Steffens, 1997). Several marine fish species are rich in n-3 polyunsaturated fatty acids (PUFAs) such as EPA or DHA. This is attributed to the lipid composition of plankton. It is well established that the presence of longer carbon chain, PUFAs in the lipid profile, especially EPA and DHA in the micro algal diet associated with high growth rates of bivalve larvae and many other juvenile aquaculture organisms. The growth of juvenile oysters was retarded if EPA and DHA were not present in the microalgal diet (Enright *et al.*, 1986).

There is strong evidence suggesting that consumption of fish containing high levels of these fatty acids is favorable for human health and has a particularly beneficial effect in preventing cardiovascular diseases (Ward and Singh, 2005). It is well known that there are two series of essential fatty acids which cannot be synthesized by animals or humans and must be supplied in the diet (Horrobin and Manku, 1990). The ω_6 series are derived from linoleic acid (LA), and the ω_3 series from α linolenic acid (ALA).

Conclusions and recommendations:

The wide spectrum of microalgae with different nutritive qualities generated through the different nutrient concentrations is responsible for these variations. Also, differences in biochemical composition of the *Artemia* produced are expected. The capacity of controlling the quality of the biomass and the produced nauplii in *Artemia* cultures could be very useful as a tool in physiological studies and in aquaculture for the nutrition of both finfish and crustacean larvae during their early development stages. The present results confirmed the potential of the microalga *T. chuii* to produce a high amount of valuable long chain ω_3PUFAs, protein, carbohydrates, lipid under different nutrient concentrations. Thus the newly formulated media was coast less media for culturing algal species for live feeds, which can be easily prepared and at the same time can offer the desired growth and production of secondary metabolites in the tested algae which can transferred through feeding organisms. Therefore, we recommend using the newly optimized medium for culturing the algal species which commonly used in marine hatcheries to produce high-quality fish, and we can use the tested species for producing fatty acids, proteins and carbohydrates for other economic important.

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أقلمة مكونات الوسط الغذائى بأستخدام تصميم بلاكت بيرمان لأنتاجية عالية من البروتين والكربوهيدرات والدهون لطحلب التتراسلمس شيياى

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تعتبر الطحالب الدقيقة من أهم العالقات النباتية المستخدمة فى تغذية يرقات الكائنات البحرية. تهدف هذه الدراسة الى تطبيق التصميم الأحصائى بلاكت بيرمان لتحديد اى من العوامل (العناصر) الموجودة فى الوسط الغذائى التى تؤثر على انتاجية البروتينات و الكربوهيدرات و الدهون والأوميجا 3 فى طحلب التتراسلمس شيياى. فقد تم زراعة الطحلب على الوسط الغذائى (F2) الوسط الكنترول لتحديد أفضل يوم للنمو وكان أعلى نمو عند اليوم العاشر. بتطبيق التصميم الأحصائى بلاكت بيرمان للحصول على وسط غذائى جديد زاد محتوى البروتين والكربوهيدرات بنسبة 43% و 50% على التوالى مقارنة بالوسط الغذائى (الكنترول) و ذلك عند تركيز عالى من النترات والفوسفات. بينما الدهون الكلية زادت بنسبة 91.5% عند تركيز منخفض من النترات والفوسفات. أيضا الأحماض الدهنية أوميجا 3 زادت 3 مرات مقارنة بالكنترول.

بعد ذلك تم تغذية الأرتيميا فرنسسكانا على طحلب التتراسلمس السابق زراعته فى كل من الوسط الجديد المعدل والوسط الكنترول. وقد أوضحت النتائج أن كمية البروتين والكربوهيدرات والدهون فى الأرتيميا التى تغذت على الطحلب السابق زراعته على الوسط الجديد زادت بنسبة 58.9, 106.6، 173 % على التوالى مقارنة بالأرتيميا التى تغذت على الوسط الغذائى (الكنترول). أما الأحماض الدهنية (أوميجا 3) فى الأرتيميا التى تغذت على التتراسلمس شيياى السابق زراعته فى الوسط الجديد كانت نسبتها 61.1% من الدهون الكلية مقارنة بالأرتيميا التى تغذت على الوسط الكنترول كانت نسبتها 34.8% وكانت هذه الأحماض C22:6 , C20:5 ,C18:3. وعلى ذلك الطحلب الغنى بالأحماض الدهنية (أوميجا 3) يغطى متطلبات الأحماض الدهنية التى تضمن أعلى نمو ومعيشه وكفاءة ليرقات الأسماك.