CHEMICAL ENGINEERING TRANSACTIONS
VOL. 32, 2013
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at: www.aidic.it/cet
Chief Editors: Sauro Pierucci, Jiří J. Klemeš

ISBN 978-88-95608-23-5; **ISSN** 1974-9791
DOI: 10.3303/CET1332163

Effect of the Culture Nutrients on the Biomass and Lipid Productivities of Microalgae *Dunaliella tertiolecta*

Teresa M. Mata*[,a], Ricardo Almeida[b], Nídia S. Caetano[a,b]

[a]LEPAE – Laboratory for Process, Environmental and Energy Engineering, Faculty of Engineering, University of Porto (FEUP), R. Dr. Roberto Frias S/N, 4200-465 Porto, Portugal
[b]Department of Chemical Engineering, School of Engineering (ISEP), Polytechnic Institute of Porto (IPP), R. Dr. António Bernardino de Almeida S/N, 4200-072 Porto, Portugal
* tmata@fe.up.pt

This work analyses the influence of macro and micronutrients on the growth, biomass and lipid productivities of *Dunaliella tertiolecta* (*D. tertiolecta*), showing that with the exception of Nitrogen (N) and Iron (Fe), the other nutrients added to the culture medium - Magnesium (Mg), Potassium (K), Manganese (Mn) and Zinc (Zn) - did not influence the studied parameters. The best maximum and average dry biomass productivities were respectively 141.8 mg/L/d and 63.1 mg/L/d, obtained in the 5th day of the test, by increasing the N concentration 10 times in comparison to the standard culture medium "artificial seawater medium with vitamins". In these conditions, the lipid content and maximum lipid productivity were respectively 33.5 % and 47.4 mg/L/d, corresponding to more than three times the value in the standard conditions (13.3 mg/L/d). Also, by increasing the Fe concentration 10 times in the culture medium, the maximum lipid productivity increased to almost the double, i.e. from 14.6 mg/L/d to 28.0 mg/L/d, obtained in the 28th day of the test. Therefore, N and Fe addition to the culture medium resulted in a significant increase in lipid productivity, suggesting that residual wastewaters rich in N or Fe could be used to cultivate *D. tertiolecta* for lipid production.

1. Introduction

Due to its rapid growth, microalgae can produce considerably larger amounts of biomass and lipids per hectare than oilseeds. For this reason they are increasingly viewed as potential alternatives to traditional biodiesel feedstocks such edible vegetable oils (Mata et al., 2012a), animal fats (Mata et al., 2010a, 2011a) or other residual materials like spent coffee grounds (Caetano et al., 2012). They have the advantages of not competing with fertile agricultural land and are an effective mean for CO_2 capture (Mata et al., 2010b). However, large-scale sustainable production of biofuels from microalgae is still a challenge, as it requires the use of much energy and nutrients (Mata et al., 2011b). Also, producing biofuels from microalgae requires a balance of tradeoffs between species with high biomass productivity and high lipid content that can be reliably manipulated and are tolerant or recover easily from a range of environmental perturbations to continue growing (Mata et al., 2012b). Likewise, it is specie-specific the photosynthetic performance and cells composition in response to a nutrients-replete or deplete medium (Mata et al., 2012c). Therefore, a combination of environmental parameters can affect microalgae growth and lipid accumulation of which, the type and availability of nutrients play a major role (Chen et al., 2011).
Several authors have reported an increase in the microalgae lipid content during a nutrients "starvation" or "stress" phase. For example, Illman et al. (2000) found that the nitrogen concentration reduction in the medium increases the lipid content in all five investigated *Chlorella* strains, among which *C. emersonii*, *C. minutissima* and *C. vulgaris* gained an increase in lipid content of respectively, 63 %, 56 % and 40 % biomass by dry weight. Macedo and Alegre (2001) demonstrated that the *Spirulina* lipid content increased approximately 3 times with the nitrogen and temperature decrease, being the nitrogen concentration decrease more effective. Liu et al. (2008) showed that high iron concentration could also induce considerable lipid accumulation in marine strain *C. vulgaris*. This suggests that some metabolic pathways

Please cite this article as: Mata T., Almeida R., Caetano N.S., 2013, Effect of the culture nutrients on the biomass an lipid productivities of microalgae dunaliella tertiolecta, Chemical Engineering Transactions, 32, 973-978 DOI: 10.3303/CET1332163

related to the lipid accumulation in *C. vulgaris* are probably modified by high level of iron concentration in the initial medium. Thomas et al. (1984) also studied the effect of nitrogen stress on algae lipid fraction, concluding that cultivation under nitrogen deprivation did indeed increase the neutral lipid contents, but it cannot be summarized as a single trend. This is observed for *Botryococcus, Isochrysis* and *Dunaliella* species grown under nitrogen stress. In *Botryococcus* the neutral lipids comprised a major proportion of the total lipids. However, the greatest neutral lipid production occurred in the resting stage and the greatest amount is formed in the conversion of the algae from the green to the brown growth phase. In contrast to the 10 % increase in the *Botryococcus* lipids, there was a drop in the lipid fraction in *Dunaliella bardawil* and *Dunaliella salina* to about 10 % of the organic weight. These halotolerant green algae shifted towards carbohydrate storage under nitrogen stress. On the other hand, *Isochrysis* accumulated higher fractions of lipids and carbohydrates under nitrogen deficiency, with lipids comprising about one-fourth of the algal organic cell weight following 10 days of nitrogen starvation. In general, the effects of nitrate deficiency were that the protein content and the chlorophyll level decreased while carbohydrate and lipids exhibited a species-specific change. The neutral lipid content is expressed in the algae that shift to lipid storage when under environmental stress. These neutral lipids are not predominantly straight chain saturated hydrocarbons but multi-branched and/or polyunsaturated components. In which respects *D. tertiolecta*, Jiang et al. (2012) observed that, with nitrogen depletion, the lipid content increased but the biomass and growth rates significantly lowered. However, Fábregas et al. (1995) reported that the lipid content of *D. tertiolecta* increased, although less than the proteins, together with increasing growth rate as a result of an increase in nitrogen availability.

This work aimed to study the influence of a nutrients change in the culture medium of the saltwater microalga *D. tertiolecta* on its growth, biomass and lipid productivities, having in mind its use for biodiesel production. Thus the concentration of macronutrients (Magnesium, Potassium and Nitrogen) and micronutrients (Manganese, Zinc and Iron) was increased by 10 and 20 times those of the standard cultivation medium (Artificial Seawater Medium with Vitamins (SAG, 2008)).

2. Materials and methods

2.1 Preparation of the culture mediums

A culture of *D. tertiolecta* SAG 13.86 from the German SAG (Sammlung von Algenkulturen Göttingen) collection was used for the purpose of this study, cultivated in the “artificial seawater medium with vitamins”, as proposed by SAG (2008) for this microalga’s standard medium. The pH of the culture medium was set at 7.7 ± 0.15 within the optimal range (between 7.5 and 8.5) for this microalga.

The reagents for the culture medium preparation were all analytical grade, from MERCK, to reduce the variability and improve accuracy in controlling the growth conditions.

In order to prevent contamination by other microorganisms, all the laboratorial material and glassware (test tubes, and glass flasks) were washed with water and detergent, rinsed with distilled water, autoclaved at 121 ºC for 20 min and placed in a laminar flow cabinet (CRUMA, 870-FL), under UV radiation for 60 min, prior to use. Disposable pipettes and sterile loops were used.

2.2 Routine microalgae culturing and acclimatization

Test tubes were inoculated with *D. tertiolecta* in approximately 10 mL of the standard medium, to start the cells acclimatization, and closed with cotton plugs (allowing air diffusion). About 25 mL of the dense test tube cultures (with 25 days from the inoculum) were transferred to 250 mL Erlenmeyer flasks and supplemented with fresh culture medium. Then, 150 mL of these cultures were transferred, after more 25 days, from the 250 mL Erlenmeyer flasks to 1,000 mL Erlenmeyer flasks and supplemented with fresh culture medium. Finally, mother cultures were prepared using about 750 mL of these pure dense cultures, after 25 days more, and supplemented to 5,000 mL with fresh culture medium. These were allowed to grow until reaching dense cultures at stationary phase, i.e. up to stabilization of the absorbance value.

The algae growth was followed in time by keeping constant the culture conditions such as room temperature (25 ºC ± 2 ºC), light/dark (L/D) photoperiod (12/12 h), and light intensity (2,500 Lux for the test tubes and 5,000 Lux for the 250, 1,000 and 5,000 mL Erlenmeyer flasks) provided by fluorescent lamps (36 W, Sylvania Aquastar T8) and measured with a luxmeter (Lutron LX-1102). Air sterilized by filtration (0.2 µm pore diameter) was supplied to the microalgae cultures at an average flowrate of 2 mL/s (for 250 mL Erlenmeyer flasks) and 4 mL/s (for 1,000 and 5,000 mL flasks) by air pumps (Pacific AP6), through the perforated rubber stopper of the flasks.

All the experimental tests were performed in triplicate, in the 1 L Erlenmeyer flasks. At the same time it was monitored the existence of any potential contamination in the cultures under study, which was done

by visual inspection and by using an optical microscope (Trinocular microscope 3B Scientific Physics, Model 400, U30712).
The stationary growth phase was attained essentially at the same time for all the assays, after 25 days from the inoculation.

2.3 Evaluation of the microalgae growth
For evaluating the microalgae growth, first the calibration curves (absorbance at 680 nm versus dry weight biomass concentration) were traced for *D. tertiolecta* according to the following procedure: (1) 1,000 mL Erlenmeyer flasks cultures were prepared in triplicate with about 200 mL from the 5,000 mL mother cultures (described in session 2.2) and allowed to grow until reaching dense cultures; (2) Absorbance measurements were done daily in a UV-Vis spectrophotometer (Shimadzu UV-160A) by taking 6 mL samples from the 1000 mL cultures for performing the reading in duplicate (with about 3 mL for each reading). The stabilization of the absorbance values indicated that the cultures reached the stationary phase as dense cultures; (3) Biomass was harvested by centrifugation (in a Sorvall superspeed centrifuge SS-3 automatic) at 4,000 rpm for 20 min and then lyophilized (in a BT6K EL Virtis freeze dryer); (4) Eight standard solutions of known concentration were prepared by weighing different amounts of the lyophilized biomass (in a Kern ALJ 220-4 digital balance, with $\pm 5x10^{-5}$ g accuracy) and adding distilled water; (5) The absorbance of these eight solutions was read in triplicate at 680 nm (maximum absorbance peak) using a cuvette with a light path length of 1 cm (in a Shimadzu UV-160A UV-Vis spectrophotometer); (6) Finally, the calibration curves were traced for each microalgae.
Second, the assays were performed in triplicate (in 1 L flasks inoculated with 200 mL of the mother culture), varying the nutrients concentration (10 and 20 x). The macronutrients assayed were magnesium (Mg), potassium (K), nitrogen (N, in the form of nitrate) and phosphorus (P), and the micronutrients were manganese (Mn), zinc (Zn), iron (Fe) and cobalt (Co). All the cultures growth was evaluated by daily reading the absorbance in a UV-Vis spectrophotometer at 680 nm. The biomass concentrations were obtained this way based on the calibration curve of absorbance versus dry weight biomass concentration. The stationary phase was reached when the absorbance values stabilized, proceeding then to the biomass harvest.

2.4 Harvest of the microalgae biomass, lipids extraction and quantification
After the culture reached the stationary phase the microalgae biomass was harvested by centrifugation at 3,000 revolutions per minute (rpm), for 15 min, using a Super-speed Automatic Centrifuge (SORVALL SS-3). Then, the biomass was cataloged and stored in cold (at -20 ± 5 º C) until the lipids extraction.
For the lipids extraction and quantification it was chosen the Bligh and Dyer (1959) method, modified in this work as follows: (1) First, the biomass sample obtained by centrifugation was weighed in a centrifuge pre-weighed glass tube; (2) Then, the co-solvents were added at ratios of 1, 2 and 0.8 (v/v) for chloroform (Riedel de Haën, p.a.), methanol (Riedel de Haën, p.a.) and distilled water, respectively. Since it was used a centrifuged biomass (with a known water content) and not lyophilized biomass, these proportions were corrected taking into account a fraction of water in the wet biomass of about 70 %; (3) The centrifuge tube containing the biomass sample with co-solvents was subjected to ultrasounds for 30 min (in a Baldelin Sonorex TK30 equipment); (4) A second extraction step was then performed by adding the co-solvents at ratios of 2, 2 and 1.8 (v/v) for chloroform, methanol and distilled water, respectively; (5) The sample was again subjected to ultrasounds for more 30 min and then centrifuged at 3,000 rpm, for 15 min (in a ECCO Tvp 25 No. 8601 centrifuge). (6) After centrifugation three layers became visible: an upper layer rich in water and methanol, a middle layer consisting of extracted biomass, and a lower layer rich in lipids and chloroform. The upper layer rich in water and methanol was discarded and the lower layer rich in lipids and chloroform was recovered to a previously weighed glass tube; (7) The chloroform was evaporated to dryness (in a laboratorial hood at room temperature of about 25 ºC) and the purified lipids extract remained in the glass tube; (8) The tube containing the lipids (pre-weighed when empty) was weighed again in order to determine the microalgae lipid content by gravimetry.

3. Results and discussion

3.1 Evaluation of the effect of the nutrients concentration on microalgae growth
Figure 1 shows the growth curves of *D. tertiolecta* cultivated in the standard medium and in the mediums with the modified concentration of macro and micro nutrients, compared to the standard medium, which served as the control culture. The macronutrients assays are designated by 10xMg, 10xK, 10xN, 20xMg, 20xK and 20xN respectively, in which the concentrations of magnesium, potassium and nitrogen were increased by 10 and 20 times compared to standard medium.

Figure 1a shows that all assays reached the stationary growth phase at about the same time (after 26 days). In the assays with nitrogen addition the biomass concentration was higher than in the standard medium. In the assay with addition of 10xN, 26 days after it started, the biomass concentration was the highest (1,497 mg/L of biomass dry weight) about 59 % greater than in the control test (942 mg/L of biomass dry weight). In the assays with magnesium and potassium addition, the biomass concentration was lower than in the control test and, the lowest concentration (712 mg/L of biomass dwt) was obtained in the assay with 20xMg. Figure 1b shows that for all the assays the stationary growth phase was reached at about the same time (after 25 days). The highest biomass concentration (1,257 mg/L of biomass dry weight) was reached in the assay with addition of 10xFe, which is about 34 % greater than in the control test, and greater than the 20xFe culture (1042 mg/L of biomass dwt). In the assays with manganese and zinc addition, the biomass concentration was lower than in the control test. The lowest biomass concentration was reached in the 10xZn assays (601 mg/L of biomass dwt). Therefore, from the assays realized it was clear that N and Fe addition at 10 times concentration in the standard culture medium favored microalgae growth rate.

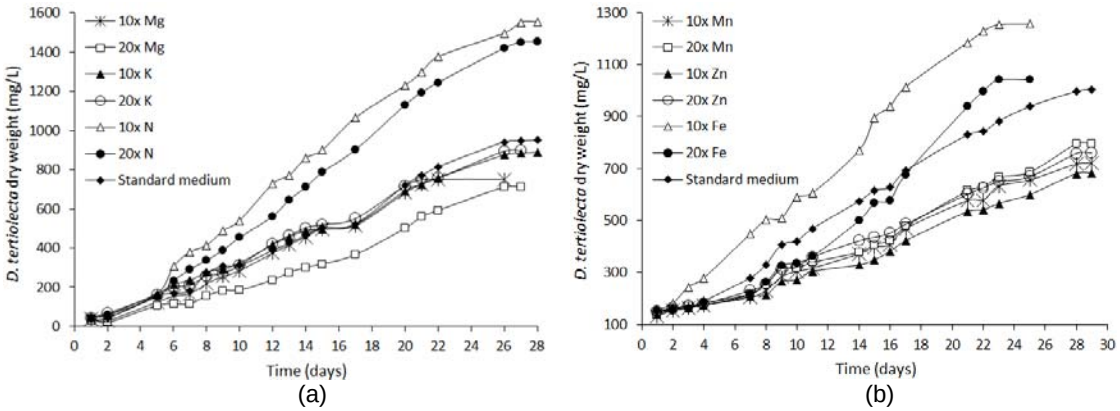

Figure 1: Growth curves of D. tertiolecta, cultivated in the standard medium and in the mediums with the modified concentration of (a) macronutrients and (b) micronutrients

3.2 Biomass and lipid productivities versus the culture medium nutrients concentration

Table 1 shows the maximum and average biomass and lipid productivities obtained by changing the macro and micro nutrients concentration in the culture medium by comparison with the standard medium, which serves as the control culture. The biomass productivity is given by the slope of the tangent to the growth curve formed by the set of biomass dry weight concentration values over time. The average biomass productivity was calculated during the exponential growth phase. The maximum biomass productivity is the maximum productivity value obtained in a 24 h period.

Results in Table 1 show that, concerning the macronutrients concentration change in the culture medium, the 10xN are the assays in which the highest maximum and average biomass productivities were obtained (respectively, 141.8 mg/L/d, from day 5 to 6, and 69.5 mg/L/d during the exponential growth phase) and these values are above the ones of the standard medium (respectively, 90.8 mg/L/d, from day 7 to 8, and 36.6 mg/L/d during the exponential growth phase). In the same 10xN assays the highest lipid content was obtained (33.45 mg/L/d) and thus, also the highest maximum and average lipid productivities (of respectively, 47.4 and 23.2 mg/L/d). In comparison with the standard medium, for which the lipid content was 14.6 mg/L/d and the maximum and average lipid productivities were 13.3 and 5.3 mg/L/d. Therefore, using a culture medium with 10 times N concentration not only induced microalgae growth but also the lipids accumulation.

Concerning the micronutrients concentration change in the culture medium, the 10xFe are the assays in which the highest maximum and average biomass productivities were obtained (respectively, 124.4 mg/L/d, from day 14 to 15, and 50.5 mg/L/d during the exponential growth phase) and these values are above the ones obtained using the standard medium (respectively, 76.4 mg/L/d, from day 8 to 9, and 38.2 mg/L/d during the exponential growth phase). In the same 10xFe assays the highest lipid content was obtained (22.6 mg/L/d) and thus, also the highest maximum and average lipid productivities (of respectively, 28.0 and 11.4 mg/L/d). In comparison with the standard medium, for which the lipid content was 19.1 mg/L/d and the maximum and average lipid productivities were 14.6 and 7.3 mg/L/d. Thus, using

a culture medium with 10 times the Fe concentration not only induced microalgae growth but also the lipids accumulation.

Table 1: Maximum and average biomass and lipid productivities (mg/L/d) versus the macro and micro nutrients concentration in comparison with the standard medium

Culture medium	Biomass productivity		Lipid content	Lipid productivity	
	Maximum	Average		Maximum	Average
Macronutrients concentration versus standard medium:					
Standard medium	90.77	36.60	14.60	13.25	5.34
10x Mg	51.67	35.10	13.08	6.76	4.59
20x Mg	59.36	24.91	16.23	9.63	4.04
10x K	48.33	30.19	14.50	7.01	4.38
20x K	49.62	29.13	14.26	7.08	4.15
10x N	141.79	69.47	33.45	47.43	23.24
20x N	83.08	64.18	20.45	16.99	13.12
Micronutrients concentration versus standard medium:					
Standard medium	76.41	38.24	19.12	14.61	7.31
10x Mn	61.54	27.68	15.85	9.75	4.39
20x Mn	62.69	27.82	14.91	9.35	4.15
10x Zn	51.92	22.59	15.09	7.83	3.41
20x Zn	60.13	24.69	13.77	8.28	3.40
10x Fe	124.36	50.49	22.55	28.04	11.38
20x Fe	100.00	44.35	17.50	17.50	7.76

4. Conclusions

In this study the influence of the culture medium composition, in terms of macro and micro nutrients, was studied in the microalgae *D. tertiolecta* growth, lipid content, and on the biomass and lipid productivities.
For that purpose the concentration of macro and micronutrients was increased 10 and 20 times compared to the standard culture medium “artificial seawater medium with vitamins”. It is concluded that, concerning the macronutrients, the addition of nitrogen (in the form of nitrate) increases the biomass productivity and the addition of magnesium reduces the biomass productivity in comparison with the standard medium. The addition of potassium does not significantly affect biomass concentration and productivities when compared to culture with standard medium. In this case, the highest biomass concentration was reached in the medium with a nitrogen concentration increased by 10 times the standard medium. Also, in this medium it was obtained the highest values of lipid content, biomass and lipid productivities of 33.5 %, 141.8 mg/L/d and 47.4 mg/L/d, respectively. Concerning the micronutrients, it is concluded that the addition of iron increases the biomass productivity and the addition of zinc and manganese reduces the biomass productivity in comparison with the standard medium. In this case, the highest biomass concentration was reached in the medium with an iron concentration increased 10 times the standard medium. Also, in this medium it was obtained the highest values of lipid content, biomass and lipid productivities of 22.6 %, 124.4 mg/L/d and 28.0 mg/L/d, respectively.
These results allow us to conclude that using residual wastewaters rich in nitrates and/or iron for cultivating *D. tertiolecta* rich in lipids could be of interest, contributing to reduce the eutrophication potential of these wastewaters while decreasing the costs of biodiesel production from microalgae.

References

Bligh E, Dyer W, 1959. A rapid method of total lipid extraction and purification, Can J Biochem Physiol., 37, 911-917.

Caetano, N.S., Silva, V.F.M., Mata, T.M., 2012. Valorization of coffee grounds for biodiesel production, Chemical Engineering Transactions 26, 267-272, DOI: 10.3303/CET1226045.

Chen M., Tang H., Ma H., Holland T.C., Ng K.Y.S., Salley S.O., 2011. Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*, Bioresour. Technol., 102, 1649-1655.

Fábregas J., Patino M., Arredondo-Vega B.O., Tobar J.L., Otero A., 1995. Renewal rate and nutrient concentration as tools to modify productivity and biochemical composition of cyclostat cultures of the marine microalga *Dunaliella tertiolecta*, Appl. Microbiol. Biotechnol., 44, 287-292.

Illman A.M., Scragg A.H., Shales S.W., 2000. Increase in *Chlorella* strains calorific values when grown in low nitrogen medium, Enzyme Microb. Technol., 27, 631-635.

Jiang Y., Yoshida T., Quigg A., 2012. Photosynthetic performance, lipid production and biomass composition in response to nitrogen limitation in marine microalgae, Plant Physiol. Biochem., 54, 70-77.

Liu Z.Y., Wang G.C., Zhou B.C., 2008. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*, Bioresour. Technol., 99, 4717-4722.

Macedo R.V.T., Alegre R.M., 2001. Influence of Nitrogen Content in the Cultivation of *Spirulina maxima* in two temperature levels - Part II, Produção de Lipídios Ciênc. Tecnol. Aliment. Campinas, 21, 183-186 (in Portuguese).

Mata T.M., Cardoso N., Ornelas M., Neves S., Caetano N.S., 2010a. Sustainable production of biodiesel from tallow, lard and poultry fat and its quality evaluation, Chemical Engineering Transactions 19, 13-18, DOI: 10.3303/CET1019003.

Mata T.M., Martins A.A., Caetano N.S., 2010b. Microalgae for biodiesel production and other applications, Renew. Sust. Energ. Rev. 14, 217-232.

Mata T.M., Cardoso N., Ornelas M., Neves S., Caetano N.S., 2011a. Evaluation of two purification methods of biodiesel from beef tallow, pork lard, and chicken fat, Energy Fuels 25, 4756-4762.

Mata T.M., Martins A.A., Sikdar S., Costa C.A.V., 2011b. Sustainability considerations of biodiesel based on supply chain analysis, Clean Technol. Envir. Policy 13, 655-671.

Mata T.M., Sousa I., Caetano N.S., 2012a. Transgenic corn oil for biodiesel production via enzymatic catalysis with ethanol, Chemical Engineering Transactions, 27, 19-24, DOI: 10.3303/CET1227004

Mata T.M., Martins A.A., Caetano N.S., 2012b. Microalgae processing for biodiesel production. In: Melero, J.A., Luque, R. (Eds.) Advances in biodiesel production: Processes and technologies, Woodhead Publishing Series in Energy No. 39, Ch.9, 204-231, Cambridge, UK.

Mata T.M., Melo A.C., Simões M., Caetano N.S., 2012c. Parametric Study of a Brewery Effluent Treatment by Microalgae *Scenedesmus obliquus*, Bioresour. Technol., 107, 151-158.

SAG (Sammlung von Algenkulturen Göttingen), 2008. Artificial Seawater Medium with Vitamins, Germany, <sagdb.uni-goettingen.de/culture_media/25 Artificial Seawater Medium.pdf>, accessed 27.11.2012.

Thomas W.H., Tornabene T.G., Weissman J. 1984. Screening for lipid yielding microalgae: Activities for 1983, SERI/STR-231-2207.