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The effect of various salinities and light intensities on the growth performance of five locally isolated microalgae [*Amphidinium carterae*, *Nephroselmis* sp., *Tetraselmis* sp. (var. red pappas), *Asteromonas gracilis* and *Dunaliella* sp.] in laboratory batch cultures

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Abstract: After a 1.5 year screening survey in the lagoons of Western Greece in order to isolate and culture sturdy species of microalgae for aquaculture or other value added uses, as dictated primarily by a satisfactory potential for their mass-culture, five species emerged and their growth was monitored in laboratory conditions. *Amphidinium carterae*, *Nephroselmis* sp., *Tetraselmis* sp. (var. red pappas), *Asteromonas gracilis* and *Dunaliella* sp. were batch cultured using low (20 ppt), sea (40 ppt) and high salinity (50 or 60 or 100 ppt) and in combination with a low (2000 lux) and high (8000 lux) intensity of illumination. The results exhibited that all these species can be grown adequately in all salinities and with best growth in terms of maximum cell density, specific growth rate (SGR) and biomass yield (g dry weight/L) at high illumination (8000 lux). The five species examined exhibited different responses in the salinities used, *Amphidinium* clearly does best in 20 ppt far better than 40 ppt and even more than 50 ppt. *Nephroselmis* and *Tetraselmis* grow almost the same in 20 and 40 ppt and less well in 60 ppt. *Asteromonas* does best in 100 ppt although it can grow quite well in both 40 and 60 ppt. *Dunaliella* grows equally well in all salinities (20-40-60 ppt). Concerning productivity as maximum biomass yield at the end of the culture period, first rank is occupied by *Nephroselmis* with ~3.0 g d.w./L, followed by *Tetraselmis* (2.0 g/L), *Dunaliella* (1.58 g/L), *Amphidinium* (1.19 g/L) and *Asteromonas* (0.7 g/L) with all values recorded at high light (8000 lux).

Keywords: salinity, light, growth, microalgae, *Amphidinium*, *Nephroselmis*, *Tetraselmis*, *Asteromonas*, *Dunaliella*

1. Introduction

As microalgae have established a foothold in our civilization in terms of an effective source of products for various uses, the quest for novel species suitable for culture or the improvement of existed culture techniques are both welcomed. In fact we are nowadays in the start of the adventure delving in algal culture and new challenges emerge as to how we can extract the most of the benefits microalgae can offer. The modern days problems of tackling increasing fossil fuel emissions, struggle for fresh water in an expanding human population, finding cheap energy alternatives to fossil fuels sources, creating sea farms as a relief to crop land and replacing meat with plant-like equivalent organisms, all lead to algae and their culture. Leaving apart for the moment the case of macroalgae whose semi-natural sea culture-based harvest in East Asia comprises about one third of global aquaculture output, we focus on microalgae as having the potential to be cultured everywhere and in numerous ways of cultivation [1].

In order to focus on target species that offer ease of culture and valuable products, the first is of paramount importance. In fact, what is the benefit if the tested species is cumbersome in its biomass production? Moreover, the effectiveness of its culture must be seen as a prerequisite for all aspects of its exploitation. Proteins, lipids, pigments, antioxidants and other special minor constituents of its cell, in a larger or smaller degree part of its biochemical profile, can offer materials

usable in several industry sectors. So, notwithstanding the already existed large catalogue of cultured species of microalgae e.g. [1, 2] a lot remains to be improved, both in the existed methods and (most important) to examine new candidate species that have not yet been trial-cultured.

The first step in the culture procedure is the small scale laboratory culture under the most basic conditions that their manipulation is easily accomplished. Salinity and light are the primary factors for marine species and can be altered and maintained at desirable levels without upsetting dramatically the environment of either the culture medium or its exterior. Temperature is of course of paramount importance and is well known that higher temperatures of 25-30 °C can boost the growth rate [3, 4] but such high temperatures are costly maintained in temperate regions and only temperatures in the region of 18-20 °C are economically feasible indoors during the cold months. So we consider the temperature of around 20 °C as a default value in testing all other parameters and if the experiments turn out to be promising, there is no reason to doubt that the species under experimentation can do better in higher temperatures during the warm months. The manipulation of pH has little to offer because its control is extremely cumbersome and practically of no decisive importance, the culture will create its own pH. Nutrients are of paramount importance for algal growth but once a widely accepted recipe is selected that covers all the needs of microalgae, it can be also considered as a default parameter.

Light is the most essential and critical factor because affects directly the photosynthesis from which biosynthesis of biomass ensues [5]. Low lighting has a limiting effect on the growth of microalgae, so, increasing the light intensity in mass cultivation of algae is a common practice to enhance growth [6] but care should be taken as too much of it can cause photoinhibition [7, 8]. LED light is preferable for use because it does not create extra heat that other light sources (e.g. fluorescent) do, disturbing in that way the desirable temperature regime for the experimentation (personal observation).

Salinity affects the growth of microalgae acting directly on the osmoregulatory mechanism of the cell. All marine species of microalgae can endure several ranges of salinity but the existing information in the literature is rather complicated on conclusions about both the range of tolerance and the optimum value. There are studies suggesting that the elevated salinities affect negatively the growth of microalgae acting directly on their photosynthetic apparatus [9, 10].

Only after reaching conclusions about the ensued growth under various salinities and light intensities it is logical to proceed to more elaborate experimentation on the influence of temperature, nutrient composition, pH, addition of extra CO₂, level of aeration etc, at the optimum salinity and light. And beyond that, the influence of these on the biochemical composition.

In the present study 4 chlorophytes (*Nephroselmis* sp., *Asteromonas gracilis*, *Tetraselmis* sp. (var. red pappas) and *Dunaliella* sp.) and 1 dinoflagellate (*Amphidinium carterae*) were selected for culture from a wealth of species isolated from the lagoons of Western Greece after a screening program of 1.5 years during 2019-2020. The above species after numerous renovations of their culture medium exhibited a remarkable ability to adapt easily to indoor conditions and dominate the culture. This is a sign of feasibility for mass culturing, as in large scale cultures, especially in open ponds, it is very difficult to attain pure monoculture of the desired species unless the species is able to dominate in terms of growth over other undesired species (that finally find their way to intrude). As lagoons are among the harshest water environments due to intense seasonal fluctuations of salinity, light, temperature, nutrient input and pollution agents, it is logical to assume that species encountered there are sturdy because of their adaptability to such constantly changing water bodies. Additionally, locally isolated and exploited species of microalgae are best suited for responding well to environmental conditions prevailing in the same region when their culture is attempted. The estuarine or lagoonal species can differ in adaptability from their pelagic counterparts [11] and in terms of locality if proved to be easily cultured, can freed the local producers from the usage of imported species [12]. Furthermore these local strains can enrich the collections of algae preserved worldwide in order to create an expanding deposit of strains of sibling or novel species.

From the five species of the present microalgae, three of them (*Asteromonas gracilis*, *Nephroselmis* sp. and *Amphidinium carterae*) has not been studied in a concise manner in laboratory cultures and only fragmented information can be detected on them. So our study aspires to pave the way for future attempts on more elaborate approaches. On the other hand *Dunaliella* sp. has been extensively studied in every possible way and our study aims to add information for this local strain. The case of *Tetraselmis* sp. (var. red pappas) is quite peculiar as on the one hand belongs to the family of *Tetraselmis*, a well studied one, but on the other, its unique feature of coloring red the culture medium suggests that is a novel strain of *Tetraselmis* with its own potential and worth studying.

2. Materials and Methods

2.1 Isolation and purification

After many monthly collections of water samples from various lagoons of W. Greece such as Messolonghi and its saltworks (prefecture of Etoloakarnania), the lagoon of Kalogria and Pappas (pref. of Achaia) and the lagoon of Kotyhi (pref. of Ilia), the samples were transported to the laboratory and 200 mL of each sample were put in glass 1-L conical Erlenmeyer flasks containing 800 mL of 40 ppt sterilized water enriched with Walne's nutrient formula [13]. The so called maintenance flasks were left for one week to mature, supplied through a 1-mL pipette with filtered air (~ 0.5 flask volume/min) fed by a central blower, exposed to continuous light of 3000 lux emitted by white light LED tubes, in a air-conditioned room of 20-22 °C temperature. The maintenance flasks were left 2-3 weeks to develop microalgae population evident by coloration of the water and microscopic examination. Those with no sign of coloration after 3 weeks were discarded. By this practice we deliberately focused on algae species that can either solely or in companion with other species be fully adapted to seawater salinity and dominate the culture. After the confirmation of the well establishment of one or more species in the maintenance flasks, serial dilutions were performed in successive steps using 20-mL glass Erlenmeyer flasks filled with sterilized and fertilized as above water of the same salinity (40 ppt). The inoculated flasks were left to mature for 20 days in a special thermo-regulated chamber at 19 °C in low ambient continuous illumination of 1300 lux and daily mildly hand agitated. After 20 days they were examined microscopically and if a monoculture was observed the content of this flask was transferred in 500-mL flasks prepared with new fertilized medium and left to mature (20-22 °C, continuous 3000 lux) until used for inoculation of various bigger flasks.

2.2. Experimental conditions

All microalgae were batch-cultured using either 2-L conical Erlenmeyer flasks or 1-L cylindrical plastic bottles in 2 or 3 replicates for each treatment. Saline water of 20, 40 and 50 or 60 or 100 ppt, and two light intensities of 2000 and 8000 lux from 20 watt 1600 lm LED lamps, measured at the middle of the outer surface of the vessel (Lux meter BIOBLOCK LX-101), were combined so as to create 6 treatments (3 salinities x 2 light intensities). In the case of *A. carterae* the salinities were 20, 40 and 50 ppt and for *A. gracilis* 40, 60 and 100 ppt because the former species cannot tolerate too high salinities and the latter too low as recorded in preliminary trials. The photoperiod of 16 hL:8 hD was timer-controlled for all treatments throughout the experiments. The temperature was maintained at 20–21.5 °C by a 18,000 BTU air condition.

All quantities of 40 ppt water used were first enriched with Walne's medium of nutrients, autoclaved, and according to the desired salinity either were diluted with enriched and sterilized distilled water to achieve salinity of 20 ppt or by the addition of the proper quantity of sterilized artificial salt (Instant Ocean®, Blacksburg, Virginia, USA) the desired higher salinities of 50, 60 or 100 ppt were obtained. In all the vessels the suspension of the cells and the supply of CO₂ were accomplished using coarse air bubbling through 2-mL glass pipettes (one in every vessel with a supply of half culture volume/min) connected through sterilized plastic hoses to the 0.45 µm filtered central air supply system fed by a blower.

2.3. Tested parameters and statistical analysis

The progress of the cultures was monitored by daily measurements of optical density at 750 nm of the medium in each vessel using a visible-UV spectrophotometer (Shimadzu UVmini-1240 UV-visible). This was accomplished by using the

proper equation from the calibration curve of number of cells vs absorbance using a dense premeasured (by haematocytometer) culture of each species with serial dilutions and additionally more couples of direct cell counts from culture samples taken every 3 days using a Fuchs-Rosenthal haematocytometer.

The maximum specific growth rate (SGR as doublings day⁻¹) was estimated during the early exponential (log) phase of the culture's growth curve using the equation [14]:

$$\text{SGR} = (\ln C_2 - \ln C_1) / (t_2 - t_1)$$

where C_1 and C_2 stand for cells/mL at days t_1 and t_2 , respectively ($t_2 > t_1$).

From the above equation the generation time t_g of the culture was calculated as days required for duplication using the formula [14]:

$$t_g = 0.6931 / \text{SGR}$$

The calculation of the dry weight was made by filtering a known amount of culture through 0.45 µm GF/C filters in a vacuum pump (Heto-SUE-3Q), washing the filter with ammonium formate and drying the filter in an oven to 100 °C for 2 h. Then, the filter was weighted to the fourth decimal and the dry weight was calculated as g/L after subtraction of the pre-weighted filter's tare weight.

The pH was daily measured by a digital pH-meter (HACH-HQ30d-flexi). Statistical treatment of the different variables was done with ANOVA and pair-wise Tukey's test for comparison of the means at a 0.05 level of significance using the free PAST3 software.

3. Results

3.1. *Amphidinium carterae* (Figure 1)

Figure 1. The cells of *Amphidinium carterae* at 1000x magnification

Preliminary experiments have shown that this species is sensitive to excessive stress caused by intense aeration, so instead of wide Erlenmeyer containers that require intense aeration to achieve uniformity in stirring water, which would cause cellular strain, 1-L plastic bottles (Figure 2) were preferred. Thus with the minimum but sufficient ventilation (flow ~ 0.3 L / min) the needs of photosynthesis are ensured (continuous exposure of the whole volume of the culture to light) and also the minimal stress of the cells.

Figure 2. The bottles of the culture of *Amphidinium carterae* at differed stages of growth. A: 2nd day, B: 8th day, C: 13th day.

The cultures lasted 15 days and from the 8th day the drop of the growth rate and the "entry" in the static (stationary) phase of each culture became visible in all conditions (although in a different way in each). At the salinity of 20 ppt (Figure 3-left) the evolution of the culture showed a much more intense increase in high light (8000 lux) compared to low light (2000 lux). In both lights there was a very short (2 days) initial phase of adaptation (lag phase) after which, especially in high light, the increase became strongly exponential (log phase). In the high light culture the final cell density ($\sim 6.5 \times 10^6$ cells/mL) was almost triple that of the low light ($\sim 2.3 \times 10^6$ cells/mL). The pH fluctuated in the alkaline region with values 8.3 -9.3 and from the beginning it showed higher values in the high light (a sign of more intense photosynthesis) and then (from the 11th day), an abrupt synchronized drop (sign of aging) in both lights. At salinities of 40 and 50 ppt (Figure 3-middle & right) the increase of the growth curves and the final densities attained after an initial adaptation phase of 2 and 3 days respectively, were much lower compared to 20 ppt and the stationary phase was

reached on the 10th day (on 14th day at 20 ppt). At the salinity of 40 ppt the final density in high light was $\sim 3.1 \times 10^6$ cells/mL almost triple that of low light ($\sim 1.4 \times 10^6$ cells/mL). At the salinity of 50 ppt even lower values of final densities were recorded in both high ($\sim 2.0 \times 10^6$ cells/mL) and low light ($\sim 0.65 \times 10^6$ cells/mL). The pH at these salinities followed the same fluctuation pattern observed in 20 ppt with alkaline higher values (>9.0) in high light as compared to low light (<9.0) and near the end of the culture period a synchronized drop to almost identical lower values (8.0 - 8.5).

Figure 3. The growth curves (in cells/mL) of the culture of *Amphidinium carterae* at salinities of 20 (left), 40 (middle) and 50 ppt (right) and at each light intensity (L: 2000 lux, XL: 8000 lux). Also depicted are the pH daily values in each condition.

For the calculation of the specific growth rate (SGR) and the doubling or generation time (t_g) the interval 3 - 8th day was chosen as it showed similarity in the upward trend of the growth curve in all salinities. There was a clearly higher growth rate in high light (0.295 - 0.232 - 0.302) for salinities 20 - 40 - 50 ppt respectively, compared to the values (0.189 - 0.167 - 0.143) for low light at the corresponding same salinities (Table 1). Statistically the values differed from each other except those of 20 and 50 ppt which in high light were statistically equal. As a reflection of the above growth rates, the generation times (t_g) were shorter in the high light condition (2.35 - 2.98 - 2.29 days for the salinities of 20 - 40 - 50 ppt respectively) compared to the values (3.67 - 4.16 - 4.85) for low light at the corresponding same salinities.

Table 1. Data on specific growth rate (SGR) and generation or doubling time (t_g) of *Amphidinium carterae* cultures at salinities of 20, 40 and 50 ppt and at each light intensity (L: 2000 lux, XL: 8000 lux).

Conditions	20ppt-L	20ppt-XL	40ppt-L	40ppt-XL	50ppt-L	50ppt-XL
SGR $\pm$ SE	0.189 ^a $\pm$ 0.0077	0.295 ^b $\pm$ 0.0064	0.167 ^c $\pm$ 0.0025	0.232 ^d $\pm$ 0.0022	0.143 ^e $\pm$ 0.0074	0.302 ^f $\pm$ 0.0023
t_g (days) $\pm$ SE	3.67 $\pm$ 0.185	2.35 $\pm$ 0.049	4.16 $\pm$ 0.062	2.98 $\pm$ 0.028	4.85 $\pm$ 0.323	2.29 $\pm$ 0.018

- The period for the calculation of SGR and t_g was from the 3rd to the 8th day.
- Number of measurements = 27.
- The different superscripts indicate a statistically significant difference at the 0.05 level of confidence (statistical processing with ANOVA and then pair-wise comparison with Tukey's test). Where there is a second superscript it means statistically equal to the value of the condition of the corresponding letter.

For the yield of the cultures (Figure 4) in biomass as dry weight per liter of culture (g/L), the values were calculated on the 13th day. It is clearly shown and in agreement with the increase curves of Figure 2 that concerns the salinity of 20 ppt compared to the curves for the salinities of 40 and 50 ppt respectively, that the salinity of 20 ppt and the high light (8000 lux) causes the highest biomass production (1.19 g/L) with all other conditions giving values below 0.8 g/L. Also noteworthy in all salinities is the much higher production in high light conditions (8000 lux) compared to their counterparts in low light (2000 lux).

Figure 4. Dry weight yield (g/L) ± SE of *Amphidinium carterae* at salinities of 20, 40 and 50 ppt and at each light intensity (L: 2000 lux, XL: 8000 lux). The existence of a statistically significant difference at the 0.05 level is indicated by a different letter. Statistically equal values are indicated by repeating the corresponding indicators on the bars (statistical processing with ANOVA and then pair-wise comparison with Tukey's test).

3.2. *Nephroselmis* sp.(Figure 5.)

Figure 5. The cells of *Nephroselmis* sp. at 1000x magnification.

For the cultures 2-L glass Erlenmeyer conical flasks in 2 duplicates for each combination of salinity and light intensity (Figure 6) were used and a continuous air supply of half container volume/min ($\sim 1\text{L/min}$) was provided. The cultures lasted 22 days (Figure 7) and after an initial adaptation-delay period of 3-4 days, all showed an intense and prolonged exponential phase which was more intense in high light (8000 lux). The "entry" in the static phase of each culture varied in each condition, after the 17th day for cultures in high light, while in the low light (2000 lux) at salinities of 20 and 40 ppt practically for the entire period there was not observed a decrease in the exponential growth curve, but it was observed at the salinity of 60 ppt from day 17 (Figure 7-right).

At 20 ppt salinity (Figure 7-left) the evolution of the culture was evident which showed a much more intense increase in high light (8000 lux) reaching densities of about 75×10^6 cells/mL on the 22nd day compared to the low light (2000 lux) where on the 20th day it reached 35×10^6 cells/mL (70×10^6 cells/mL that day in high light).

Figure 6. The vessels of the culture of *Nephroselmis* sp. at differed stages of growth. A: 1st day, B: 4th day, C: 15th day.

The pH fluctuated at the alkaline level with values initially ~ 7.9 which quickly rose to the level of ~ 8.5 and from the beginning it already showed higher values in high light (a sign of more intense photosynthesis) and then (from day 17) a synchronized drop (sign of aging) in both lights. At 40 ppt salinity (Figure 7-middle) also the culture increase showed a much more intense increase in high light (8000 lux) reaching a density of about 67×10^6 cells/mL on the 22nd day compared to the low light (2000 lux) which reached 41×10^6 cells/mL on the same day. In both light intensities after a very short initial phase (3-4 days) of delay-adaptation, the increase became strongly exponential especially in high light. The pH fluctuated at the alkaline level with values $\sim 8.2 - 8.8$ and from the beginning it already showed higher values in high light and then (from the 17th day) a synchronized drop in light intensities. At the salinity of 60 ppt (Figure 7-right) also the increase in culture showed a much more intense increase in high light (8000 lux) reaching a density of about 50×10^6 cells/mL on the 22nd day compared to the low light (2000 lux) which reached 30×10^6 cells/mL on the same day. In both light intensities after a very short initial phase (3-4 days) of delay-adaptation, the increase became strongly exponential especially in high light but lasted only until the 11th day while in the low light up to the 17th day. The cultures then entered the static phase which was most evident in bright light.

Figure 7. The growth curves (in cells/mL) of the culture of *Nephroselmis* sp. at salinities of 20 (left), 40 (middle) and 60 ppt (right) and at each light intensity (L: 2000 lux, XL: 8000 lux). Also depicted are the pH daily values in each condition.

For the calculation of the specific growth rate (SGR) and the doubling or generation time (t_g) the interval 4th- 8th day was chosen as it showed similarity in the upward trend of the growth curve in all salinities. There was a clearly higher growth rate in all salinities with high light (0.401 - 0.370 - 0.462) for salinities 20 - 40 - 60 ppt respectively, compared to the values (0.338 - 0.341 - 0.336) for low light at the corresponding same salinities (Table 2). Statistically the values differed from each other except those of 20, 40 and 60 ppt which in low light were statistically equal. As a reflection of the above growth rates, the generation times (t_g) were shorter in the high light condition (1.729 - 1.875- 1.499 days for the salinities of 20 - 40 - 60 ppt respectively) compared to the values (2.051 - 2.032 - 2.063) for low light at the corresponding same salinities.

Table 2. Data on specific growth rate (SGR) and generation or doubling time (t_g) of *Nephroselmis* sp. cultures at salinities of 20, 40 and 60 ppt and at each light intensity (L: 2000 lux, XL: 8000 lux).

Conditions	20ppt-L	20ppt-XL	40ppt-L	40ppt-XL	60ppt-L	60ppt-XL
SGR $\pm$ SE	0.338 ^a $\pm$ 0.001	0.401 ^b $\pm$ 0.0021	0.341 ^{c.a.c} $\pm$ 0.0016	0.370 ^d $\pm$ 0.0006	0.336 ^{e.a.} $\pm$ 0.0008	0.462 ^f $\pm$ 0.0023
t_g (days) $\pm$ SE	2.051 $\pm$ 0.003	1.729 $\pm$ 0.009	2.032 $\pm$ 0.01	1.875 $\pm$ 0.003	2.063 $\pm$ 0.005	1.499 $\pm$ 0.011

- The period for the calculation of SGR and t_g was from the 4th to the 8th day.
- Number of measurements = 18.
- The different superscripts indicate a statistically significant difference at the 0.05 level of confidence (statistical processing with ANOVA and then pair-wise comparison with Tukey's test). Where there is a second superscript it means statistically equal to the value of the condition of the corresponding letter.

For the yield of the cultures (Figure 8) in biomass as dry weight per liter of culture (g/L), the values were calculated on the 17th day. It is clearly shown and in agreement with the increase of the curves in Figure 7 that the salinities of 20 and 40 ppt resulted in much higher yield (2.88 and 2.99 g/L respectively) compared to the salinity of 60 ppt (1.61 g/L) at high light (8000 lux). In all salinities at low light the yields were much lower around 1 g/L and statistically equal.

Figure 8. Dry weight yield (g/L) $\pm$ SE of *Nephroselmis* sp. at salinities of 20, 40 and 60 ppt and at each light intensity (L: 2000 lux, XL: 8000 lux). The existence of a statistically significant difference at the 0.05 level is indicated by a different letter. Statistically equal values are indicated by repeating the corresponding indicators on the bars (statistical processing with ANOVA and then pair-wise comparison with Tukey's test).

3.3. *Tetraselmis* sp. (var. red pappas) (Figure 9)

Figure 9. The cells of *Tetraselmis* sp. (var. red pappas) at 1000x magnification. Many cells are getting reddish.

Figure 10. The culture vessels of *Tetraselmis* sp. (var. red pappas) in various phases of color change. **A:** 15th day, cultures with aeration, the color has already become dull dark green. **B:** 16th day, cultures at rest, many cells have precipitated the color begins to take on a reddish tinge. **C:** 17th day, cultures without aeration, the front row comes from light of 2000 lux, the back row from 8000 lux and is clearly reddened. **D:** 17th day, cultures of 20-40-60 ppt (from left to right) and from bright lighting (8000 lux) without aeration with their reddish color clearly visible.

The cultures of *Tetraselmis* sp. (var. red pappas) in all salinities (20-40-60 ppt) were done in 2-L glass conical Erlenmeyer flasks (Figure 10) receiving 1L of air/min and with 3 replicates for each combination of salinity and light intensity, low and high (2000 and 8000 lux respectively). The culture period lasted 17 days (Figure 11) and in all treatments after an initial delay-adaptation period of 2-4 days an intense and continuous exponential growth, much more pronounced in the high light, was observed. The only culture that entered the stationary phase was that of 60 ppt-high light which resulted in substantially lower final densities in both high and low light (5.8×10^6 and 2.2×10^6 cells/mL respectively) compared to 20 ppt (10×10^6 and 4.3×10^6 cells/mL respectively) and 40 ppt (9.6×10^6 and 3.3×10^6 cells/mL respectively). Nevertheless the specific growth rate (SGR) calculated for the period from the 3rd till the 9th day for all treatments, did not exhibit a huge difference between all salinities at high light (Table 3) with values bigger than 0.3, while in low light was much lower at all salinities (0.18-0.203). It seems that high light intensity has a profound positive affection on the speed of multiplication enhancing the overall metabolism of the cells.

The fluctuation of pH over the culture period is indicative of a more intense photosynthetic activity in the high light cultures in all salinities as it kept substantially over 8.5 while the low light cultures kept at 8.0 or lower.

Figure 11. The growth curves (in cells/mL) of the culture of *Tetraselmis* sp. (var. red pappas) at salinities of 20 (left), 40 (middle) and 60 ppt (right) and at each light intensity (L: 2000 lux, XL: 8000 lux). Also depicted are the pH daily values in each condition.

Table 3. Data on specific growth rate (SGR) and generation or doubling time (t_g) of *Tetraselmis* sp. (var. red pappas) cultures at salinities of 20, 40 and 60 ppt and at each light intensity (L: 2000 lux, XL: 8000 lux).

Conditions	20ppt-L	20ppt-XL	40ppt-L	40ppt-XL	60ppt-L	60ppt-XL
SGR $\pm$ SE	0.181 ^a $\pm$ 0.0035	0.323 ^b $\pm$ 0.0069	0.180 ^{c,a} $\pm$ 0.0023	0.331 ^{d, b} $\pm$ 0.0037	0.203 ^e $\pm$ 0.0031	0.310 ^f $\pm$ 0.0019
t_g (days) $\pm$ SE	3.860 $\pm$ 0.074	2.163 $\pm$ 0.046	3.870 $\pm$ 0.052	2.097 $\pm$ 0.023	3.429 $\pm$ 0.052	2.236 $\pm$ 0.013

- The period for the calculation of SGR and t_g was from the 3rd to the 9th day.
- Number of measurements = 18.
- The different superscripts indicate a statistically significant difference at the 0.05 level of confidence (statistical processing with ANOVA and then pair-wise comparison with Tukey's test). Where there is a second superscript it means statistically equal to the value of the condition of the corresponding letter.

For the yield of the cultures (Figure 12) in biomass as dry weight per liter of culture (g/L), the values were calculated on the 17th day. It is clearly shown and in agreement with the increase of the curves in Figure 11 that at high light (8000 lux) the salinities of 20 and 40 ppt resulted in much higher yield (2.0 and 1.8 g/L respectively, and statistically equal) compared to the salinity of 60 ppt (0.92 g/L). In all salinities at low light the yields were much lower around 0.9 g/L statistically equal between 20 and 40 ppt and also equal to the relevant one of 60 ppt-XL. The lowest yield (0.78 g/L) was recorded in 60 ppt-L.

Figure 12. Dry weight yield (g/L) $\pm$ SE of *Tetraselmis* sp. (var. red pappas) at salinities of 20, 40 and 60 ppt and at each light intensity (L: 2000 lux, XL: 8000 lux). The existence of a statistically significant difference at the 0.05 level is indicated by a different letter. Statistically equal values are indicated by repeating the corresponding indicators on the bars (statistical processing with ANOVA and then pair-wise comparison with Tukey's test).

3.4. *Asteromonas gracilis* (Figure 13).

Figure 13. The cells of *Asteromonas gracilis* with the majority of them in a star-like appearance.

The case of the chlorophyte *Asteromonas gracilis* is very special as its natural habitat is exclusively ultra-salty areas in many parts of the Earth. It is a species that can, prefers, and grows in salinities much higher than seawater. In our study we isolated it from the saltern basins of the Messolonghi lagoon and it is easily maintained at a salinity of about 100 ppt [11]. From the literature [12] and from preliminary tests it was found that this species does not grow at all in salinities around 20 ppt, so our cultures were formed at 40, 60 and 100 ppt.

Figure 14. The bottles of the culture of *Asteromonas gracilis* at differed stages of growth. A: 1st day, B: 4th day, C: 11th day.

The cultures of *A. gracilis* (Figure 14) in all salinities (40-60-100 ppt) were conducted in triplicate with two replicates of 2-L glass conical Erlenmeyer flasks receiving ~1L of air/min and one of a 1-L plastic cylindrical vessel receiving ~0.5L of air/min for each combination of salinity and light intensity (low-L and high-XL, 2000 and 8000 lux respectively). The culture period lasted 17 days till just when the cultures entered the stationary phase.

The most prominent characteristic of the cultures of *A. gracilis* in all salinities is the rather long initial lag phase that lasted 5-7 days in the lower salinities (40 and 60 ppt) absolutely in a similar manner for both high and low light, compared to 100 ppt that lasted only 2 days for high light and 4 days for low light (Figure 15).

The highest value of cell density was recorded in the salinity of 100 ppt at high light on the 17th day (6.4×10^5 cells/mL) clearly higher than the relevant values of the 60 and 40 ppt at high light (4.9×10^5 and 5.5×10^5 cells/mL respectively). It is worth noting however that these maximum values in the salinities of 60 and 40 ppt can be considered as equivalent due to the high amount of variation in the values of 40 ppt as evidenced by the width of the error bars in Figure 15 (left). This was further certified as the pace of growth at high light shown in all three diagrams of Figure 15 presented uniformity till at least the 12th day when cell densities were identical around 4×10^5 cells/mL for both 40 and 60 ppt cultures.

The maximum densities in the condition of low light were much lower compared to high light in all salinities with the maximum value recorded at 60 ppt (3.9×10^5 cells/mL) and the lowest at 40 ppt (1.8×10^5 cells/mL) and 2.9×10^5 cells/mL at 100 ppt.

Figure 15. The growth curves (in cells/mL) of the culture of *Asteromonas gracilis* at salinities of 40 (left), 60 (middle) and 100 ppt (right) and at each light intensity (L: 2000 lux, XL: 8000 lux). Also depicted are the pH daily values in each condition.

The values of pH fluctuated greatly at all treatments in the region of 8.0 - 8.5 during the whole culture period for the cultures of 40 and 60 ppt, with higher values steadily recorded at high light over those of low light but after the 11th day the values of high light decreased substantially and in the salinity of 60 ppt dropped below those of low light. This phenomenon was much exaggerated at the salinity of 100 ppt where the drop started from the 9th day and additionally was manifested for both light intensities and with values plummeted to values below 8.0 (Figure 15-right).

The specific growth rate (SGR) calculated for the period from the 3rd till the 9th day based on the findings of the growth curves of Figure 15 in order to obtain values containing on the one hand a part of the initial lag phase (3rd day) and on the other a representative day of the exponentially (log) phase around its middle period (9th day). The highest growth rate of 0.280 doublings/day was recorded in 100 ppt-XL and in 60 ppt-XL (0.273) which were statistically equal (Table 4) followed by 100 ppt-L (0.205) and 60 ppt-L (0.190), while the lowest salinity of 40 ppt resulted in the lowest values (0.143 and 0.106) for both high light (XL) and low light (L) respectively. As of these values, the shortest generation time (t_g) of 2.551 days was recorded in 100 ppt-XL and the longest (8.034 days) in 40 ppt-L.

Table 4. Data on specific growth rate (SGR) and generation or doubling time (t_g) of *Asteromonas gracilis*. cultures at salinities of 40, 60 and 100 ppt and at each light intensity (L: 2000 lux, XL: 8000 lux).

Conditions	40ppt-L	40ppt-XL	60ppt-L	60ppt-XL	100ppt-L	100ppt-XL
SGR $\pm$ SE	0.106 ^a $\pm$ 0.007	0.143 ^b $\pm$ 0.01	0.190 ^c $\pm$ 0.005	0.273 ^d $\pm$ 0.014	0.205 ^e $\pm$ 0.009	0.280 ^f $\pm$ 0.01
t_g (days) $\pm$ SE	8.034 $\pm$ 0.773	5.674 $\pm$ 0.426	3.711 $\pm$ 0.101	2.768 $\pm$ 0.154	3.607 $\pm$ 0.175	2.551 $\pm$ 0.07

- The period for the calculation of SGR and t_g was from the 3rd to the 9th day.
- Number of measurements = 30.
- The different superscripts indicate a statistically significant difference at the 0.05 level of confidence (statistical processing with ANOVA and then pair-wise comparison with Tukey's test). Where there is a second superscript it means statistically equal to the value of the condition of the corresponding letter.

The yield in g dry weight/L was measured at the final day of the culture period (17th day) and from the very first glance becomes evident that the high light condition resulted in much more production of biomass compared to the low light counterpart in each salinity. The maximum value (0.70 g/L) was recorded in the treatment of 100 ppt-XL and the lowest (0.25 g/L) in 40 ppt-L (Figure 16). Worth-noting are the statistically equal values of both 40 ppt-XL and 60 ppt-XL (0.52 and 0.51 g/L respectively) and also those of 40 ppt-L and 60 ppt-L (0.38 and 0.36 g/L respectively).

Figure 16. Dry weight yield (g/L) $\pm$ SE of *Asteromonas gracilis* at salinities of 40, 60 and 100 ppt and at each light intensity (L: 2000 lux, XL: 8000 lux). The existence of a statistically significant difference at the 0.05 level is indicated by a different letter. Statistically equal values are indicated by repeating the corresponding indicators on the bars (statistical processing with ANOVA and then pair-wise comparison with Tukey's test).

3.5. *Dunaliella* sp. (Figure 17).

Figure 17. The cells of *Dunaliella* sp. at 630x magnification

The cultures of *Dunaliella* sp. in all salinities (20-40-60 ppt) were conducted in triplicate (Figure 18) with two replicates of 2-L glass conical Erlenmeyer flasks receiving ~1L of air/min and one of a 1-L plastic cylindrical vessel receiving ~0.5L of air/min for each combination of salinity and light intensity (low-L and high-XL, 2000 and 8000 lux respectively). The culture period lasted 22 days as all cultures had already entered the stationary phase around the 14th day.

Figure 18. The bottles of the culture of *Dunaliella* sp. at differed stages of growth. A: 1st day, B: 4th day, C: 11th day.

In all treatments (Figure 19) the lag phase was very short (2-3 days) after which a sharp elevation of the growth curve characterized the log phase especially in the high light of the 40 and 60 ppt salinities in contrast to the low light curves where the transition to the log phase was long and smooth.

Figure 19. The growth curves (in cells/mL) of the culture of *Dunaliella* sp. at salinities of 20 (left), 40 (middle) and 60 ppt (right) and at each light intensity (L: 2000 lux, XL: 8000 lux). Also depicted are the pH daily values in each condition.

In all salinities at high light cell densities measured on the 22nd day recorded values over 25×10^6 cells/mL while their counterparts at low light fluctuated around half that value. The maximum densities were recorded in the salinity of 40 ppt with 29×10^6 cells/mL and 16×10^6 cells/mL for high and low light respectively. pH fluctuated intensively in the salinities of 20 and 40 ppt with values around 7.9 at start and then rising to 8.6 at high light where it stabilized for the most of the culture period while the values for low light remained continuously substantially lower in the salinity of 20 ppt. Only after the 13th day the pH values of both light intensities were equated till the termination of the experiment. In the salinity of 60 ppt the pattern of pH fluctuation was similar to those of 20 and 40 ppt with the only difference the drop of the values for high light below those for low light after the middle of the culture period.

Table 5. Data on specific growth rate (SGR) and generation or doubling time (t_g) of *Dunaliella* sp. cultures at salinities of 20, 40 and 60 ppt and at each light intensity (L: 2000 lux, XL: 8000 lux).

Conditions	20ppt-L	20ppt-XL	40ppt-L	40ppt-XL	60ppt-L	60ppt-XL
SGR	0.239 ^a ±0.01	0.350 ^b ±0.015	0.279 ^{c.a} ±0.004	0.387 ^{d.b} ±0.0037	0.215 ^e ±0.003	0.405 ^{f.d} ±0.007
t _g (days)	3.020±0.111	2.09±0.095	2.495±0.036	1.815±0.042	3.246±0.052	1.774±0.027

- The period for the calculation of SGR and t_g was from the 3rd to the 8th day.
- Number of measurements = 27.
- The different superscripts indicate a statistically significant difference at the 0.05 level of confidence (statistical processing with ANOVA and then pair-wise comparison with Tukey's test). Where there is a second superscript it means statistically equal to the value of the condition of the corresponding letter.

The specific growth rate (SGR) calculated for the period from the 3rd till the 8th day was based on the uniformity of the shape of the growth curves of all treatments in Figure 19. The highest growth rate of 0.405 doublings/day was recorded in 60 ppt-XL and in 40 ppt-XL (0.387) which were statistically equal (Table 5) followed by 20 ppt-XL (0.350). The values at low light in all salinities were substantially lower (0.215 - 0.279). As of these values the shortest generation time (t_g) of 1.77 days was recorded in 60 ppt-XL and the longest (3.24 days) in 60 ppt-L.

The yield in g dry weight/L was measured at the final day of the culture period (22nd day) and from the very first glance becomes evident that the high light condition resulted in much more production of biomass compared to the low light counterpart in each salinity. The maximum values (1.58 and 1.56 g/L) were recorded in the treatments of 40 and 60 ppt-XL respectively and the lowest (0.59 and 0.45 g/L) in 20 and 60 ppt-L respectively (Figure 20).

Figure 20. Dry weight yield (g/L) $\pm$ SE of *Dunaliella* sp. at salinities of 20, 40 and 60 ppt and at each light intensity (L: 2000 lux, XL: 8000 lux). The existence of a statistically significant difference at the 0.05 level is indicated by a different letter. Statistically equal values are indicated by repeating the corresponding indicators on the bars (statistical processing with ANOVA and then pair-wise comparison with Tukey's test).

4. Discussion

A considerable bulk of data exists in the literature that concern the effect of various manipulation of physicochemical parameters on the growth and biochemical output of microalgae cultures. Two major drawbacks are recognizable on reviewing this issue in the literature. First, the immense variation in the tools (e.g. vessels) and techniques (e.g. batch culture or continuous, nutrients, light, temperature, etc) used. Second, the fragmented information that the results of each work transport to the reader. An example of this is the calculation of specific growth rate (SGR) which in the majority of papers is presented with no reference of the exact culture time on which was calculated upon. This can ensue in great discrepancies of the calculated values and comparisons are greatly hindered. In our study we calculated SGR from around the end of lag (stationary) phase till the middle of log (exponential) phase. Have it be otherwise (i.e. from the well advanced log phase) our values would have been much higher. This has the meaning of a more realistic approach each particular tested species presents in reality. It makes little sense to record SGR only during the short log phase whilst a long lag phase has preceded it, e.g. [17].

It is logical and essential to rank the priorities on the path to investigate and know well every species needs and potentials in culture terms. With no doubt the first priority is to investigate the maximum possible biomass that can be produced under economically feasible conditions. For this purpose a kind of default set of parameters should be implemented in order to be considered as a starting point. In the present paper an ordinary indoor temperature of 20-21 °C, a classic nutrient medium (Walne), a sufficient range of illumination (2000-8000 lux), bubble aeration with no addition of CO₂, not too small not too big vessels of 1-2 L and non manipulated pH were chosen for our purpose which was to investigate the evolution of the growth curve, the growth rate and the dry weight yield of each species in salinities of brackish, sea, and hypersaline waters. Only after gathering results from experimentation with similar to the above mentioned conditions the procedures for further manipulations in order to achieve maximum biochemical products

(e.g. pigments, lipids, etc) can have meaning. It is of little usefulness to find that a certain species produces a lot of a valuable substances in conditions that otherwise result in minimum biomass from a very small growth rate and a very long culture period.

Of the 5 species of microalgae cultured in the present paper only one in terms of Genus (*Dunaliella* sp.) is represented quite well in the relevant literature followed by *Tetraselmis*. *Amphidinium* has only limitedly been studied and even less *Nephroselmis*. *Asteromonas gracilis* is almost neglected at all although its importance as feed for the rotifer *Brachionus plicatilis* is well documented [18, 19] and presents also some other advantageous features [15].

4.1. *Amphidinium carterae*

The interest for culture of this species and in general of the dinoflagellates is based on their capacity to produce bioactive metabolites (amphidinolides and amphidinols) and possibly biofuels although there are concerns about their ability to withstand shear stress caused by turbulence in photobioreactors [20-23]. From the existed limited data concerning the effects of light and salinity on its culture growth no solid conclusions can be drawn as either the used vessels were extremely different ranging from minute 25 mL [24] to small (200 mL, [25]), to medium (1L, [26]), to Photobioreactors [17]), or the methods applied in calculation of specific growth rate were not fully clarified. Only the optimum salinity for the best growth can be indirectly set with certainty at a value of less than 35 ppt which was the case in our study and was documented [24] for another relative species (*Amphidinium klebsii*). The very low yield in our work both in density of cells and g/L in our 40 and 60 ppt cultures as compared to the one in 20 ppt, strongly indicates that *A. carterae* responds much better in lower salinities. Concerning the effect of light, the situation is even more complicated in the literature as it is not only the various light intensities used and their units (e.g. what is $4000 \mu\text{W cm}^{-2}$ [20] and how this can be transformed to lux based on the well known formula: $5 \mu\text{mol photons m}^{-2} \text{ sec}^{-1} = 250 \text{ lux} = 1 \text{ watt m}^{-2}$?), but also the various temperatures used either much higher than ours (e.g. 25-30 °C, [26]) or similar (~20 °C) to ours [17, 24], the addition of CO₂ [25] and a puzzling combination of them and others when looked upon trying to decipher the existed topic for the optimum conditions. We found far better growth in the cultures of *A. carterae* in high light intensity (8000 lux) compared to low light (2000 lux) and this occurred in all salinities tested. The same was documented in all previous studies but as yields are highly variable in them we consider our results in growth rate and cell density (SGR = ~0.3 at high light, ~0.18 at low light and 6.5×10^6 cells/mL and 2.3×10^6 cells/mL respectively) and in yield (1.19 g/L at 20 ppt-XL vs 0.59 g/L at 20 ppt-X) far more realistic than those of [17] (0.473, 0.226 g/L and 69×10^3 cells/mL with more than 10 days lag phase at 2000 lux) or [26] (0.41 and 100×10^3 cells/mL at ~3000 lux).

4.2. *Nephroselmis* sp.

The chlorophyte *Nephroselmis* sp. has drawn interest due to its potential for production of lipids, carotenoids and various antioxidants [27-29] and as an excellent feed for *Artemia* [30]. Its documented mixotrophic ability [29] is an advantage for its mass culture as it can overcome occasionally shortage of nutrients and even benefit from the bacterial load usually present in old cultures. Because of its special ability for mixotrophy the cultures of *Nephroselmis* almost never collapse (personal observation) and keep on alive for many weeks in the stationary phase, subjected only to slight decoloration (fade green) in comparison to its exponential (log) phase color (green). After renewal of the medium with enriched water, the growth and the color are revived. From the outcome of our cultures it is shown that it is highly productive both in terms of final cell density and yield in dry weight. It grows well in a wide range of salinities from half to full strength sea water and reaches densities on the order of $\sim 70 \times 10^6$ cells/mL and yields close to 3.0 g d.w./L in about 20 days provided that light intensity is at least 8000 lux. Both were attained in batch culture at temperature ~20 °C, with no addition of extra CO₂ and compared to the relevant values of [28] (0.5 g/L at 25 °C, 6000 lux and addition of CO₂) or [27] ($17\text{-}30 \times 10^6$ cells/mL, 0.35-0.56 g/L at 26.5 °C and continuous illumination of 30,000 lux) are impressively higher. In addition, based on the fact that cell growth enhances with rising temperature in the range of 14-35 °C that represents the viable range for microalgae [31], it is logical to expect even higher yields if we culture *Nephroselmis* using higher temperatures (e.g. 25-30 °C) than 20-21.5 °C used in the present study. A lot of future research is needed for the culture

of *Nephroselmis* concerning the effect of many factors on its growth characteristics for establishing a reliable and trustworthy culture protocol.

4.3. *Tetraselmis* sp. (var. red pappas)

This strain of the species *Tetraselmis* sp. was given the arbitrary name "var. red pappas" due to its originating place of the lagoon pappas in W. Greece. It is a chlorophyte that differs from other species of genus *Tetraselmis* in its peculiar and astonishing characteristic of imparting a dark-red coloration to its culture medium (Figure 10) in conditions of illumination of 8000 lux when it reaches the stationary phase, as happened in our culture. This unique phenomenon has never been reported in the literature and we cannot give a plausible explanation other than it should be due to the accumulation of extracellular substances excreted by the senescent cells. Definitely cannot be attributed to carotenoids as the absorbance spectrum of the devoid of cells supernatant of centrifuged culture samples did not present any peaks corresponding to any kind of pigments, being almost absolutely zero-valued and flat along the range of 350-750 nm. So we assume that a naturally excretion of organic matter by its intact or lysed cells can be the cause for this phenomenon as such excretions are known to occur in microalgae in various intensities depending on species, conditions, and phase of the culture [32-37]. As the red coloration occurs only late in the stationary phase it is probable to be due to high molecular substances (carbohydrates or humic species from decomposition of cells) [36, 38, 39] than to low molecular ones (peptides or small proteins) that are known to be excreted during the exponential phase of the culture [32, 38, 39]. In the literature there are various studies on culture of several species of the genus *Tetraselmis* (*Tetraselmis* sp., *T. suecica*, *T. chui*) but they vary extremely in their conditions used and the output data. What seems from all of them to be in accordance with our data, is that *Tetraselmis* is growing best at salinities of 35 ppt or lower and that the high illumination is more productive [40-47]. This was confirmed in our study as the maximum density of *Tetraselmis* reached the level of $\sim 10 \times 10^6$ cells/mL in the salinities of 20 and 40 ppt and high illumination (8000 lux), far higher than the corresponding one of 60 ppt ($\sim 6 \times 10^6$ cells/mL) and even higher from their counterparts of low (2000 lux) illumination ($\sim 4.1 \times 10^6$, 3.2×10^6 and 2.1×10^6 cells/mL at salinities 20, 40 and 60 ppt respectively). Based on our experience of many years in culturing *Tetraselmis suecica* and now the present *Tetraselmis* sp. (var. red pappas), we never experienced densities over 10×10^6 cells/mL. In this respect the value of 35×10^6 cells/mL in [45] using low intensity light of 1588 lux is in our opinion questionable. We consider also as questionable the notation of [43] that their culture of *T. suecica* entered the stationary phase on the 4th day and presented a growth rate of 0.8 yielding biomass dry weight of 0.57 g/L as in our cultures, *Tetraselmis* for 17 days kept its exponential phase at salinities of 20 and 40 ppt and entered the stationary phase on day 14 and only at salinity of 60 ppt. Our higher yield in biomass of about 2.0 g d.w./L is supporting the overall published merits of genus *Tetraselmis* as an ideal species for aquaculture feed [48], candidate for biodiesel production [49] and easy to manipulate due mainly to its high tolerance to extreme salinities [50, 51].

4.4. *Asteromonas gracilis*

This extremely halotolerant (in respect to higher than seawater salinities) chlorophyte [15, 16, 52] which has been proven as a suitable live food for rotifers in marine fish hatcheries [18, 19] and a candidate for biofuel production [53] has drawn little attention for mass culture. To the best of our knowledge our study is the first one focused on the basics of its growth in batch culture in order to be considered as a starting point for future more elaborated studies. Compared to other cultured microalgae, it is the biggest in cell size (18-22 μ m) and because of this its maximum density is around 6.5×10^5 cells/mL (Figure 15-right) which was attained at the highest (100 ppt) of salinities tested and in high illumination (8000 lux). The salinity of 100 ppt in high light presented also the highest yield of 0.7 g/L as compared to 40 and 60 ppt (both ~ 0.5 g/L). All these values are higher from the only reported in the literature [53] of ~ 0.4 g/L in the culture of which he conducted the experiment in higher temperature (25 °C, ours 20-21.5 °C) but with weaker illumination (2500 lux, ours 8000 lux for the above mentioned values) and recorded the end of exponential (log) phase on day 9 while in our culture even on day 17 the exponential (log) phase had not been ended. We feel that our results apart from corroborating (at least in part) the findings of [53], must be considered as a more realistic approach due to the meticulous planning of our

experimentation that had the sole purpose to study the parameters of growth of *A. gracilis*. Definitely we can conclude that this species grows best at salinities over 60 ppt and apart from its highest cell density and yield attained, an indirect proof of it is the shortest lag phase in 100 ppt (3 days) as compared to 5 and 8 days recorded in 60 and 40 ppt respectively. From all the above however, the opinion that *A. gracilis* cannot be cultured in lower than 100 ppt salinities must be discarded because as it is shown in Figure 15 (left and middle) grows well also in both 40 and 60 ppt.

4.5. *Dunaliella* sp.

Species of the genus *Dunaliella* have been the subject of numerous studies over the years as this chlorophyte is notorious for its high salt tolerance by producing excessive amounts of glycerol and carotenoids [54, 55] and from long ago has found a positive response by aquaculturists aiming to the production of value added products [56, 57]. Being so, it comes of no surprise to meet in the literature highly variable data concerning its growth performance in terms of density of culture (cells/mL), growth rate and biomass yield as a consequence of the various volumes and conditions used in each particular experimentation. In the present study our batch culture was an experiment using medium volumes (2L), average easily attainable temperature (20-21.5 °C) and light intensities (2000 and 8000 lux), simple aeration with no extra CO₂, a well balanced nutrient mixture (Walne's), and 3 salinities covering the range from brackish to double strength seawater. We found that our local strain of *Dunaliella* grows well in all salinities (20-40-60 ppt) reaching more than 25×10^6 cells/mL after 3 weeks in light conditions of 8000 lux and to this point, concerning in particular the effect of light, we are in accordance with most of other studies [58 - 62] but in contrast to [63] in which growth decreased with increasing light intensity. Concerning the salinity we demonstrated that this local strain has an impressive ability to osmoregulate and adapt in both low and high salinity growing the same fast in the range of 20-60 ppt. This clear-cut conclusion supported by our results in high light (8000 lux) of final densities ($>25 \times 10^6$ cells/mL), SGR (0.350-0.405 doubl./day) and yield (1.25-1.58 g d.w./L) ranks our tested strain of *Dunaliella* among the most productive between its counterparts in the literature.

5. Conclusions

In all the five species cultured, two things were common to all and deserve special attention. First, that growth was far better in high light (8000 lux) as compared to low light (2000 lux) and second, the elevated values of pH in high light throughout the exponential phase and then its plummeting as the culture advanced in maturation. Intensity of photosynthesis is enhanced as light intensity increases and this was reflected in elevated pH values due to intense proton absorbance by the active cells [64]. The five species examined exhibited different response to the salinities used, *Amphidinium* clearly does best in 20 ppt far better than 40 ppt and even more than 50 ppt. *Nephroselmis* grows almost the same in 20 and 40 ppt and less well in 60 ppt although the SGR for the first 10 days were almost the same for all salinities. Almost the same as *Nephroselmis* performance was shown by *Tetraselmis*. *Asteromonas* does best in 100 ppt although it can grow quite well in both 40 and 60 ppt. *Dunaliella* grows equally well in all salinities (20-40-60 ppt). Concerning productivity as maximum biomass yield at the end of the culture period, first rank is occupied by *Nephroselmis* with ~3.0 g d.w./L, followed by *Tetraselmis* (2.0 g/L), *Dunaliella* (1.58 g/L), *Amphidinium* (1.19 g/L) and *Asteromonas* (0.7 g/L). All these values were resulted from batch cultures in a moderate temperature of 20 -21.5 °C and we speculate that at higher temperatures yields could be even higher. For all 5 species the literature has accumulated a lot of works only for various species of *Dunaliella* and *Tetraselmis*, very few for *Nephroselmis* and *Amphidinium* and almost none for *Asteromonas*. But in order to extract useful data from the existed bibliography for comparison with the data of the present work, the situation is very puzzled as data on growth, SGR and yield greatly varied, and often contradicted with each other. This obviously results from the different conditions selected for their experimentation, the methodology and time selected for the measurements. It is advised here for future works that the calculation of SGR should be made in a clearly indicated time period (i.e. from day "a" till day "b" of the culture), preferably from the day of the end of the lag (adaptation) phase till a day close to the middle of the log (exponential) phase..

Supplementary Materials: The following are available online at:

<https://youtu.be/3Vew3G9IRUE>, Video S1: *Tetraselmis* sp. (var. red pappas)

<https://youtu.be/R8ue4H6zuYQ>, Video S2: *Amphidinium carterae*

<https://youtu.be/giZ430t15Sc>, Video S3: *Nephroselmis* sp.

https://youtu.be/_t8HNZ457XQ, Video S4: *Asteromonas gracilis*

https://youtu.be/a7X_0walwRQ, Video S5: *Dunaliella salina*

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