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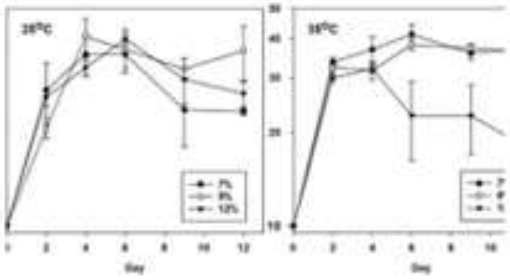

Indrayani Indrayani, Navid R. Moheimani ... Michael A. Borowitzka

OriginalPaper | Published: 30 January 2020 | Pages: 977 - 987

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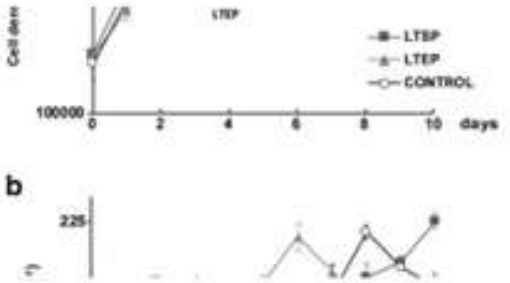

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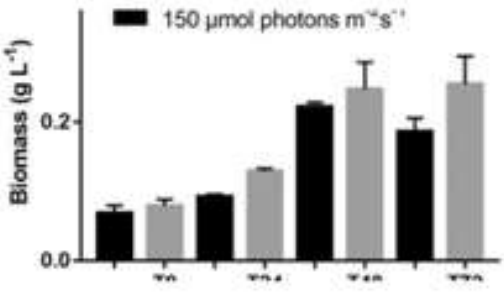
OriginalPaper | Published: 04 February 2020 | Pages: 989 - 1001

Vital parameters for biomass, lipid, and carotenoid production of thraustochytrids

Mohamad Nor Azzimi Sohedein, Wan Abd Al Qadr Imad Wan-Mohtar ... Phang Siew-Moi

ReviewPaper | Published: 03 January 2020 | Pages: 1003 - 1016

The effect of light intensity on the production and accumulation of pigments and fatty acids in *Phaeodactylum tricornutum*



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Temperature and salinity effects on growth and fatty acid composition of a halophilic diatom, *Amphora* sp. MUR258 (Bacillariophyceae)

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Abstract

Diatoms are of great interest for large-scale cultivation due to their high lipid content. The ability to grow over a wide range of salinities is also of great advantage. We studied the effect of temperature and salinity on the growth, lipids and fatty acid profiles of a newly isolated halophilic diatom *Amphora* sp. MUR 258. *Amphora* sp. MUR 258 is unusual in that it grows over a wide range of temperatures (24–35 °C) and salinities (7–12% (*w*/*v*) NaCl). The highest specific growth rate (SGR; 0.607 ± 0.017 day^{-1}) was achieved at 7% NaCl at 35 °C, and the lowest SGR (0.433 ± 0.087 day^{-1}) was obtained at 9% NaCl at 25 °C. The cells contained more lipids in the exponential phase, except when grown at 12% NaCl where the lipid content was higher in the stationary phase. The alga achieved its highest lipid content (57.69 ± 2.039% ash-free dry weight (AFDW) when grown at 7% NaCl at 25 °C and the lowest (34.43 ± 3.955% AFDW) obtained at 12% NaCl at 35 °C. The highest biomass productivity (0.171 ± 0.017 g_{AFDW} L^{-1} day^{-1}) and the lipid productivity (0.062 ± 0.017 g_{AFDW} L^{-1} day^{-1}) were achieved when the *Amphora* were grown at 9% NaCl at 35 °C and 7% at 25 °C, respectively. Irrespective of the growth conditions, the predominant fatty acids of *Amphora* sp. MUR 258 were palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1) and oleic acid (C18:1), as well as low quantities of eicosapentaenoic acid (C20:5).

References

Al-Hasan RH, Ali AM, Hana H, Radwan SS (1990) Effect of salinity on the lipid and fatty acid

Temperature and salinity effects on growth and fatty acid composition of a halophilic diatom, *Amphora* sp. MUR258 (Bacillariophyceae)

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Abstract

Diatoms are of great interest for large-scale cultivation due to their high lipid content. The ability to grow over a wide range of salinities is also of great advantage. We studied the effect of temperature and salinity on the growth, lipids and fatty acids profiles of a newly isolated halophilic diatom *Amphora* sp. MUR 258. *Amphora* sp. MUR 258 is unusual in that it grows over a wide range of temperatures (24-35ºC) and salinities (7-12% (w/v) NaCl). The highest specific growth rate (SGR; 0.607±0.017 day^{-1}) was achieved at 7% NaCl at 35ºC and the lowest SGR (0.433±0.087 day^{-1}) was obtained at 9% NaCl at 25ºC. The cells contained more lipids in the exponential phase, except when grown at 12% NaCl where the lipid content was higher in the stationary phase. The alga achieved its highest lipid content (57.69±2.039% ash-free dry weight (AFDW) when grown at 7% NaCl at 25ºC and the lowest (34.43±3.955% AFDW) obtained at 12% NaCl at 35ºC. The highest biomass productivity (0.171±0.017 g_{AFDW} L^{-1} day^{-1}) and the lipid productivity (0.062±0.017 g_{AFDW} L^{-1} day^{-1}) were achieved when the *Amphora* were grown at 9% NaCl at 35ºC and 7% at 25ºC, respectively. Irrespective of the growth conditions, the predominant fatty acids of *Amphora* sp. MUR 258 were palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1), and oleic acid (C18:1), as well as low quantities of eicosapentaenoic acid (C20:5).

Keywords: Bacillariophyta, microalgae, halophilic diatom, lipid, fatty acids, PUFA

Introduction

Species selection is the first and most important aspect in bio-prospecting microalgae for any commercial application (Borowitzka 2013). New microalgae species capable of reliable growth under outdoor conditions over a wide range of salinities and temperatures are of specific interest for large-scale production for biofuels or other products (Borowitzka and Moheimani 2013).

When grown in outdoor open pond systems cultures are exposed to varying temperatures, both diurnally and seasonally. For instance, in temperate locations with high insulation such as southern Western Australia, temperature can vary between -1^{o}C at night and up to 42^{o}C in the day (Bureau Meteorology, http://www.bom.gov.au/climate/data/?ref=ftr). Therefore, it is important that the alga of interest can grow optimally over a wide range of temperatures so that it can be cultured throughout the year. Almost all successful large-scale outdoor open pond cultures (e.g., *Dunaliella salina*, *Arthrospira* spp and *Chlorella* spp) experience broad temperature ranges (Béchet et al. 2013; Belay 2013; Borowitzka 2016). Optimal algal growth over a wide salinity range is another important criterion for successful cultivation in outdoor open ponds because salinity variation will occur due to evaporation and dilution caused by rain. Furthermore, in order to replace evaporation losses and maintain salinity, very large amounts of fresh water are needed. On the other hand, if saline water is used to replace evaporative losses, the salinity of the cultures will gradually increase (Borowitzka and Moheimani 2013). Therefore, microalgae capable of grow over awide salinity range and at high temperatures are highly desirable for successful and sustainable long-term culture in saline water in open pond systems. Furthermore, species that can grow at highly selective environment such as high salinity (*D. salina*), are less prone to contamination enhancing long-term culture stability (Borowitzka and Vonshak 2017).

Amphora sp. MUR 258 is a newly isolated halophilic diatom that contaminated and took over a *D. salina* culture grown in a 10 m^2 raceway pond at Murdoch University (Indrayani 2017). As a newly isolated species, there was no information available regarding limits to growth, lipid and fatty acids composition of this species. The aim of this study was to determine how temperature and salinity affect growth as well as lipid and fatty acid composition of *Amphora* sp. MUR 258.

Materials and Methods

Algal Species: *Amphora* sp.MUR 258 was originally isolated from a culture of *Dunaliella salina* in a 10 m^2 raceway pond at the Algae R&D Center at Murdoch University, Perth, Western Australia (31.9554° S, 115.8585° E) in April 2011. The salinity of the *D. salina* culture was 14% NaCl. *Amphora* was isolated by agar plating (Andersen and Kawachi (2005) in f+Si medium (Guillard and Ryther 1962) at three different salinities (10, 12 and 15% (*w/v*) NaCl). Uni-algal colonies were isolated after repeated streaking on fresh agar plates at 10% NaCl (*w/v*) salinity. This strain is unusual in that it is 'non-sticky' and can be maintained suspended in the water column by minimal mixing which prevents settling, a feature important for culture in raceway ponds.

Growth conditions: The cultures were grown in triplicates in 500 mL Schott bottles containing 300 mL of f+Si medium at three different salinities (7, 9 and 12% NaCl) and two different temperatures, 25±1°C and 35±1°C, under a 12 h:12 h light:dark cycle and 65±5 µmol photons m^{-2} s^{-1} irradiance provided by daylight fluorescence tubes. The inoculum cell concentration was $10x10^4$ cells mL^{-1}. The salinity range was selected to reflect the likely salinity range which the alga would be exposed to in outdoor continuous cultures using seawater as the main water source (Fon Sing and Borowitzka 2016). The two temperatures were selected as representing the range of temperatures that arelikely to be encountered during the day in outdoor algae cultures in Western Australia (Boruff et al 2015). The cultures were shaken manually daily (morning and afternoon) and grown in batch mode for at least two weeks. To make sure that the cultures were receiving the same amount of light, the culture flasks were rotated randomly every day using a table of random numbers. The pH of the cultures was not controlled during the experiments.

Analytical methods: Algal growth was monitored by counting cells every second day using Neubauer haemocytometer and calculating the specific growth rate (Moheimani et al. 2013). The specific growth rate (µ) was calculated using the following equation:

$$\boldsymbol{\mu = Ln\,(N_2\,/N_1\,)/(t_2 - t_1)}$$

Where N_1 and N_2 are the cell density at time 1 (t_1) and 2 (t_2) within the exponential phase. Dry weight (DW), ash-free dry weight (AFDW) and lipid content were determined on day 2 (logarithmic phase) and day 9 (stationary phase) using the methods described Moheimani et al. (2013). For DW, 5 mL of culture was filtered through pre-weighed and pre-combusted

Whatman GF/C, 25 mm filter paper. The filters then dried in an oven at 75ºC for 5 h and weighed. Dry weight was calculated using the following equation:

$$Dry\ Weight(gram\ per\ liter) = weight\ of\ filter\ plus\ algae - weight\ of\ filter$$

The filters were then transferred to a furnace at 450ºC and ashed for 5 h. Organic dry weight (Ash-free dry weight) was calculated by the following equation:

$$Ash - Free\ Dry\frac{Weight}{Biomass}Yield\ (gram\ per\ liter) = DW - weight\ of ash$$

Biomass productivity was calculated as a function of yield and specific growth rate at exponential phase (Moheimani et al. 2013):

Biomass Productivity (g L^{-1} day^{-1}) = μ x Biomass Yield

Lipid extraction followed the Bligh and Dyer method modified by Kates and Volcani (1966). The lipid samples were then used for fatty acid analysis following a method adapted from Christie (1989). Fatty acid components were analysed with a Varian CP3800 Gas Chromatograph. The instrument was equipped with a split/split less injector and a Flame Ionisation Detector (FID) with analysis performed using an Agilent VF-5MS (30 m, 0.32 mm ID) fused silica column coated with a 0.1 μm phenyl film. Samples (1 μL) were injected via a split/split less (5:1) injector held at 280°C. The oven temperature was initially held at 80°C, then heated at 7°C min^{-1} until 310°C and held isothermally for 3 min. The carrier gas was hydrogen at 1.2 mL min^{-1} and the detector was held at 280°C with a hydrogen flow rate of 30 mL min^{-1} and air flow rate of 300 mL.min^{-1}. The total run time was 35.86 min. The fatty acid profile was determined by dividing the area of the fatty acid of interest by the sum of all fatty acid areas present in the sample. All analyses were carried out in duplicate.

FAME identification was performed on a HP 6890 GC-MS using the same column and conditions as outlined above. Component identification was by retention time and MS data. 60 μL of samples were spiked with alkane mix (5 μL dodecane, pentadecane, nonadecane, docosane, octacosane, and dotriacontane) to calculate the Kovats Retention Index (KRI) of the FAMES identified on the GC-MS. The KRI was then used to identify the residence time of the FAMES in the GC-FID data.

Data analysis: Differences between treatments were analysed with a two-way analysis of variance (ANOVA). Pairwise multiple comparison procedures (Holm-Sidak Method) were then used to precisely test differences between conditions. All statistical analyses were performed using Sigma-Plot 13 package (Systat Software Inc., USA).

Results

Growth and biomass productivity. The growth curves are shown in **Figure 1**. Under all temperature and salinity conditions, there was no apparent lag phase indicating that the cells quickly adapted to the new conditions. At 25ºC the maximum cell density of $44x10^4$ cells mL^{-1} was reached on day 4 except for the cultures grown at 12% NaCl that reached a maximum density of $34x10^4$ cells mL^{-1} on day 6. All cultures at the higher temperature (35ºC) grew rapidly in the first 2 days before entering the stationary phase. It took 4 days for the cultures grown at 25ºC to reach the same cell density on day 2 of the cultures grown at 35ºC.

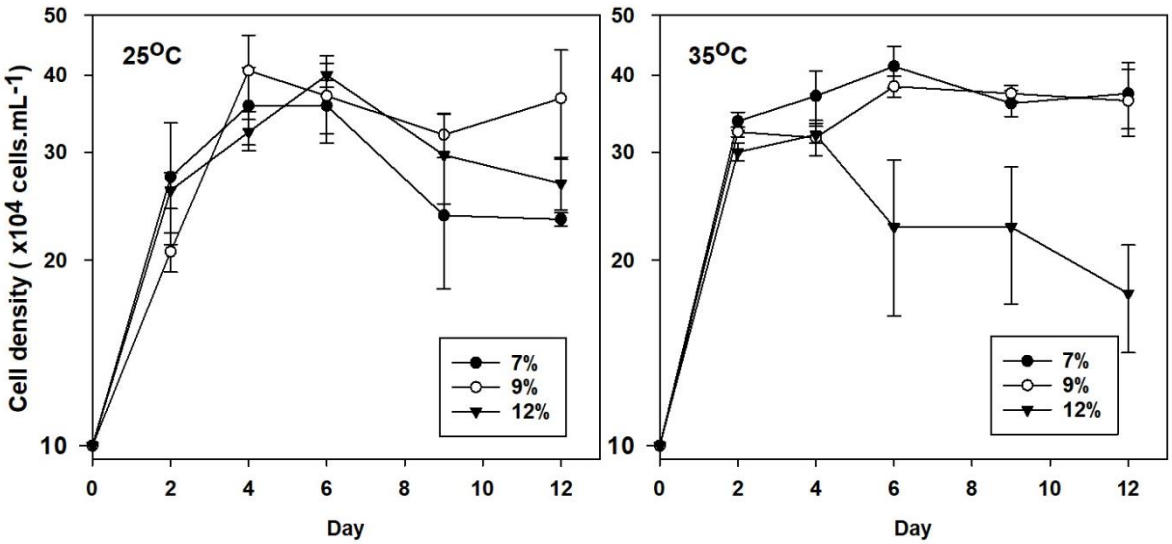

Figure 1. Growth of *Amphora* sp. MUR 258 under different temperatures (25 and 35ºC) and salinities (7, 9 and 12% NaCl). Mean ± standard deviation, n = 3.

The specific growth rates of *Amphora* sp. MUR 258 were significantly affected by the temperature (Two-Way ANOVA, $F_{(1,12)}$=12.75; P=0.004)being higher at high temperature (35ºC) (Figure 2). However, there was no significant difference in the specific growth rate of the between salinity (Two-Way ANOVA, $F_{(2,12)}$=0.75; P=0.496) and no significant interaction between temperature and salinity was observed (Two-Way ANOVA, $F_{(2,12)}$=1.33; P=0.302). The SGRs at 25ºC and 7, 9 and 12% salinities were 0.495±0.109 day^{-1}, 0.433±0.087 day^{-1} and 0.477±0.033 day^{-1}, respectively, whereas, the SGRs at 35ºC and 7, 9 and 12% salinities were 0.607±0.017 day^{-1}, 0.587±0.008 day^{-1} and 0.549±0.017 day^{-1}, respectively.

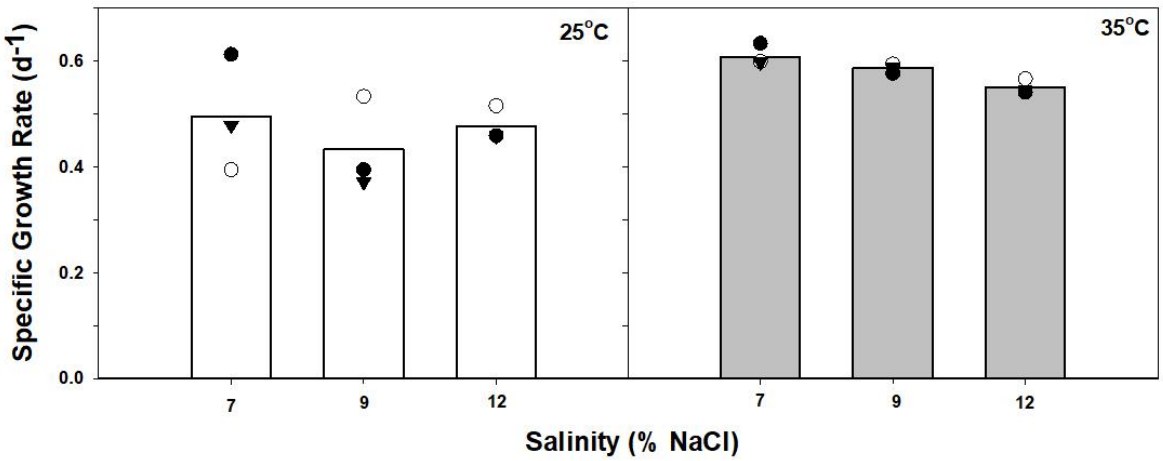

Figure 2. Specific growth rate (d^{-1}) of *Amphora* sp. MUR 258 under different temperatures (25 and 35ºC) and salinities (7, 9 and 12% NaCl). There was a significant difference in the specific growth rate between the temperatures (Two-Way ANOVA, $F_{(1,12)}$=12.75; P=0.004), but not the salinity (Two-Way ANOVA, $F_{(2,12)}$=0.75; P=0.496) . The different symbols represent the three replicate cultures, bars show the means (n=3)

The biomass yields (g_{AFDW} L^{-1}) of *Amphora* at exponential phase (**Figure 3-upper panel**) were not affected by temperature (Two-Way ANOVA, $F_{(1,12)}$=0.120; P=0.735) and there was no significant interaction between temperature andsalinity (Two-Way ANOVA, $F_{(2,12)}$=0.096; P=0.909). However, statistically significant differences in the biomass yields at different salinities were observed (Two-Way ANOVA, $F_{(2,12)}$=9.85; P=0.003). There was a statistical significant difference in the biomass yield between 12 and 9% salinity (Holm-Sidak, P=0.005) and between 12 and 7% (Holm-Sidak, P=0.006), but no significant difference in the biomass yield was observed between salinity 7 and 9% (Holm-Sidak, P=0.752). The highest biomas yield at exponential phase (0.258±0.017 g_{AFDW} L^{-1}) was achieved at 25ºC and 12% NaCl and the lowest biomass yield of 0.212±0.02 g_{AFDW} L^{-1} was obtained at 25ºC and 9% NaCl. At stationary phase, temperature affected the biomass yield (Two-Way ANOVA, $F_{(1,12)}$=11.722; P=0.005) but not with the salinity (Two-Way ANOVA, $F_{(2,12)}$=0.924; P=0.423). No significant interaction between temperature and salinity on the biomass yield was observed (Two-Way ANOVA, $F_{(2,12)}$=1.829; P=0.203) (**Figure 3-upper panel**). The highest biomas yield at stationary phase (0.300±0.026 g_{AFDW} L^{-1}) was achieved at 35ºC and 12% NaCl and the lowest biomass yield of 0.224±0.049 g_{AFDW} L^{-1} was obtained at 25ºC and 9% NaCl.

Temperature and salinity affected the the biomass per cell at exponential phase (Two-Way ANOVA, $F_{(1,12)}$=45.236; P<0.001 and $F_{(2,12)}$=6.269; P=0.014, respectively) (**Figure 3-lower panel**), but the interaction between salinity and temperature was not significant (Two-Way ANOVA, $F_{(2,12)}$=2.526; P=0.121). In exponential phase the biomass per cell was significantly higher at 35ºC than at 25ºC (Holm-Sidak, P<0.001), but temperature had no effect on the biomass per cell in stationary phase (Two-Way ANOVA, $F_{(1,12)}$=12.462; P=0.143). Interestingly, we also observed that after 1 week of growth at 35ºC the algal cells were noticeably bigger and more rounded than their counterparts growing at 20 and 25ºC. In the exponential phase the cells were heavier at 9 and 12% salinity compared to 7% salinity (Holm-Sidak, P=0.040 and P=0.017, respectively). However, salinity and interaction between temperature and salinity affected the biomass per cell (Two-Way ANOVA, $F_{(2,12)}$=8.441; P=0.005 and $F_{(2,12)}$=14.839; P<0.001, respectively). In stationary phase at 25ºC salinity had no effect on the biomass per cell, whereas at 35ºC the cells at 12 % salinity were significantly heavier than the cells at the other two salinities (Holm-Sidak, P<0.001).

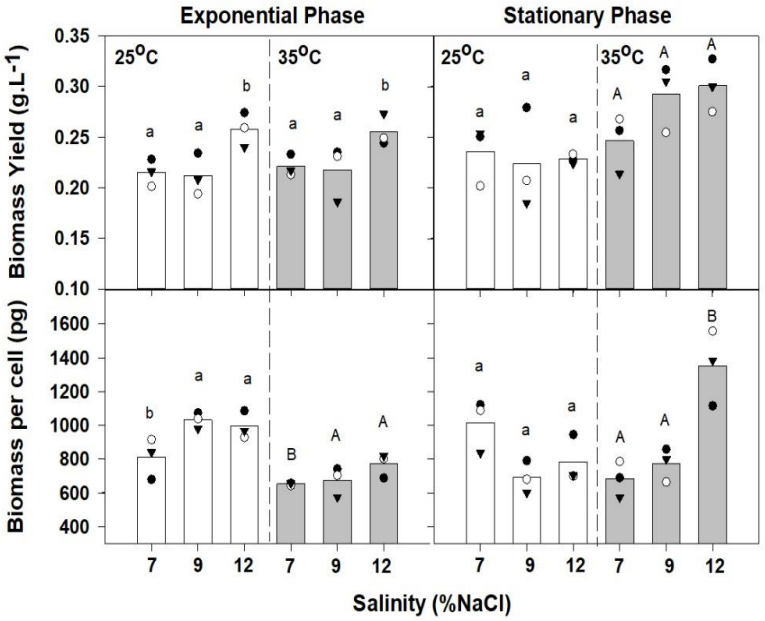

Figure 3. *Amphora* sp. MUR 258 biomass yield (g_{AFDW} L^{-1}) and cell weight (pg_{AFDW} $cell^{-1}$) in different growth phases when grown at different temperature and salinity. Different letters above the bars indicate statistical difference (p<0.05). The different symbols represent the three replicate cultures, bars show the means (n=3)

Biomass productivities were affected by temperature (Two-Way ANOVA, $F_{(1,12)}$=28.53; P<0.001), but not salinity (Two-Way ANOVA, $F_{(2,12)}$=0.044; P=0.958) (Figure 4). There was no significant interaction between temperature and salinity on the biomass productivity of the

alga (Two-Way ANOVA, $F_{(2,12)}$=1.548; P=0.252). A significant difference in biomass productivity between 25 and 35ºC was observed (Holm-Sidak, P<0.001). The highest biomass productivity was observed at 35ºC and 9% salinity (0.171±0.017 g_{AFDW} L^{-1} day^{-1}) and the lowest at 25ºC and 9% salinity (0.096±0.024 g_{AFDW} L^{-1} day^{-1}).

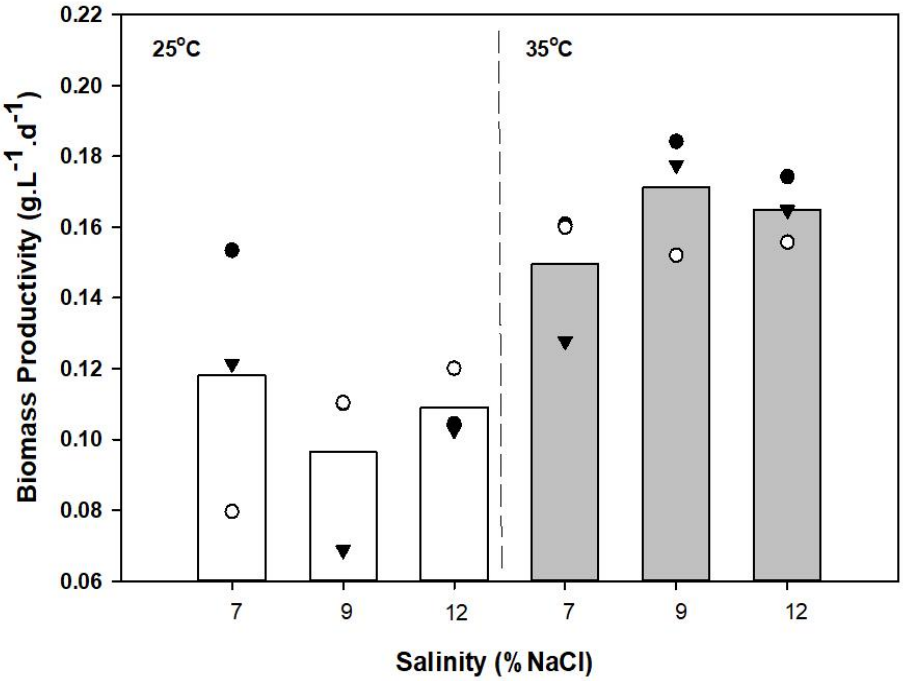

Figure 4. Ash-free dry weight biomass productivity of *Amphora* sp. MUR 258 growing under different temperatures and salinities. Temperature has a significant effect on biomass productivity (Two-Way ANOVA, $F_{(1,12)}$=28.53; P<0.001) but not the salinity (Two-Way ANOVA, $F_{(2,12)}$=0.044; P=0.958) . The different symbols represent the three replicate cultures, bars show the means (n=3)

Lipid content and productivity. The lipid content of *Amphora* sp. MUR 258 at exponential phase was significantly affected by temperature (Two-Way ANOVA, ($F_{(1,12)}$=53.66; P<0.001) and salinity (Two-Way ANOVA, $F_{(2,12)}$=28.57; P<0.001) (Figure 5). A significant difference in the lipid content was observed between 25 and 35ºC (Holm-Sidak, P<0.001), between salinity 7 and 12% (Holm-Sidak, P<0.001) and between salinity 9 and 12% (Holm-Sidak, P<0.001), but no significant difference in the lipid content between salinity 7 and 9% was observed (Holm-Sidak, P=0.476). Similarly, there was no significant interaction between the temperature and salinity on the lipid content of the alga (Two-Way ANOVA, $F_{(2,12)}$=2.475; P=0.126). The alga achieved its highest lipid content (57.69±2.039% AFDW) when grown at 7% NaCl at 25ºC and the lowest (34.43±3.955% AFDW) obtained at 12% NaCl at 35ºC .

Temperature affected the lipid content of the alga at stationary phase (Two-Way ANOVA, $F_{(1,12)}$= 11,335; P=0.006), but not the salinity (Two-Way ANOVA, $F_{(2,12)}$=0.714; P=0.509) (Figure 5). There was no significant interaction between temperature and salinity on the lipid content of the species at stationary phase (Two-Way ANOVA, $F_{(2,12)}$=1.540; P=0.254). It is important to note that the lipid content was higher in exponential phase than in stationary phase, except for the cultures grown at 12% salinity at both temperatures, where the lipid content was higher at stationary phase. The highest lipid content of 57.69±2.04% AFDW achieved at 7% at 25ºC and the lowest (31.49±3.42% AFDW) obtained at 9% at 35ºC .

Both temperature and salinity affected the lipid content per cell at exponential phase (Two-Way ANOVA, $F_{(1,12)}$=79.180; P<0.001 and $F_{(2,12)}$= 4.783; P=0.032, respectively) and there was a statistically significant interaction between the two on the lipid per cell of the *Amphora* (Two-Way ANOVA, $F_{(2,12)}$=6.429; P=0.013). Significant difference in the lipid per cell was observed between salinity 9 and 12% (Holm-Sidak, P=0.002), and between 9 and 7% salinity (Holm-Sidak, P=0.022) at25ºC, but no significant difference in the lipid content per cell between 7 and 12% salinity (Holm-Sidak, P=. 0.127) was observed. The highest lipid per cell (583±72 pg) and the lowest lipid per cell (285±35 pg) at 25ºC were obtained at 9% and 12% salinity, respectively. At 35ºC no significant difference in the lipid content between the different salinities tested was observed (Holm-Sidak, P>0.005). In relation to temperature, there was a significant difference in the lipid per cell between 25 and 35ºC at 7% salinity (Holm-Sidak, P<0.001), 9% salinity (Holm-Sidak, P<0.001) and 12% salinity (Holm-Sidak, P=0.016). The highest lipid content per cell (319.57 pg) and the lowest lipid content per cell (249.84 pg) at 35ºC were obtained at 12% and 9% salinity, respectively (**Figure 5**).

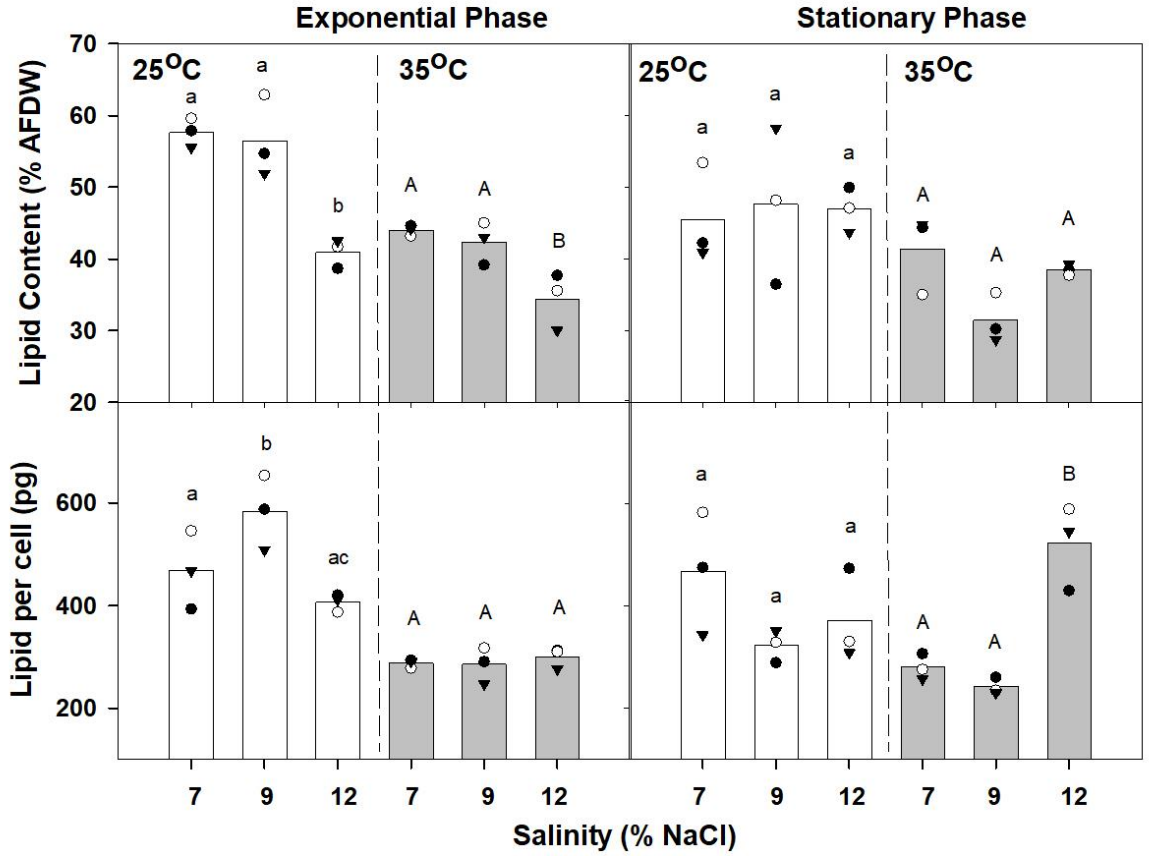

Figure 5. Lipid content (% AFDW) and lipid per cell (pg) of *Amphora* sp. MUR 258 under different temperature, salinity and growth phase. Different letters above the bars indicate statistical difference (p<0.05). The different symbols represent the three replicate cultures, bars show the means (n=3)

At stationary phase, there was no significant difference in the lipid content per cell between the temperatures (Two-Way ANOVA, $F_{(1,12)}$=1.332; P=0.271) (Figure 5). However, a significant difference was observed between salinities (Two-Way ANOVA, $F_{(2,12)}$=7.8; P=0.007) and there was a significant interaction between temperature and salinity (Two-Way ANOVA, $F_{(2,12)}$=8.66; P=0.005). When grown at 25°C no significant difference in the lipid per cell was observed (Holm-Sidak, P>0.005). However, at 35°C, there was a significant difference in the lipid per cell was observed between salinity 12 and 9% (P=0.001) and between 12 and 7% salinity (Holm-Sidak, P=0.003), but no significant difference was observed between 7 and 9% salinity (Holm-Sidak, P=0.530). There was a significant difference in the lipid per cell between 25 and 35°C at 7% salinity (Holm-Sidak, P=0.008) and 12% salinity (Holm-Sidak, P=0.025), but no significant difference in the lipid per cell was observed between temperature 25 and 35°C at 9% salinity (Holm-Sidak, P=0.193). The highest lipid per cell (521±82 pg) and the

lowest lipid per cell (242±16 pg) at stationary phase were obtained at 12% salinity at 35ºC and 9% at 35ºC, respectively (**Figure5**). It is important to note that the cellular lipid content of the cultures grown at 12% salinity and 35ºC was more than double in stationary phase, whereas their counterparts grown at 12% salinity and 25ºC had a lower lipid content. On the other hand, the cellular lipid content of the cultures grown at 9% salinity and 25ºC decreased dramatically in the stationary phase, whereas their counterparts grown at 9% and 35ºC showed only a small decrease in stationary phase (**Figure 5**). It is also interesting to note that the cellular lipid profiles of the *Amphora* are associated with the biomass per cell as shown in **Figure 3**.

The lipid productivity of *Amphora* sp. MUR 258 was not affected by the temperature (Two-Way ANOVA, $F_{(1,12)}$=0.009; P=0.926) and the salinity (Two-Way ANOVA, $F_{(2,12)}$=1.832; P=0.202) (Figure 6). Also, there was no significant interaction between temperature and salinity (Two-Way ANOVA, $F_{(2,12)}$=0.094; P=0.911). The lipid productivity at 25ºC ranged from 0.04 to 0.081 g_{AFDW} L^{-1} d^{-1} and the lipid productivity at 35ºC ranged from 0.045 to 0.062 g_{AFDW} L^{-1} d^{-1}.

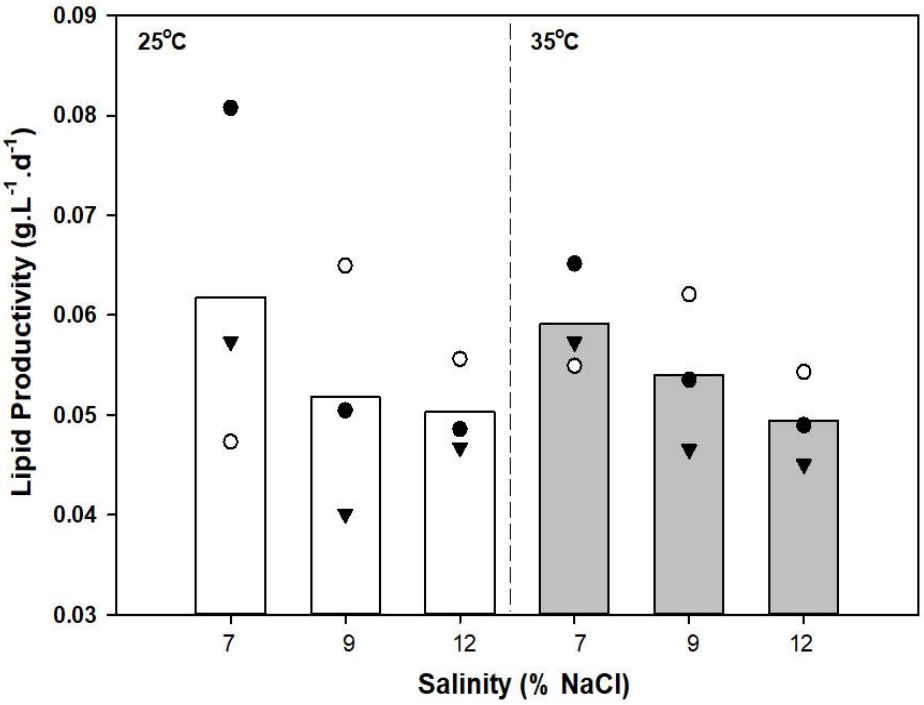

Figure 6. Lipid productivity on an ash-free dry weight basis of *Amphora* sp. MUR 258 growing under different temperatures and salinities. The lipid productivity was not affected by temperature (Two-Way ANOVA, $F_{(1,12)}$=0.009; P=0.926) or salinity (Two-Way ANOVA, $F_{(2,12)}$=1.832; P=0.202). The different symbols represent the three replicate cultures, bars show the means (n=3)

Fatty acid composition. The fatty acid composition of *Amphora* sp. MUR 258 grown at different temperatures (25 and 35ºC) and salinities (7, 9 and 12% NaCl) was analysed during exponential and stationary phases (**Table 1**). The fatty acids consisted of saturated fatty acids (SFAs) (67.2-79.5% of total FAMEs), monounsaturated fatty acids (MUFAs) (17.3-28.3% of FAMEs) and polyunsaturated fatty acids (PUFAs) (3-4.5% FAMEs). The total SFAs tended to decrease as the temperature increased from 25 to 35ºC, whereas total MUFAs and PUFAs were higher at the higher temperature.

Table 1. Fatty acid profiles (% of total FAMEs) of *Amphora* sp MUR 258 growing under different temperatures (ºC), salinities (%NaCl) at different growth phases (exponential phase (EP) and stationary phase (SP). Data are the mean of two replicates.

Fatty acids	**Temperature (25±1ºC)**						**Temperature (35±1ºC)**					
	7% NaCl		**9% NaCl**		**12% NaCl**		**7% NaCl**		**9% NaCl**		**12% NaCl**	
	EP	**SP**	**EP**	**SP**	**EP**	**SP**	**EP**	**SP**	**EP**	**SP**	**EP**	**SP**
Saturated fatty acids (SFAs)												
Methyl Laurate (C12:0)	0.6	0.5	0.7	0.5	0.7	0.5	0.0	0.5	0.7	0.5	0.7	0.5
Methyl Myristate (C14:0)	4.2	4.4	4.4	4.8	4.2	5.0	4.8	5.2	4.4	5.6	4.2	5.2
Methyl Pentadecanoate (C15:0)	0.6	1.0	0.7	0.8	0.3	0.8	0.8	1.7	0.4	1.8	0.4	1.6
Methyl Palmitate (C16:0)	40.3	37.6	42.1	40.0	39.9	37.7	39.1	35.8	37.0	37.4	38.2	37.5
Methyl Stearate (C18:0)	32.2	30.3	31.6	28.9	32.0	29.1	27.8	26.0	29.7	21.6	29.4	23.1
Arachnic acid methyl ester (C20:0)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0
Methyl Lignocerate (C24:0)	0.5	0.6	0.0	0.3	0.0	0.5	0.0	0.5	0.0	0.3	0.0	0.0
Total SFA	**78.4**	**74.4**	**79.5**	**75.3**	**77.1**	**73.6**	**72.5**	**70**	**72.2**	**67.2**	**72.9**	**67.9**
Monounsaturated fatty acids MUFAs)												
Methyl Palmitoleate (C16:1)	7.7	12.6	7.2	12.1	6.4	12.6	14.1	16.7	12.5	18.9	11.2	18.2
Methyl Oleate (C18:1)	9.1	8.4	8.5	7.9	10.2	8.3	8.7	7.9	9.5	7.8	9.9	8.0
Methyl Eicosanoate (C20:1)	0.8	0.7	0.8	0.7	1.0	0.7	0.0	0.5	0.8	0.6	0.7	0.5
Methyl Erucate (C22:1)	1.1	1.0	0.8	1.1	2.3	1.0	1.1	0.8	1.4	1.0	1.1	1.0
Total MFAs	**18.7**	**22.7**	**17.3**	**21.8**	**19.9**	**22.6**	**23.9**	**25.9**	**24.2**	**28.3**	**22.9**	**27.7**
Polyunsaturated fatty acids (PUFAs)												
Methyl Linoleate (C18:2)	2.8	2.5	2.8	2.5	3	2.7	2.7	2.7	3.2	2.8	3.4	2.9
Methyl 5,8,11,14,17 Eicosapentaenoate (C20:5)	0.2	0.4	0.5	0.5	0.0	1.0	0.9	1.5	0.6	1.7	0.9	1.5
Total PUFAs	**3**	**2.9**	**3.3**	**3**	**3**	**3.7**	**3.6**	**4.2**	**3.8**	**4.5**	**4.3**	**4.4**
Total FAs	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

Irrespective of temperature, salinity, and growth phase, the predominant fatty acids of *Amphora* sp MUR 258 were palmitic acid C16:0 (35.8-42.1% of total FAMEs), followed by stearic acid C18:0 (21.6-32.2% of total FAMEs), palmitoleic acid C16:1 (6.4-18.9% of total FAMEs) and oleic acid C18:1 (7.8-10.2% of total FAMEs). In addition, the contents of the major fatty acids were higher at exponential phase than in stationary phase, except for palmitoleic acid (C16:1), myristic acid (C14:0) and EPA (C20:5) whose content was higher at the stationary phase.

Interestingly, the EPA (C20:5) content of *Amphora* sp MUR 258 cultured at 9% salinity at 35ºC increased nearly threefold, from 0.6% at exponential phase to 1.7% of total FAMEs at stationary phase, whereas the EPA content of the cultures grown at 25ºC remained the same at around 0.5% at both exponential and stationary phase.

Discussion

Temperature is one of the key environmental factors affecting the growth of microalgae (Yu et al. 2018). Considering the environmental changes experienced by large-scale outdoor cultures, the ability to grow well over a wide range of temperatures is a prerequisite characteristic of microalgae to be cultured successfully outdoors over the whole year (Borowitzka 2013). In this study we found that *Amphora* sp. MUR 258 grew well over a wide range of temperatures (25-35ºC) similar to those expected to be experienced in the field in Western Australia. There are relatively few reports of diatom growth at temperatures higher than 30ºC, although this may due in part to the small number of studies on tropical species and species from desert salinas. Some examples are *Chaetoceros* sp. CS256 isolated from North Queensland (max. temp 35ºC (Renaud et al. 2002)); *Chaetoceros gracilis* S/CHAET-1 (grows at 35ºC, optimum temp 30-32ºC (Mortensen et al. 1988)); *Chaetoceros muelleri* isolated from Mexico, (35ºC (McGinnis et al. 1997)); In the present study, *Amphora* sp. MUR 258 grew at temperatures up to 36ºC, the highest temperature tested. In addition, *Amphora* sp. MUR258 grew very well at much higher temperature up to 42.2ºC during summer in outdoor raceway ponds in Perth, Western Australia (Indrayani et al. 2019).

Although *Amphora* sp. can grow well at high temperature, long-term exposure to constant high temperature and at a high salinity over two weeks of culturing had a slight detrimental effect on the cultures. The most affected cultures were those at 35ºC and 12% salinity where cell density slowly declined after day 4. However, unlike the constant conditions in the experiment, in outdoor cultures such high temperatures are not maintained for long periods with the culture temperature declining at night.

Apart from acclimation issues, reduced growthof *Amphora* sp. MUR258 at constant high temperature and salinity can also be attributed to the fact that CO_2 is less soluble at high temperatures and high salt concentrations (Duan et al. 2006) resulting in CO_2 limitation in

thecultures. Several studies have also shown that short-term exposure to high temperatures has a detrimental effect on photosynthesis (Salleh and McMinn 2011; Barati et al. 2018) which in turn can lead to reduced growth. Another potential factor for the cell decline at high temperature is that the dark respiration rate increases with increasing temperature (Richmond 1986). Thus, high night-time temperatures will lead to greater night-time biomass loss (Grobbelaar and Soeder 1985; Torzillo et al. 1991).

Temperature also affected the per cell biomass and the morphology of the *Amphora* cells. Montagnes and Franklin (2001) also observed an enlargement of diatom size with increasing temperatures. The big and round cells observed at 35ºC are similar in appearance to dormant resting cells which have been observed in diatoms under unfavourable conditions (McQuoid and Hobson 1996).

The diatom *Amphora* sp. MUR 258 grew well over a very wide range of high salinities (7-12% NaCl) confirming that it is a hypersaline alga requiring an environment with salt concentrations higher than seawater of at least 7% of NaCl for optimum growth. Although there are many studies of diatoms as part of the flora of saline lakes (e.g., Herbst and Blinn 1998, Roberts and McMinn 2004), there are very few studies on the salinity tolerance and growth of these diatoms. Clavero et al. (2008) studied the growth of 34 benthic diatom strains isolated from thalassic hypersaline lakes over a salinity range of 0.5 to 17.5% total salts, with the 5 most halotolerant taxa growing at up to 15% total salts, and Mercz (1994) studied the growth of an *Amphora coffaeoformis* at up to 7% NaCl. The upper salinity limit for diatoms is about 15% NaCl and this is probably due to limits in their ability to osmoregulate and in algae the type of osmoregulatory compatible solute appears to set this limit (MacKay et al. 1984; Reed et al. 1986). Diatoms mainly use proline as a compatible solute (Krell et al. 2007; Scholz and Liebezeit 2012; Hagemann 2016) although, in addition to proline, cyclohexanetetrol (Fujii et al. 1995) and mannose (Paul 1979) have been identified. The exact osmoregulatory mechanism in halophilic diatoms such as *Amphora* sp. MUR 258 remains to be elucidated.

Many studies have evaluated the lipid and fatty acids composition of algae, but little information is available regarding the fatty acid profiles of hypersaline diatoms. The quantity and quality of microalgal lipids also are influenced by environmental conditions including temperature (Renaud et al. 2002; Chen 2012; Pasquet et al. 2014) and salinity (Al-Hasan et al. 1990; Scholz and Liebezeit 2013). The lipid content of *Amphora* sp. MUR 258 ranged between

30-63% AFDW (11-24% DW) or 0.08-0.132 g_{AFDW} L^{-1} which is comparable with other microalgal species (Griffiths and Horrison 2009).

In the present study, the lipid content of *Amphora* sp. MUR 258 was significantly lower at higher growth temperatures in agreement with previous studies on diatoms; i.e. *Chaetoceros calcitran*s and *C. simplex* (Thompson et al. 1992), *Nitzschia* spp. (Renaud et al. 1995) and *Chaetoceros* sp. (Renaud et al. 2002). In relation to salinity, the only comparable study is that of Al-Hasan et al. (1990) who studied the effect of different salinity (0.5, 1.7 and 2.5 M NaCl) on the lipid and fatty acid composition of the diatom *Navicula* sp. They found that the lipid content increased with increasing salinity from 0.5 M (5.3 % of DW) to 1.7 M (13.4% of DW), but then declined at 2.5 M (4.3% DW). The lipid content of the *Amphora* sp MUR 258 in the present study was very high, generally decreasing as salinity increases.

Unlike most studies which report high lipid accumulation in the stationary phase (e.g., McGinnis et al. 1997; Hu et al. 2008; Su et al. 2011), this study showed that *Amphora* sp. MUR 258 accumulated more lipids during exponential phase when grown at lower salinity (7 and 9%) and at lower temperature (25^{o}C). However, when grown at higher salinity (12% NaCl) and higher temperature (35^{o}C), the cells accumulated more lipids at stationary phase. As pointed out by Hu et al. (2008), many algae modify their lipid biosynthetic pathways towards neutral lipid accumulation that serve as a storage material under unfavourable conditions for growth. Beside strains and growth condition variations, another possible explanation for our unusual observations is the complex evolutionary history of diatoms which differs substantially from many other photosynthetic organisms in terms of intracellular compartmentation and organization of carbon partitioning pathways that play a substantial role in the regulation of carbon flux into lipid/fatty acids biosynthesis (Smith et al. 2012). However, the exact mechanisms underlying the lipid metabolic pathways under combination of two stressors (temperature and salinity) in *Amphora* sp. MUR 258 are unknown.

The observation of a higher lipid content in exponential growth phase is important as this phase results in high lipid productivity. Furthermore, it is likely that the lipid content and productivity can be further increased through manipulation of culture conditions. For example, it may be possible to optimize lipid productivity by employing a temperature and/or salinity shift strategy: i.e. first, grow the alga at 7% NaCl and 35^{o}C to achieve the maximum growth rate in the exponential phase. Second, when *Amphora* sp. enters the early stationary phase the

temperature could be decreased to 25ºC for high lipid accumulation within the cells. By such a two-stage method, one could potentially increase the lipid productivity two-fold. However, it must also be recognised that such a strategy is difficult to implement in large-scale ponds, especially the change in temperature, although a change in salinity is theoretically possible.

In terms of the fatty acid composition, the fatty acid composition of the *Amphora* sp. was dominated by palmitic, stearic, palmitoleic and oleic acids and remained similar irrespective of the growth conditions. It has been observed in many microalgae that saturated fatty acids increase with increasing temperature and unsaturated fatty acids increase with decreasing temperature (Renaud et al. 2002; Rousch et al. 2003; Jiang and Gao 2004; Guschina and Harwood 2013). Furthermore, at low temperature, algae tend to produce more PUFAs to maintain membrane fluidity in a state necessary for biological functioning (Guschina and Harwood 2013). Contrary to these findings, the total saturated fatty acids of *Amphora* sp MUR 258 tended to decrease slightly as the temperature increased, whereas the unsaturated fatty acids, both mono- and polyunsaturated fatty acids, increased as the temperature increased. This is clearly a species-specific response, which may also depend on the preferable growing condition of the alga. The unexpected cell response at high temperature was probably due to the differences in the optimum growth temperature of the algae. The algae tested in the other studies have relatively low optimum temperature, whereas our alga has high optimum temperature for growth (35ºC). The results will possibly be different if the tested temperature is much lower i.e below 20ºC. As pointed out by Piepho et al. (2011) changes in biochemical composition of microalgae are less likely to occur under optimum growth conditions. Major changes in fatty acid composition would only be expected if the temperature was clearly sub-optimal. For example, Jiang and Chen (2000) reported that changing the temperature from 25°C (for 48 h) to 15°C (for 24 h) resulted in an 19.9% increase in cellular DHA content and a 6.5% increase in productivity of *Crypthecodinium cohnii* compared to that maintained at 25°C (for 72 h). Similarly, Rousch et al. (2003) who studied the effect of heat stress on the fatty acid composition of a thermo-intolerant (*Phaeodactylum tricornutum*) and a thermo-tolerant (*Chaetoceros muelleri*) marine diatom found that both short duration (2 h) and long duration heat-treatments (24 h) affected the levels of fatty acids to a greater degree in *C. muelleri* than *P. tricornutum*. In addition, irrespective of the treatments, saturated fatty acids of both diatom species increased with increasing temperature whereas the polyunsaturated fatty acids decreased with increasing temperature.

Although diatoms are well known as PUFAs producers, their fatty acid composition is specific specific. The fatty acids profiles of the *Amphora* sp.MUR258 was unusual compared to most diatom species in that the level of PUFAs was very low less than 5% of TFA. However, several diatoms have also been reported to have similar low level of PUFAs (i.e EPA). For example, EPA content of *Amphora bigibba* in summer and winter were 3.68 and 3.98% TFA, respectively . The EPA contents of *Cocconeis scutellum* in summer and winter were 2.47 and 0.74% TFA, respectively, and the EPA content of *Skeletonema costatum* in summer and winter were 0.47 and 0.31% TFA, respectively (Chen 2012). Low percentages of EPA at exponential phase were also found in the diatoms *Chaetoceros* sp. and *Thalassiosira* sp. (1.95±0.43% and 0.55±0.001%, respectively) (Costard et al. 2012). In contrast, Martin et al (2016; 2018) found that EPA was one of the major fatty acids in *Halamphora coffeaoformis*.

The fact that C16 and C18 were the major fatty acids in *Amphora* sp MUR 258 coupled with a low amount of PUFAs suggests *Amphora* sp. MUR 258 as a potential biodiesel feedstock since these fatty acids are the most common fatty acids contained in biodiesel (Knothe 2013).

In summary, this study has determined some of the key growth factors including temperature, salinity and growth phase affecting the growth, lipid content, lipid productivity and fatty acid composition of the newly isolated halophilic diatom *Amphora* sp. MUR 258. Optimum growth conditions were 7% salinity and 35^{o}C. High growth rate and high lipid content at exponential phase resulted in higher lipid productivity. The high biomass productivity (0.171±0.017 gAFDW L^{-1} day^{-1} at 35^{o}C and 9% NaCl) and high lipid productivity (0.062±0.017 g*AFDW* L^{-1} day^{-1} at 25^{o}C and 7% NaCl) makes this algal strain attractive for mass culture. Furthermore, the ability to grow at high salinities is another advantage as it limits potential contamination since not many microorganisms can tolerate such high salt concentrations. The predominant fatty acids of *Amphora* sp. MUR 258 were C16 and C18 indicating its potential as a suitable raw material for biodiesel production. This information is useful for further research aiming to optimise its productivity as well as to tailor its fatty acid composition to different applications.

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