

Fatty Acid Composition in Cultured *Amphiascoides neglectus* (Copepoda: Harpacticoida)

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Abstract. This study investigates the effect of salinity on the fatty acid profile of a harpacticoid copepod *Amphiascoides neglectus* exposed to constant laboratory lighting and temperature (25-26°C) at pH7 for 40 days. The results were compared with those cultured in the common open hatchery environment which is almost the same as in the natural ecosystem. Different fatty acid levels were determined using Gas Chromatography and Mass Spectrometry (GC-MS). Results showed that there were no significant differences ($p > 0.05$) in fatty acid content for both 20ppt and 30ppt treatment but the percentage of EPA was higher in 20ppt. Harpacticoid copepods in the hatchery showed significantly ($p < 0.05$) more fatty acids in 30ppt than 20ppt. EPA was also higher in harpacticoids cultured under 30ppt. Manipulation of salinity and temperature in combination of light factor for copepod rearing system could improve the deposition of fatty acids in their body.

Keywords: Harpacticoid culture, fatty acid, EPA, culture environment

1 Introduction

Harpacticoid copepods fit the criteria of being a model animal by having a short generation time, being flexible in diet and tolerant towards a wide range of environmental factors [1][2][3]. They also have a small adult size [4][5], as well as being readily available by having their population widespread across the globe and capable of producing large amounts of offspring in a short time. They were reported to be the major attraction for small fishes to enter the intertidal zone during high tide [6]. They are highly adapted in many coastal ecosystem [7] thus become the most available diet for fish larvae [8].

In the field of aquaculture, temperate harpacticoid copepod is well recognized as important live feed for newly hatched larval of fishes and shrimps in hatchery. Cutts [9] confirmed on the superiority of harpacticoid over artemia as early diet for larvae due to the ability to synthesize EFAs. *Tisbe biminiensis* was reported to be able to bio-convert fatty acid C18:0 into chains with 20 unsaturated carbons [10].

The concentration of fatty acids was reported as dependent to the environmental factors in the water body. A temperate harpacticoid, *Platychelipus littoris* showed a high degree depletion in fatty acid storage when exposed to elevated temperatures [11]. Low temperature (below 15°C) on the other hand could trigger stress in lipogenesis process thus retards growth in copepods [12]. Theoretically, increase in temperature and warm condition would cause the salinity of the seawater to be increased as the evapotranspiration process would occur. Therefore, the fatty acid composition in copepods would have changed as well as an adaptation to the changing environment [13]. The different in diet was also noted to affect the fatty acid composition [14]. Nonetheless, some copepod species could have the capability to adjust their PUFA depending on the environmental variation unless the sudden environmental changes occurred [15].

Mimicking the natural environment in investigating the role of environmental changes towards the fatty acid content in tropical harpacticoid copepods could improve our understanding on the survival of copepods in tropic. The consistent warmer condition and more daylight could somehow play a role in the accumulation of fatty acid in the tissue. The aim of this research is to determine the fatty acid composition in harpacticoid copepod *Amphiascoides neglectus* cultured under several conditions in laboratory and hatchery.

2 Materials and Methods

2.1 Stock Culture of Harpacticoid

Stocks of benthic harpacticoid copepods for this study were collected from a copepod culture that was previously maintained in the hatchery in our research station. Harpacticoid copepods were separated from other organisms by using standard laboratory sieves with a mesh size of 62 microns. Samples were acclimated in a stock culture with autoclaved sea water of 30 ppt salinity at pH 7 for a period of one month, and was fed daily with 1 mL of baker's yeast (0.02 g L^{-1}) [16].

Stock samples were also acclimated to ambient temperature, which in this project is defined as temperature ranging between 25°C to 26°C. This was made by maintaining the air-conditioner at 25-26°C during the experiment period.

2.2 Experiment 1: Effect of Salinity onto Harpacticoids under a Laboratory Condition

Several copepod individuals were transferred from the stock culture to serially arranged glass beakers of 100 ml each. Each beaker contained a number of 100 individual copepods. Water quality parameters were maintained under ambient temperature at different salinity of 5, 20, and 30 ppt in replicates of three. Hand-held refractometer (ATAGO) was used to measure the desired salinity. The experiment was then repeated under different temperatures of 20°C and 28°C. All cultures were maintained at pH 7. Each of the glass beakers was covered with aluminium foil in order to prevent contamination. The cultures were done entirely under laboratory conditions, and were exposed to artificial light sources for 24 hours per day.

Temperature for each culture was maintained using air-conditioned rooms at 20°C and 28°C, while cultures for ambient temperature are placed in an air-conditioned room operating between 25°C to 26°C. Each culture was fed daily with 1 mL of baker's yeast (0.02 g L^{-1}). After 40 days of experimental period, the samples were prepared for counting and lipid extraction. Counting of density was carried out under a stereo microscope (Leica).

2.3 Experiment 2: Effect of Salinity onto Harpacticoids under a Hatchery Condition

Glass beakers of 250 mL were marked for different salinity of 30ppt and 20ppt. Five replicates beakers were prepared in order to produce large number of copepods. Using a Pasteur pipette, approximately 100 individuals of copepods were transferred from stock culture into each beaker. Filtered sea water and distilled water were added until 150mL and until desired salinity were reached. The cultures were maintained at common temperature in the hatchery and at pH7. Photoperiod was following the natural environmental condition. Each beaker of copepods was fed with 1mL of diluted baker's yeast daily in 40 days period before lipid extraction.

2.4 Experiment 3: Fatty Acids Extraction and Determination from Harpacticoid

The method used was modified Dole's extraction method [17]. About 200 to 300 individuals of harpacticoid copepods were taken from the cultured copepods and were put into micro-centrifuge tubes. The samples were then freeze dried for 2 days. Meanwhile, extraction solvent was prepared by mixing iso-propanol-n-heptane-phosphoric acid (40:10:1, v/v) and was thoroughly stirred. After that, the freeze dried samples were used for fatty acid analysis. Each sample was transferred from micro-centrifuge tubes into screw-capped test tubes. Then, the samples were mixed with 2.5mL of extraction solvent and the tubes were thoroughly vortexed. The tubes were then immersed in a sonicator water bath and were sonicated in 30 seconds intervals for 2 minutes. The samples were then vortexed rigorously and allowed to stay at room temperature for 10 minutes. 1mL of heptane and 1.5mL of distilled water were added and the tubes were thoroughly vortexed and sonicated again for 1 minute. Tubes were centrifuged at 1,000 rpm for 10 minutes at 4°C. 1.5mL aliquot of the top layer was transferred carefully using micropipette to another test tube and dried under a stream of nitrogen using nitrogen generator leaving lipid sample in it.

Methylation method is carried out by acid methylation following Ichihara and Fukubayashi [18]. 9.7mL of 35% HCl was diluted with 41.5mL of methanol to make 50mL of 8% HCl also known as HCl-methanol reagent. The lipid samples were dissolved in 0.2mL of toluene. Into the lipid solution, 1.5mL

of methanol and 0.3mL of the 8% HCl solution were added respectively. The test tube was vortexed and was heated at 95°C for 1 hour in water bath. After cooling to room temperature, 1mL of hexane and 1mL of water were added into the samples. The tubes were vortexed, and then the hexane layer was transferred into another tubes followed by drying under stream of nitrogen. The lipid left was diluted with 100µL of hexane, transferred into vials. The concentrated sample was then injected into Gas chromatography-mass spectrometry (GC-MS) to read the spectra using caprylic acid ($\text{CH}_3(\text{CH}_2)_6(\text{COOH})$) as an internal standard.

Percentage of fatty acid detected in each treatment was expressed in Mean±SD. Analysis of Variance (ANOVA) test was used to check the difference in FAs percentage between treatments. The names of fatty acid displayed by the library were recorded. Area under the peak of chromatogram was used to calculate the percentage of each fatty acid. The percentage is used to determine the levels of fatty acids in copepod samples.

2.5 Statistical Analysis

Data analysis was done using a software IBM SPSS Statistics 20 and Microsoft Office Excel 2013. Data were tested for their mean in Microsoft Office Excel 2013. Using the IBM SPSS Statistics 20, normality test was perform using Shapiro Wilk Test, the variance of homogeneity was tested by using Levene's Test, and significant difference (P-value) by using Wilcoxon-Mann-Whitney Test.

3 Results

3.1 Population Density of *A. neglectus* under Laboratory Condition

The population density of *A. neglectus* was calculated by observing and counting all the living individuals present in each culture. The mean number and standard deviation for each treatment of different salinity and temperature was calculated, and the results were illustrated in Figure 1. The *A. neglectus* samples were unable to survive in 5 ppt cultures.

There was a statistically significant effect ($p < 0.05$) of salinity on the mean number of copepod density ($p=0.000369$). However, there was no significant effect ($p > 0.05$) of temperature on the mean number of copepod density ($p=0.391489$). The two-way ANOVA results also indicated that there is no significant interaction ($p > 0.05$) between salinity and temperature ($p= 0.699673$). Since there was no significant effect of the chosen temperatures to copepod's density, the lipid analysis was carried out using those cultured under the ambient temperature.

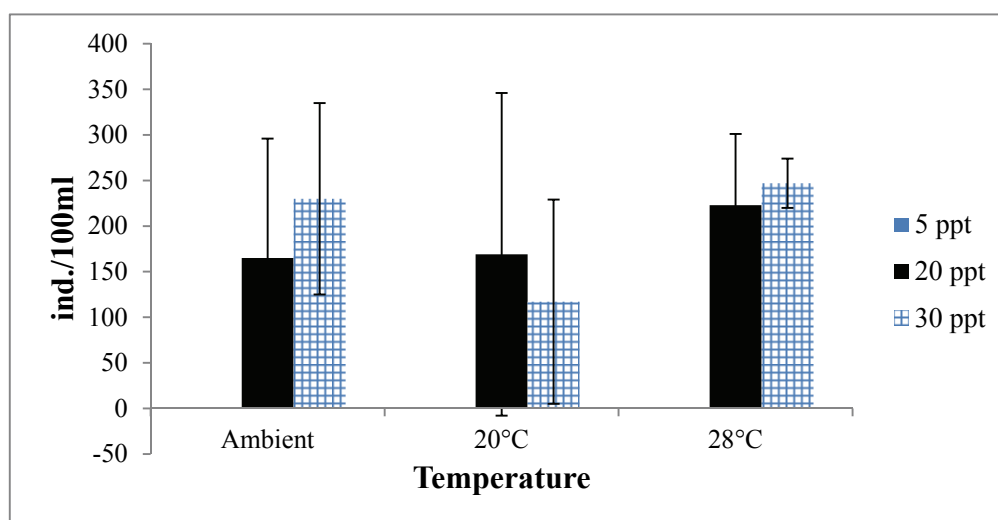


Figure 1. Mean number of *A. neglectus* under the influence of salinity and temperature in laboratory condition.

3.2 Fatty Acids Composition in *A. neglectus* for 20ppt and 30ppt Salinity at Ambient Temperature in the Laboratory

The percentage of fatty acid composition in *A. neglectus* samples were summarized and listed in Table 1. Lipid numbers are written in the form of *C:D*, where *C* is the number of carbon atoms present in the fatty acid and *D* is the number of double bonds present in the fatty acid. Fatty acids composition in *A. neglectus* copepods were characterized into saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), omega-3 polyunsaturated fatty acid (n-3 PUFA) and omega-6 polyunsaturated fatty acid (n-6 PUFA) to determine the total fatty acids percentage. The high polyunsaturated fatty acids (HUFA), arachidonic acid was only found in 30ppt treatment, while eicosapentaenoic acid occurred in high percentage in 20ppt treatment. Linoleic acid was 50% higher in 30ppt than the 20ppt treatment. Nonetheless, in overall, there is no significant effect of salinity on the percentage of fatty acids in harpacticoid cultured in the laboratory (Table 2).

Table 1. The list of fatty acid composition in the lipid extract of *A. neglectus* for both 20 ppt and 30 ppt salinity at ambient temperature in the laboratory

Types of Fatty Acid	Lipid Numbers	Percentage (%)	
		20 ppt	30 ppt
Myristic acid	14:0	8.19	7.29
Myristoleic acid	14:1	6.54	3.89
Palmitic acid	16:0	27.82	-
Linoleic acid	18:2	15.69	33.93
Oleic acid	18:1	0.33	14.68
Arachidic acid	20:0	1.35	11.38
Arachidonic acid (AA)	20:4	-	4.98
Palmitoleic acid	16:1	9.2	14.05
Eicosadienoic acid	20:2	14.73	7.46
Eicosapentaenoic acid (EPA)	20:5	16.14	2.33

Table 2. Statistical analysis for total fatty acid percentage in the lipid extract of *A. neglectus* for both 20 ppt and 30 ppt salinity at ambient temperature in the laboratory

Types of Fatty Acid	Significance	Mean Percentage (%)± SD	
		20 ppt	30 ppt
Saturated Fatty Acid (SFA)	N.S.	37.36±6.93	18.67±1.51
Monounsaturated Fatty Acid (MUFA)	N.S.	16.07±2.29	10.87±3.17
Total Omega Polyunsaturated Fatty Acid (PUFA)	N.S.	46.56±0.36	48.70±7.67
Omega-3 Polyunsaturated Fatty Acid (n-3 PUFA)	N.S.	16.14±0.30	12.33±2.21
Omega-6 Polyunsaturated Fatty Acid (n-6 PUFA)	N.S.	30.42±0.34	46.37±8.40

N.S. = No significance, * = $p < 0.05$ and ** = $p < 0.01$

3.3 Fatty Acids Composition in *A. neglectus* for 20ppt and 30ppt Salinity at Ambient Temperature in the Hatchery

Table 3. Mean percentage composition of essential fatty acids detected in harpacticoid copepods (*A. neglectus*) treated with two different salinities in hatchery condition.

No.	Trivial Name	Structure Name (Essential Fatty Acid)	Molecular Formula	Salinity		P-value
				30ppt	20ppt	
1	Arachidonic acid	5,8,11,14-eicosatetraenoic acid, (all-Z)	C ₂₀ H ₃₂ O ₂	0.04±0.06	-	0.317
2	Timnodonic acid	cis-5,8,11,14,17-eicosapentaenoic acid	C ₂₀ H ₃₀ O ₂	0.01±0.02	-	0.317

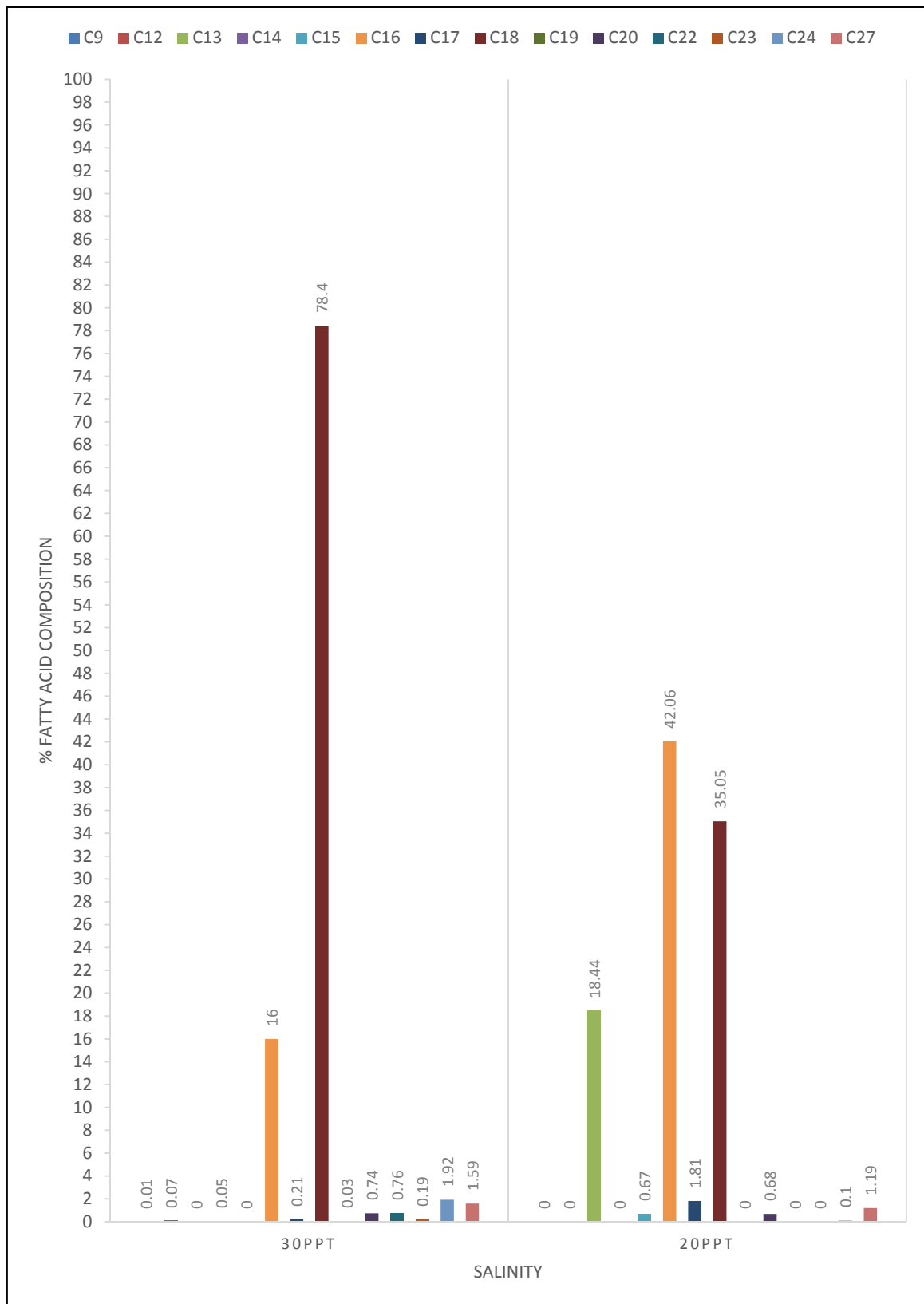


Figure 2. Comparison of percentage fatty acid composition (%) in *A. neglectus*. cultured in two different salinities under hatchery condition

EFA presented in a small amount in the harpacticoid cultured under hatchery condition (Table 3). Two EFAs were detected in this study but with very low concentration (0.01-0.04%) which is lower than the percentage found under the laboratory culture. Both of them which are arachidonic acid and timnodonic acid and they were detected only in salinity 30ppt under the hatchery condition.

There were more fatty acids detected in the samples treated with salinity 30ppt compared to 20ppt (Figure 2). The significant difference between treatments was observed for the fatty acid composition. It was observed that there were 3nEFAs with significantly varying concentration ($p < 0.05$); Azelaic acid, Erucic acid, Linoleic acid (2, 5-octadecadiynoic acid). Other fatty acids were dispersed in different treatments of varying percentages. The expression of C_{18} in salinity 30ppt showed highest composition percentage with more than 60% followed by C_{16} with approximately 20% while others were recorded with less than 2%. In contrary, the highest percentage fatty acid compositions in salinity 20ppt were C_{16} followed by C_{18} with approximately 40% and 30% respectively. Others fatty acids were recorded with less than 2% of fatty acid composition except for C_{13} with percentage of approximately 20%.

4 Discussion

There have been several reports that indicate the capability of marine copepods to survive on a low salinity such as 5ppt, but on the condition that they were acclimatized properly [19][20]. In contrary, the effectiveness of culture condition of higher salinity regime where a harpacticoid copepod, *Amphiascoides atopus* is found to grow best in salinity range of 25 to 35ppt was reported [21]. A tropical harpacticoid *Nitocra affinis* showed the maximum growth in 35 ppt compared to lower salinity [22]. The insignificant differences found in the density when cultured in 20ppt or 30ppt could indicate the optimal salinity for the harpacticoids to grow. A harpacticoid, *Pararobertsonia* sp., cultured in its optimal salinity and temperature underwent only minimal amount of stress, leading to a higher expression of fatty acids [3].

It was reported that organisms living in a high salinity environment are likely to increase their PUFA content in order to raise membrane fluidity at high salt concentrations [23]. Both cultured in 20 ppt and 30 ppt salinity contained high amount of PUFAs, which can be attributed to *A. neglectus* adapting in a high salinity environment. EFAs are crucial in stabilizing a healthy ecosystem and are primary source of natural availability of EFA [24]. Other fatty acids also play important rule, such as those in the exoskeleton which could give the intra-specific recognition in *Artemia* species [25]. The low concentration of several FA found in this study could possibly relate to the diet, in this study all harpacticoids were having yeast as their diet which is known for the low nutrient content if compared to microalgal diet [26].

Photoperiod has shown its effect on fatty acid content [27] and also marine prey [28]. In the hatchery condition lighting was not control and the cultures were exposed more to natural photoperiod. This condition might play a role in managing the expression of fatty acids in the copepod [29]. The natural light exposure during daytime could be different depending on the daily tropical weather condition. The effect could be different from the constant light condition and this might affect the reproduction as well [30]. Further investigation on photoperiod effect would give the answer.

5 Conclusion

A harpacticoid copepod *A. neglectus* was successfully cultured under a laboratory and hatchery condition where salinity was maintained at the salinity of 20 and 30 ppt, pH7 and ambient temperature in the laboratory and hatchery. Exposure to the culture condition in the laboratory with salinity 20 and 30 ppt gave no significant difference ($p > 0.05$) in the composition of fatty acids. Both treatment of 20 ppt and 30 ppt salinity contain a very high yet almost equal amount of PUFAs, which can be attributed to *A. neglectus* adapting in a high salinity environment. Nonetheless, the composition was significantly higher for the copepods cultured at the same salinities under hatchery condition. The significant difference between treatments was observed for the fatty acid composition. Hatchery condition with more natural photoperiod might play a role in managing the expression of fatty acids in the copepod.

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