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THE EFFECT OF ENRICHMENT ON THE FATTY ACID COMPOSITION OF *ARTEMIA SALINA*

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ABSTRACT

The initial feeding of small fish larvae is problematic and knowledge of their nutritional requirement is often scarce or lacking. The larvae are small and undeveloped and it is difficult to process nutritionally sound and complete dry micro-feed which is stable in water. Therefore, life feed like rotifers and *Artemia* are still essential part in marine finfish hatcheries. Standard methods have been developed for hatching and enhance the nutritional value of life feed using different enrichment product. There are various available enrichment products in the market but often a lack of knowledge how well they might fit in for a particular fish larva species. The first step is to recognize how well the life feed is absorbing and preserving nutrients from different enrichment product. *Artemia salina* was enriched with four enrichment diets including *Nanochloropsis*, *Isochrysis*, *Pavlova* and Cod liver oil for 12h and 24h. Survival, energy content and fatty acid composition of *Artemia* was determined after 12h and 24h enrichment. Survival of *Artemia* was highest in cod liver oil (95%), followed by Nanochloropsis, *Isochrysis* and *Pavlova*. However, there was not significantly different ($p > 0.05$) in survival rate among the enrichment feeds for both two enrichment time, except for 24h enriched *Artemia* with *Pavlova* (66%). The energy content of both cyst and newly hatched *Artemia* was not significantly different (15 kJ/g, $p > 0.05$). No significant differences ($p > 0.05$) in energy content among cyst, newly-hatched *Artemia* and 12h enriched *Artemia* with all feeds were detected. With 24h enrichment treatment, difference in energy contents of *Artemia* fed *Nanochloropsis*, *Isochrysis*, *Pavlova* and cod liver oil was detected ($p < 0.05$). The higher energy content was found with cod liver oil treatment (19 kJ) compared with the other feeds treatments (from 7.7 kJ to 11.6 kJ). The energy content of *Artemia* enriched with all feeds was not influenced by enrichment time. The results of survival and energy content of *Artemia* in this study indicate that all four enrichment feeds can be used successfully in the rearing of *Artemia* for short term feeding. With fatty acid composition, the results in this study did not allow a clear conclusion for the effect of enrichment on fatty acid composition of *Artemia* because of high un-identified fatty acid compositions.

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1 INTRODUCTION

Brine shrimp *Artemia* is among the most common live feeds used in cultivation of fish larvae and crustacean species due to its nutritional value and convenience to use (Sorgeloos, 1998; Sorgeloos & Dhert, 2001). All the life stages of *Artemia*, i.e. cysts (after decapsulation), nauplii and adult are used for larval cultivation. Generally, *Artemia* contains sufficient levels of protein and the 10 amino acids that are essential for fish larvae (Wan-Loy, 2004). However, freshly hatched *Artemia* contains relatively low levels of long chain poly-unsaturated fatty acids (PUFA), essential fatty acids (EFA), that the fish cannot synthesize and need to get from their food (Sargent, 1997, 1999). The improvement of biochemical composition of *Artemia* is thus considered a key issue in larval cultivation using live food.

1.1 Aquaculture in Vietnam

Vietnam aquaculture production has been increasing gradually from 2000 to 2012 to keep up with the demand of the increasing human population (Figure 1). Total aquaculture production reached 3.12 million metric tonnes (MT) in 2012 (General Statistic Office, 2013). About 68% of the total aquaculture production mainly came from fresh water fish (2.12 MT) in which *Pangasius* accounted for 52% of total fresh water fish production. Production of brackish and seawater fish accounted for 34% (1.1 MT) of total aquaculture production (General Statistic Office, 2013). Brackish shrimp and *Pangasius* are main species contributing the development of the national economy.

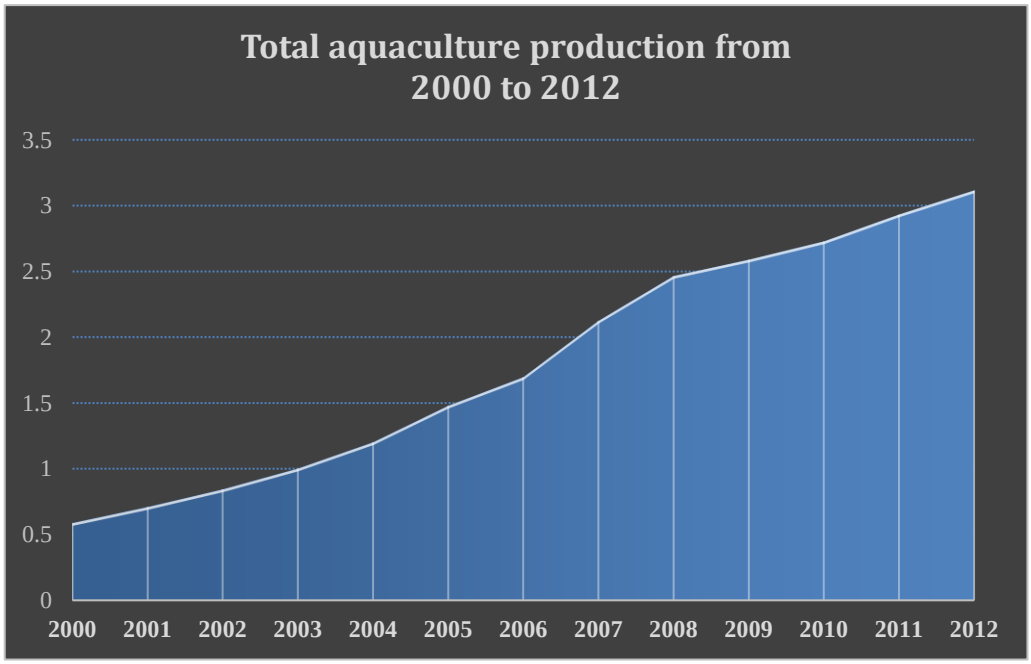

Figure 1: Vietnam total aquaculture production from 2000 to 2012.

Although the aquaculture production has been increasing year on year, aquaculture activities are facing a number of challenges in recent years. Some of the challenges are related to climate changes, degraded water quality leading to the spread of diseases, unstable brood stock quality and survival rate of new cultured species. The outbreak of diseases in shrimp farming is one of main reasons leading the expansion and diversity of cultured fish species in aquaculture. There are not only traditional fish as cobia *Rachycentron canadum*, sea bass *Lates calcarifer*, tilapia *Oreochromis* but also some new fish species such as pompano *Trachinotus blochii* and gopy *Pseudapocryptes elongates* are becoming important species for aquaculture. The new fish species, especially finfish species, is predicted to be the main cultured species contributing to the growth of Vietnam aquaculture in future.

Pompano is among of promising marine finfish species due to fast growth rate, good meat quality and high market value (Hung, 2013; Wang, 2013). Pompano, an emerging fish species, has been cultivating in Asia Pacific countries like Taiwan, Indonesia and Vietnam. Pompano larvae is cultivated successfully for the first time at Khanh Hoa province in 2011 (Hung, 2013). The culture area of pompano is increasing and mainly in central and southern Vietnam. The production of pompano in Vietnam is still unstable and faces with challenges regarding nutrient issue due to limited information about nutrient requirement for larvae cultivation (Hung, 2013). Therefore, the suitable diets for finfish generally and pompano in particular is one of the important research fields in Vietnam.

1.2 *Artemia* in aquaculture

Artemia has been seasonally cultivated in coastal salt-works in Vietnam since the 1990s to produce cyst for aquaculture activities (Anh, 2009). *Artemia* culture is now widespread over 1000 ha providing considerable socio-economic benefits (Anh, 2009). Main cultivation activities have been carried out in the coastal area in southern Vietnam (Anh, 1997a; Sy, 2011), while the cultivation in the central part of Vietnam is less developed and mainly carried out in two provinces, i.e. Ninh Thuan and Khanh Hoa (Sy, 2011).

Artemia is not only used as live feed but also to make formulated aquafeed (Hai & Nhat, 2008; Hoa, 2007). *Artemia* is considered to be a suitable ingredient for replacing fishmeal in aquafeeds because of its protein level and low production cost (Anh & Wille, 2011). High quality feeds used for aquaculture in Vietnam currently depend on fish meal (Edwards, 2004). Research on the supplement or replacement of fishmeal with frozen or dried adult *Artemia* in diets of some penaeid shrimp and giant freshwater prawn has been carried out (Anh & Hien, 2009; Naegel, 2004). The researchers indicate that formulated feed with *Artemia* is suitable for larval cultivation. *Artemia* is thus becoming a promising feed in the cultivation of aquatic species, which may reduce the use of fishmeal in feeds.

Artemia has been shown to be excellent feed for fish larvae and shrimp cultivation (Anh & Wille, 2011; Duy, 2013; Le, 2008; Sorgeloos, 1998), for mud crab (Anh & Quynh, 2011) and ornamental fish (Lim, Dhert, & Sorgeloos, 2003). Studies to develop optimal growth conditions for *Artemia* in Vietnam have mainly focused on the biomass, growth and survival rate of *Artemia* (Anh, 1997a, 1997b; Anh & Wille, 2011; Duy, 2013). Few experiments have been conducted on lipid and fatty acid composition of *Artemia* (Anh, 2009; Sy, 2011).

1.3 Objectives of project

The general objective of project is to observe the effect of different enrichments on the fatty acid composition of *Artemia salina*.
The following goals of this project are:

(a) To determine fatty acid composition and energy concentration of *Artemia salina* grown under different enrichments including diet and feeding time.
(b) To suggest suitable *Artemia salina* enrichment methods for marine fish species in Vietnam generally and finfish species such as pompano.
(c) To study method of fatty acid composition and energy content analyses.

2 LITERATURE REVIEW

2.1 The requirement of polyunsaturated fatty acids (PUFA) of marine fish larvae

The PUFA in lipid are among the most essential feed ingredients for growth and development of marine fish, particularly their larval stage (Hamre & Yúfera, 2013; Watanabe, 1993). These essential fatty acids (EFA) are eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6). EPA and DHA concentrations are high in the neutral and visual membrane that fish cannot synthesize (Rainuzzo, 1997). ARA may improve larval growth and pigmentation in several marine fish species since it provides precursors for eicosanoid production (Bell & Sargent, 2003). Therefore the absence of these EFAs from the diet leads to the reduced growth and increased mortality of fish (Glencross, 2009; Sargent, 1997).

The nutritional requirement for the essential fatty acids of fish species is different among types of fish species, for example marine *vs* fresh water fish (Bowyer, Qin, & Stone, 2013; Rainuzzo, 1997; Sargent, 1999). Freshwater species generally require C_{18} PUFA whereas marine fish have a strict requirement for long-chain PUFA such as EPA, DHA and ARA (Tocher, 2010). In contrast to freshwater species, most marine organisms does not have the capacity to biosynthesize essential fatty acids (EFA) from lower chain unsaturated fatty acids, such as linolenic acid (18:3n-3) and linoleic acid (18:2n-6) due to the low or negligible Δ-5 desaturase activity (Figure 2) (Sargent & Bell, 1999; Tocher, 2010). They therefore require long chain HUFA and PUFA in their diet.

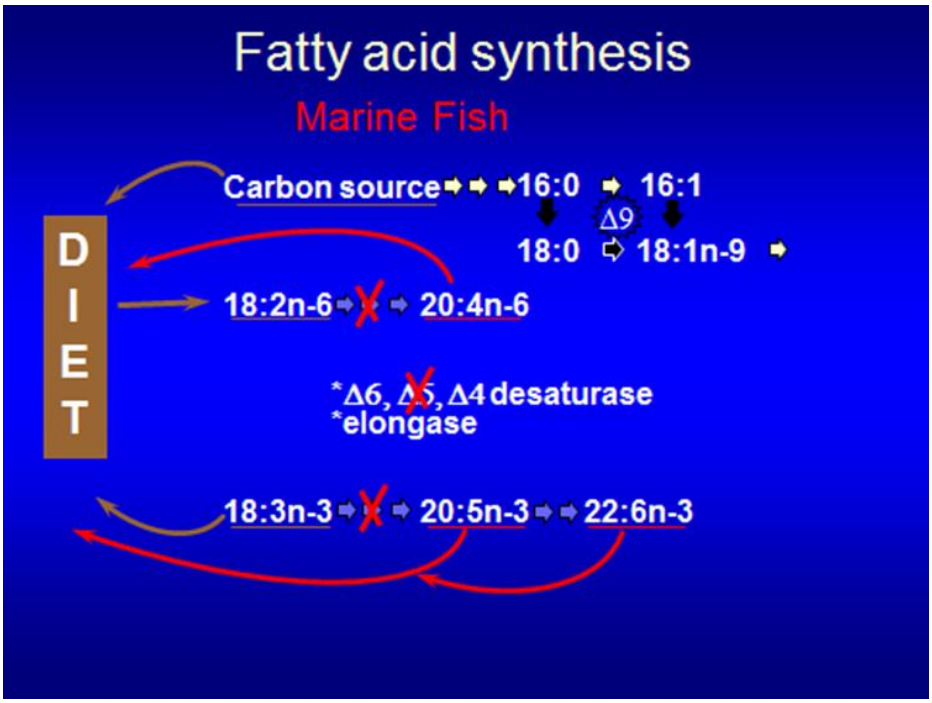

Figure 2: The inability of marine fish to biosynthesize essential fatty acids (EFA) from lower chain unsaturated fatty acids, such as linolenic acid (18:3n-3) and linoleic acid (18:2n-6).

Both the concentration and ratio of all three essential PUFA are important in larval marine fish nutrition (Tocher, 2010). The optimum ratio of DHA:EPA:ARA is species specific. A ratio of DHA:EPA equal or greater than 2 has been considered adequate for many marine fish studied (Sargent, 1999). Some authors suggested nutritional requirements of sea bass, halibut, and turbot eggs and larvae (Sargent, 1999). The optimal dietary ratio of EPA:ARA was approximately 1: 1 in sea bass larvae, but may be as high as 10**:** 1 for turbot and halibut (Sargent, 1999). In some recent studies, the EPA:ARA ratio of 4 was recommended for the growth of gillhead seabream and European seabass larvae (Atalah *et al*., 2011a; Atalah *et al.*, 2011b). Villalta (2005) reported that Senegalese sole gave 100% normal pigmentation at a ratio of EPA:ARA of 2.

Little is known about the EFAs requirement of many marine finfish species from subtropical/tropical waters, as most of them, as cobia and pompano, are emerging species in aquaculture (Hauville & Main, 2014). As a general trend, these finfish species tend to need intermediate to high DHA and ARA levels and low EPA level in their diet, leading to high DHA:EPA and ARA:EPA ratios in their diet (Hauville & Main, 2014; Ogata & Emata, 2004; Yanes-Roca & Rhody, 2009). Cobia juvenile is assumed to require high amounts of DHA, EPA and ARA because these account for approximately 80 - 90% of the PUFAs in cobia eggs and yolksac larvae (Faulk & Holt, 2003; Fraser & Davies, 2009). The ratios of DHA:EPA of 3,3:1 in yolk-sac larvae of cobia and were within the range of other larvae of marine fish and the ratio of EPA:ARA of 2,2:1 in cobia yolksac larvae was found to be lower than typically reported for marine species due to the higher concentration of ARA (Fraser & Davies, 2009). Regarding pompano, research on the requirement of essential fatty acids of pompano is still scarce. Egg of Florida pompano species have a high ARA:EPA ratio (Hauville & Main, 2014). Due to fish eggs contain all the essential nutrients required for the successful development of the embryo and the yolk-sac larvae, their composition can reflect the optimal first feeding diet (Hamre & Yúfera, 2013; Heming & Buddington, 1988).

2.2 Factors effect *Artemia* fatty acid composition, growth and survival

2.2.1 Enrichment

Enrichment is one of the main factors affecting biochemical composition, especially fatty acid composition of *Artemia*. The fatty acid content and profile of newly-hatched nauplii (instar I) are not affected by diet or environmental conditions because instar I nauplii does not take up food and replies completely on its yolk-sac (Wan-Loy, 2004).

Several studies have been revealed that the change in fatty acid composition of *Artemia* is influenced by types of diet and enrichment time (Chakraborty, 2007; Dhert & Sorgeloos, 1993; Han, 2001; Han, Geurden, & Sorgeloos, 2000; Sorgeloos & Dhert, 2001; Viciano & Monroig, 2013). Enrichment diets (live algae, algal paste, high PUFA-containing lipid emulsion and oils) and enrichment time (12h, 24h, 36h and 48h) have been commonly used in *Artemia* cultivation (Arumugam & Inbakandan, 2013; Chakraborty, 2007; Figueiredo & Woesik, 2009; Han et al., 2000; Narciso & Pousão Ferreira, 1999). Chakraborty (2007) reported that DHA, EPA and total PUFA of the enriched *Artemia* by different three microalgae for both 12h and 24h was higher than that of newly-hatched *Artemia*. However, the decrease of these fatty acids content was observed after 24h enrichment compared with 12h enrichment. Figueiredo and Woesik (2009) examined the effect of enrichment time (0h – 24h) on fatty acid profile of *Artemia franciscana.* The fatty acid content, especially DHA, was decreased with rising enrichment time and DHA content was lowest in enrichment time of 24h. However, another research showed that the PUFA content and DHA:EPA ratio of *Artemia* enriched with some commercial oils for 36h was highest compared with that of *Artemia* enriched for 9h, 24h and 48h (Narciso & Pousão Ferreira, 1999).

It has been suggested that food concentration effects growth and fatty acid profile of *Artemia.* Because *Artemia* is a continuous filter-feeding organism, highest growth is achieved when food is distributed as frequently as possible. Therefore, concentrations should be maintained above the critical minimum uptake concentration, which is specific for feeds and the developmental stage of *Artemia* (Dhont & Stappen, 2003).

Some studies about suitable feed concentration for *Artemia* have been undertaken (Han *et al.*, 2000; Olsen & Jensen, 1997). Research of Olsen and Jensen (1997) have been conducted to test the change in DHA content of *Artemia* enriched with the alga *Isochrysis galbana* which is rich in DHA at six concentrations ranging from 1 to 20 mg carbon/L. They revealed that enriched *A. franciscana* should not be incubated with high algal concentrations (> 6 mg carbon/L). With the experiment of Han et al. (2000), *Artemia* was cultivated with 5 different enrichment concentrations (0.05, 0.1, 0.2, 0.3 and 0.4 g/L) of high HUFA emulsion for 12h and 24h. The highest enrichment levels of DHA, EPA and total n-3 PUFA were observed at 0.4 g/L for 12h enrichment and 0.3 g/L for 24h enrichment.

2.2.2 Temperature, salinity and light intensity

Temperature influences on *Artemia* growth and survival (Lavens, 1991). A range of 6-30^{o}C and 30-110 ppt is favorable condition for most *Artemia* strains (Lavens, 1991). Studies have been conducted on the effects of temperature and salinity on survival, life span and reproductive characteristics of *Artemia* strains (Barata, 1996; Barata, Hontoria, & Amat, 1995). Barata (1996) showed that *A. tunisiana* died before reproductive maturity at 30°C.

There is often a complex co-relationship between temperature and salinity, where temperature can modify the effects of salinity, thereby changing the salinity tolerance range of an organism, and salinity can modify the effects of temperature accordingly (Browne & Wanigasekera, 2000). The combined effects of temperature and salinity on survival and fatty acid composition have been investigated in *Artemia* by researchers (Browne & Wanigasekera, 2000; Figueiredo & Woesik, 2009). Browne and Wanigasekera (2000) reported that interaction between temperature and salinity influenced survival of *Artemia franciscana*. Figueiredo and Woesik (2009) tested the effect of temperature (16-28°C), salinity (3-33 ppt) and enrichment time on survival, growth and fatty acid profile of *Artemia*. It was revealed that both temperature and salinity influenced survival, growth and fatty acid level of *Artemia*.

Light has been also mentioned as a factor influencing on survival of *Artemia* (Asil & Fereidouni, 2013; Sorgeloos, 1972). Sorgeloos (1972) indicated that the growth of *Artemia salina* was not significantly difference under the light intensity of 400 to 5.000 lux, but the growth rate of *A. salina* was higher in complete darkness condition than in continuous illumination. Research of Asil and Fereidouni (2013) conducted with nauplii of *A. urmiana* cultivated at four illuminations (0, 100, 2000 and 5000 lux) with 14:10 (light:dark) photoperiod. Higher growth and survival of *Artemia* were observed at higher light intensities compared with darkness or 100 lx.

2.2.3 Cultivation density

Density may be one of factors influencing *Artemia* growth and survival. Suitable densities for enrichment are around 100 nauplii/ml (for enrichment periods that may exceed 24h) and up to 300 nauplii/ml (maximum 24h enrichment period) (Lavens & Sorgeloos, 1996). When high densities are incubated, culture condition becomes suboptimal such as degraded water quality and low individual food availability. These factors influence the growth and survival of *Artemia* (Dhont, Lavens, & Sorgeloos, 1993).

3 MATERIALS AND METHODS

The experiments were carried out at Verid the Holar University College research centre in Saudarkrokur from January to February 2015.

3.1 General experiment set up

The flow chart of the experimental design is presented in Figure 3. The experiment with *Artemia* was carried out with different enrichment durations and diets. Durations of enrichment period were 12h and 24h. Cod liver oil and three instant algae such as *Nanochloropsis*, *Isochrysis* and *Pavlova* were used for enrich *Artemia*. Brine shrimp *Artemia* was cultivated in 12L buckets of seawater at 34‰ salinity and at 28°C with optimum pH level (8-8.5). The light intensity at the surface of the buckets was 1000 lux. A strong aeration was maintained at the bottom to secure mixing of the cultures and to ensure an oxygen level above 70% (about 5 mg O_2 per liter). All experimental treatments were realized in triplicate (Figure 4). The fatty acid composition and energy concentration were measured analytically after harvest.

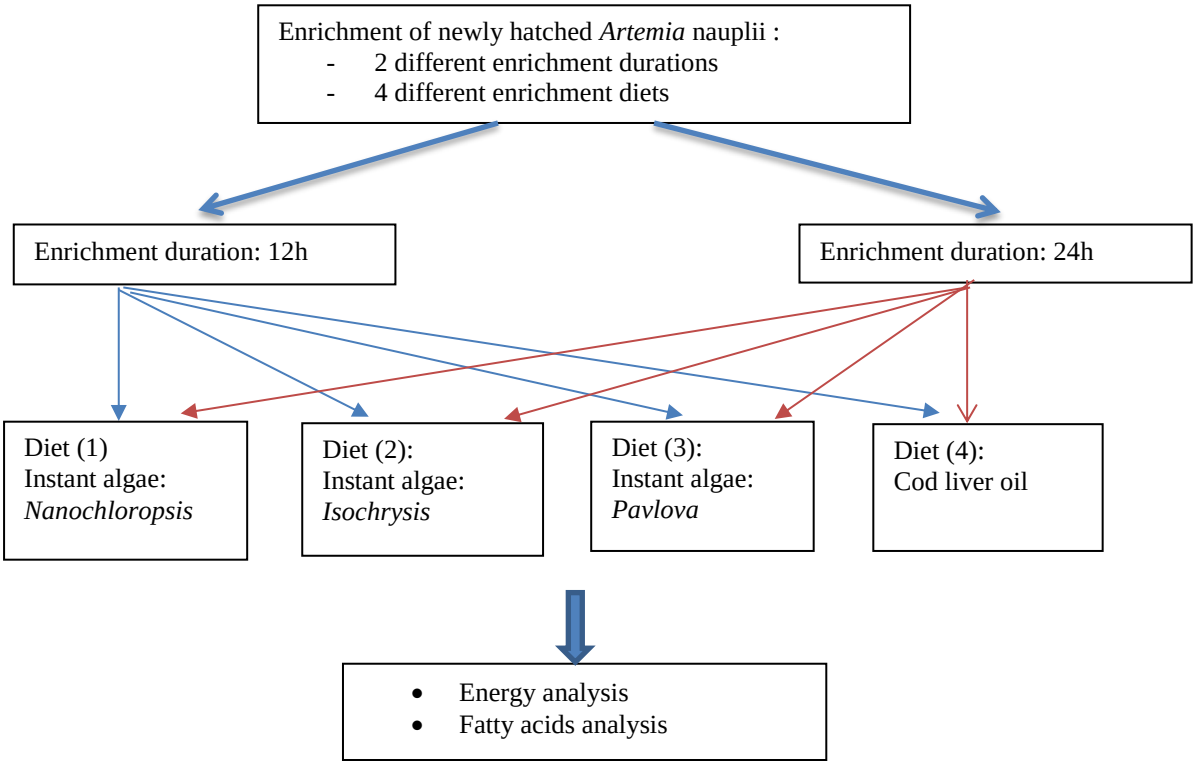

Figure 3: The flow chart of the experiment design

Figure 4: The experiment set up for Artemia enrichment.

3.2 Enrichment diets

Table 1 presents the fatty acid compositions of four enrichment diets expressed in mg/g of dry weight for three instant algae (*Nanoscholopsis, Isochrisys* and *Pavlova*) and in mg/5 ml for cod liver oil. Instant algae *Nanoscholopsis* gives a high EPA and ARA. *Isochrisys* is rich in DHA. Both *Pavlova* and cod liver oil are relative high in DHA and EPA profiles (Figure 5).

The amount of *Nanochloropsis, Isochrysis* and *Pavlova* used for *Artemia* per 12h is shown in Table 2 (adapted from Naegel (1999)). To prepare emulsified cod liver oil, the oil (5ml) was mixed with boiled egg yolk (1g) in 100 ml seawater in order to have small particles that *Artemia* can consume (Figure 6). After mixing, 10ml of the emulsion was added into 1 liter of *Artemia* cultures.

Table 3 presents the energy content of enriched diets expressed in kJ/100g dry weight for three instant algae (*Nanochlosopsis, Isochrysis* and *Pavlova*) and in kJ/5ml for cod liver oil.

Table 1: Fatty acid compositions of four enrichment diets expressed in mg/g of dry weight for three instant algae (Nanochloropsis, Isochrysi and Pavlova) and in mg/5 ml for cod liver oil according to the manufacturers (Reedmariculture and Lysi). ΣSFA is sum of saturated fatty acid, ΣMUFA is sum of mono unsaturated fatty acid, ΣPUFA is sum of poly unsaturated fatty acid.

	Fatty acid composition of instant algae (mg/g of dry weight)			Fatty acid composition of cod liver oil (mg/5 ml)
	Nanochloropsis	*Isochrysis*	*Pavlova*	
ΣSFA	40.86	43.13	48.2	1000
ΣMUFA	43.56	37.43	21	1800
ΣPUFA	70.2	91.01	102	1800
EPA	51.66	3.42	44	350
DHA	0.54	21.28	12.4	650
ARA	4.86	0.76	1.8	0

Table 2: The amount (x10^6 cells) of *Nanochloropsis*, *Isochrysis* and *Pavlova* used for *Artemia* per 12h.

	Amount of algae (x10^6 cells per individual *Artemia*)
Nanochloropsis	0.12
Isochrysis	0.05
Pavlova	0.05

Table 3: Energy content of four enrichment diets expressed in kJ/100g dry weight for three instant algae (Nanochlosopsis, Isochrysis and Pavlova) and in kJ/5ml for cod liver oil.

Energy of instant algae (kJ/100g dry weight of algae)			Energy of cod liver oil (kJ/5 ml)
Nanochloropsis	*Isochrysis*	*Pavlova*	
1.88	1.88	1.67	170

Figure 5: Enrichment diets including Nanochloropsis, Isochrysis, Pavlova and Cod liver oil used for Artemia.

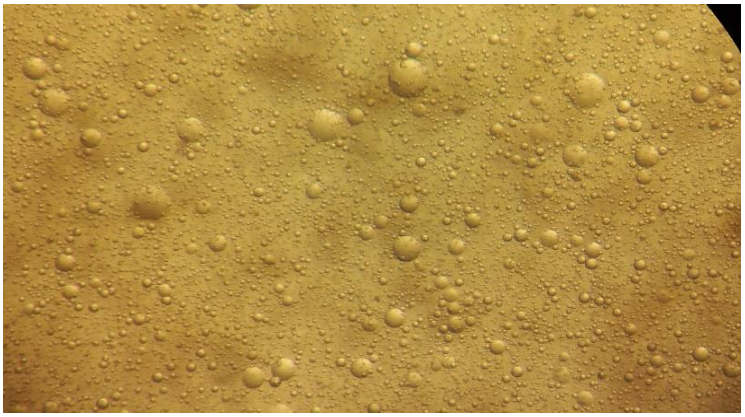

Figure 6: The emulsified cod liver oil with small particles used for Artemia.

3.3 De-capsulation and incubation of the cysts of *Artemia*

Decapsulation is the process whereby the chorion that encysts the *Artemia* embryo is completely removed by a short exposure to a hypochlorine solution (Lavens & Sorgeloos,

1996). The de-capsulation solutions were prepared with hypochlorine (NaOCl), sodium hydroxide (NaOH) and seawater.

The cysts were first hydrated in freshwater for 1 hour at 25°C. After 1 hour of hydration, the hydrated cysts were drained through a 200µm harvest net and transferred into the de-capsulation bucket containing de-capsulation solutions. During treatment time, the temperature was checked regularly and kept less than 30°C. After change in color of the cysts from brown to orange, a splash of de-foam was added into the bucket. The cysts were then transferred to a 200µm net and rinsed in running seawater for 5-7 minutes. The cysts in a harvest net was moved into a bucket added vinegar solution and rinse with seawater to remove and de-active chlorine. The de-capsulated cysts were immediately incubated in buckets for hatching and NaOH was added to keep the optimal value of pH (8-8.5). The density of the cysts in incubation buckets was 4 g/L. All cysts were hatched after 24h incubation. The newly hatched *Artemia* nauplii was collected, washed and separated from the empty shells. The density of newly hatched *Artemia* nauplii was determined before transferred into three enrichment buckets.

3.4 Enrichment of newly hatched *Artemia*

The newly hatched *Artemia* nauplii was cultivated for the enrichment at a density of 300 nauplii/mL. After enrichment period, the survival (%) of *Artemia* must be determined in all buckets. All parameters such as temperature, pH, oxygen, salinity were measured.

- **Experiment 1: 12h enrichment duration**

The *Artemia* nauplii was enriched with instant algae productions and cod liver oil and harvested after 12h (Figure 7).

- **Enrichment 2: 24h enrichment duration**

The *Artemia* nauplii was enriched with instant algae productions and cod liver oil and again after 12h. The enriched *Artemia* nauplii was harvested after 24h (Figure 8).

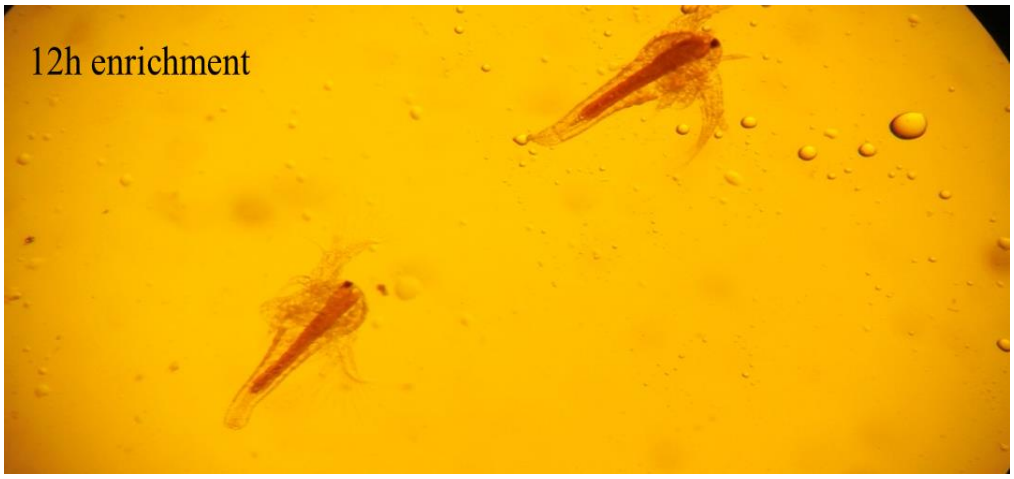

Figure 7: Artemia salina after 12h enrichment with cod liver oil.

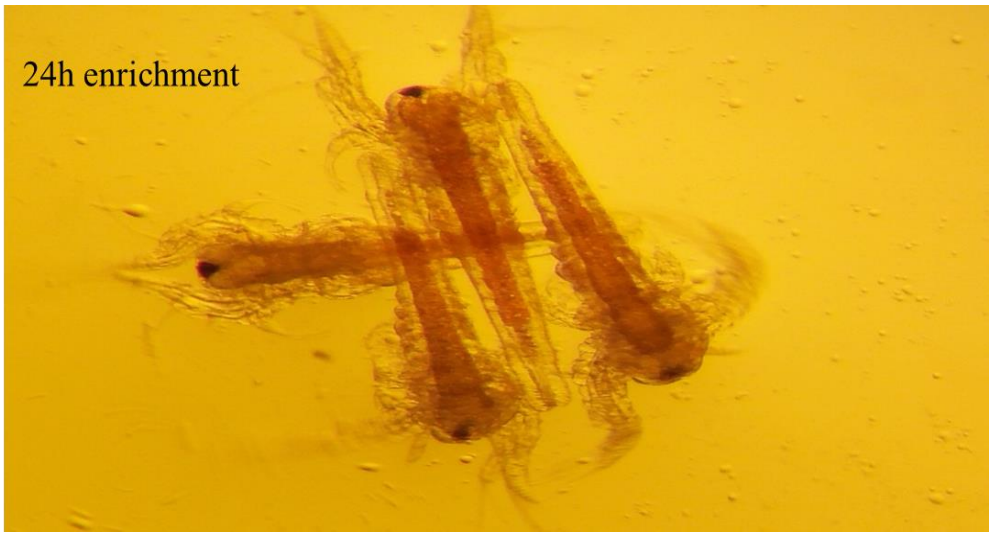

Figure 8: Artemia salina after 24h enrichment with cod liver oil.

3.5 Analytical methods and calculation

3.5.1 Determination of survival of Artemia nauplii

The density of newly hatched and enriched *Artemia* nauplii was assessed by counting the number of nauplii in six 1 mL sub-samples. To ensure that the *Artemia* density was assessed correctly, the sample was taken from different parts of the buckets. The samples must be mixed well and counted for nauplii and unhatched cysts under microscope after fixation with alcohol solution (95%).

The survival of enriched *Artemia* nauplii is calculated by Equation (1):

$$\text{Survival (\%)} = N/N_0 * 100 \qquad (1)$$

Where: N_0 is the initial number of *Artemia* nauplii introducing to the enrichment buckets
N is the number of *Artemia* nauplii after enrichment period.

3.5.2 The fatty acids analysis

Both newly hatched and enriched *Artemia* naupli samples were collected for lipid and fatty acids analyses. *Artemia* was collected on a net of 200 µm and rinsed carefully with seawater. Excess water was removed by putting the net on tissue paper. The samples were put in aluminum paper and kept freeze-dried afterwards and stored at – 80°C under an N2 atmosphere until chemical analysis. The fatty acid composition was determined using an improved method of Ichihara (1996). The fatty acid composition of fat is determined by gas chromatography as fatty acid methyl esters (FAME).

Preparation of FAME from glycerolipids: In a small glass tube were placed 0.5 – 1g of triacylglycerol, 2 ml of hexane, and 0.2 ml of 2 M methanolic KOH. The tube was vortexed at room temperature for 2 min. FAME are separated on a Varian 3900 GC equipped with a fused silica capillary column (HP-88, 100 m x 0.25 mm x 0.20 µm film), split injector and flame ionisation detector fitted with Galaxie Chromatography Data System, Version 1.9.3.2 software.

3.6 Energy analysis

Samples of newly hatched- and enriched *Artemia* were collected and dried in oven at 70^0C for 36 hours. Then, about 10 mg – 25 mg dry weight of samples was used in a bomb filled with oxygen and all organic material burned in the microbomb clorimeter. The rough of energy which is warming up water in the calorimeter is detected and reconverted into Joule (J) per gram ash-free dry weight.

3.7 Statistical analysis of data

All data are presented as mean ± standard error. The experimental data were tested for statistical significance by using one-way analysis of variance (ANOVA) with Holm - Sidak post-hoc multiple comparison test. Differences were considered as statistically significant at $p<0.05$ for all tests.

All the statistical tests are performed by Sigmaplot version 12.5 for windows.

4 RESULTS

4.1 Survival

The survival rate of *Artemia* ranged from 66% to 95% (Figure 9). With two enrichment time treatments, the survival rate of enriched *Artemia* with *Nanonochloropsis*, *Isochrysis* and cod liver oil was not significantly different ($p > 0.05$) and ranged from 77% to 95%. But a statistically significant difference ($p < 0.05$) was detected in *Pavlova* treatment. The survival rate of *Artemia* fed *Pavlova* after 24h (66%) was significantly lower ($p < 0.05$) than after 12h enrichment (86%).

Among four enrichment feeds, no statistical significantly difference ($p > 0.05$) in survival rate was detected after 12h enrichment. However, there was statistic significantly different ($p < 0.05$) in survival rate of 24h enriched *Artemia*. The survival rate of 24h enriched *Artemia* with *Pavlova* was lower ($p < 0.05$) compared with *Nanochloropsis* and cod liver oil.

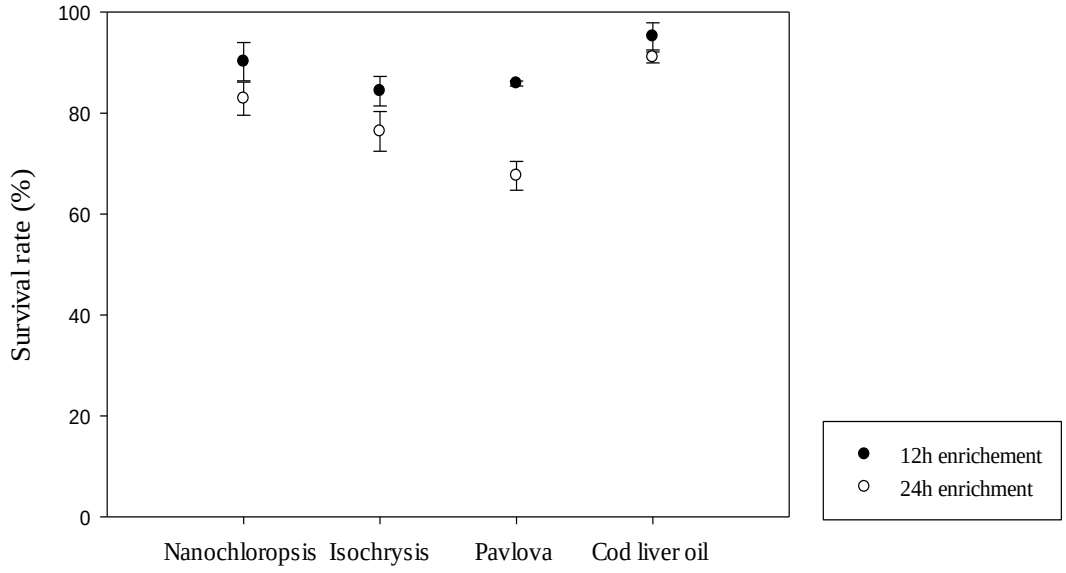

Figure 9: The survival rate (%) of Artemia after 12h and 24h enrichment with Nanochloropsis, Isochrysis, Pavlova and Cod liver oil. Values are mean ± SE of three triplicates.

4.2 Energy content of *Artemia*

The energy content (kJ/g of dry weight) of cyst, newly-hatched *Artemia* and enriched *Artemia* with *Nanochloropsis*, *Isochrysis*, *Pavlova* and Cod liver oil for 12h and 24h is presented in Figure 10 and Table 4. The energy content of cyst and newly-hatched *Artemia* was not statistically significantly different ($p > 0.05$) and about 15 kJ.

For 12h enrichment treatment, the energy content of *Artemia* with all enrichment feeds was from 13 kJ to 19.4 kJ. The highest energy content was found in cod liver oil treatment (19.4 kJ), followed by *Isochrysis* (15.8 kJ), *Pavlova* (14.6 kJ) and *Nanochloropsis* (13.6 kJ). However, no significant differences ($p > 0.05$) in energy content among cyst, newly-hatched *Artemia* and 12h enriched *Artemia* with all feeds were detected.

For 24h time treatment, the energy contents of *Artemia* with *Nanochloropsis*, *Isochrysis*, *Pavlova* and Cod liver oil were 8 kJ, 10 kJ, 11 kJ and 19 kJ, respectively. There was statistical significantly higher ($p < 0.05$) energy content in enriched *Artemia* with cod liver oil compared with three algae treatments. No significant differences were observed ($p > 0.05$) in energy content among three algae treatments.

The energy content of enriched *Artemia* with all feeds was not significantly different ($p > 0.05$) between 12h and 24h enrichment.

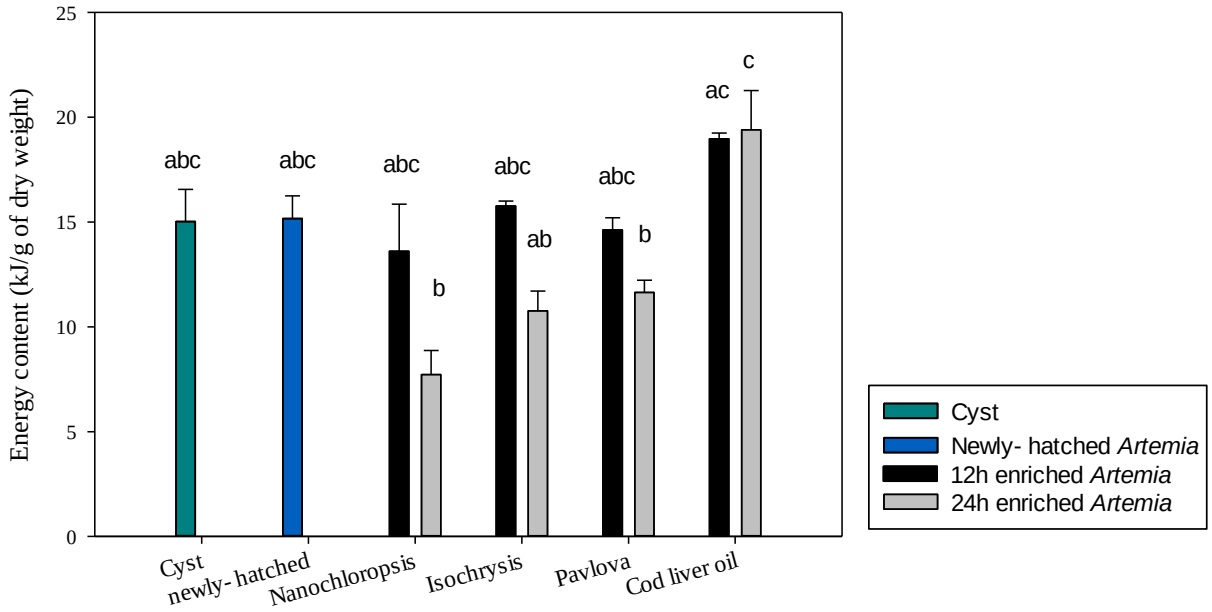

Figure 10: Energy content (kJ/g of dry weight) of cyst, newly-hatched *Artemia* and enriched *Artemia* with *Nanochloropsis*, *Isochrysis*, *Pavlova* and Cod liver oil for 12h and 24h. Values are mean ± SE of two replicates. Significant differences are indicated with different superscript letters (Holm-sidak post-hoc test, $p < 0.05$).

Table 4: Energy content (kJ/g of dry weight) of Cyst, newly-hatched Artemia and enriched Artemia with Nanochloropsis, Isochrysis, Pavlova and Cod liver oil for 12h and 24h. Values are mean ± SE of two replicates. Significant differences are indicated with different superscript letters (Holm-sidak post-hoc test, $p<0.05$).

Treatment	*Nanochloropsis* enriched	*Isochrysis* enriched	*Pavlova* enriched	Cod liver oil enriched	Newly-hatched *Artemia*	Cyst
12h	13.6 ± 2.25^{abc}	15.8 ± 0.24^{abc}	14.6 ± 0.58^{abc}	19 ± 0.28^{ac}	15.2 ± 1.09^{abc}	15 ± 1.53^{abc}
24h	7.7 ± 1.15^{b}	10.8 ± 0.94^{ab}	11.6 ± 0.58^{b}	19.4 ± 1.87^{c}		

4.3 Fatty acid composition

Figure 11 shows the composition of saturated (SFA), mono-unsaturated (MUFA) and poly-unsaturated fatty acids (PUFAs) after 12h enrichment (Fig.11A) and after 24h enrichment (Fig. 11B). In general, the PUFA contents of *Artemia* varied from 9.42% to 33.74% of total fatty acid. The highest of PUFA percentage obtained in newly-hatched (33.74% of total fatty acid), followed by *Artemia* grown on *Isochrysis* (in the range of 27.84% and 29.14%) and *Nanochloropsis* (from 24.21% to 24.38%). The lowest PUFA percentage was found in cod liver oil (from 10.84% to 14.98%) and *Pavlova* (from 9.42% to 19.31%) treatments. However, higher level of MUFA was found in cod liver oil and *Pavlova* treatments compared with the other feeds.

The PUFA levels in enriched *Artemia* with *Nanochloropsis*, *Isochrysis* and cod liver oil after 12h and 24h were relative stable (Fig. 11A and 11B). While PUFA percentage in *Artemia* grown on *Pavlova* after 12h enrichment (9.42%) was lower than after 24h enrichment (19.31%).

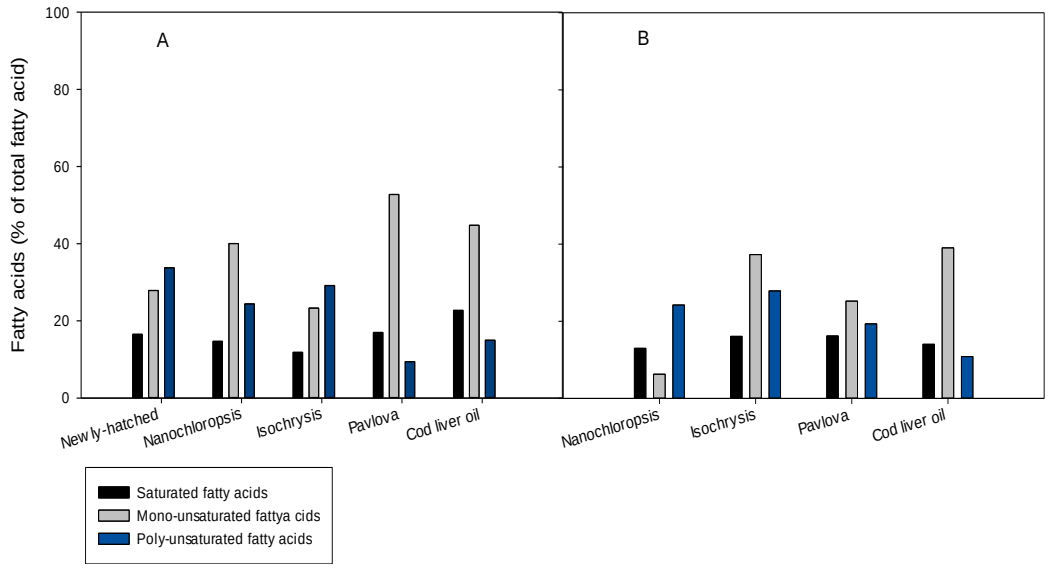

Figure 11: The compositions of saturated (SFA), mono-unsaturated (MUFA) and poly-unsaturated fatty acids (PUFA) (% of total fatty acid) in newly hatched and Artemia fed Nanocholoropsis, Isochrysis, Pavlova and Cod liver oil after 12h enrichment (Figure 11A) and after 24h enrichment (Figure 11B).

Both newly-hatched and enriched *Artemia* showed similar fatty acid profiles. The dominate fatty acids were 16:0, 16:1 and 18:1. While 18:4n-3 and 20:5n-3 (EPA) were found in lower quantities (Table 5).

Higher (n-3) PUFA levels of enriched *Artemia* were found in *Nanochloropsis* (21% of total fatty acid) and *Isochrysis* (17%-20%) treatments, compared with the other treatments including cod liver oil (1.06%-2.66%) and *Pavlova* (0%-11%). But, *Artemia* enriched with cod liver oil and *Pavlova* contained high (n-6) PUFA. The (n-3) PUFA composition of *Artemia* enriched with *Nanochloropsis*, *Isochrysis* and cod liver oil was not changed after 12h and 24h enrichment, except for *Pavlova* treatment.

Ratio of (n-3) PUFA and (n-6) PUFA ranged from 0.1% to 6.26% of total fatty acid. The highest (n-3):(n-6) ratio was found in enriched *Artemia* with *Nanochloropsis* (6.06%-6.26%), followed by newly-hatched *Artemia* (5.38%). The lowest was in *Isochrysis* (1.5%-2.5%), *Pavlova* (1.3%) and cod liver oil (0.1%-0.34%) treatments.

Essential fatty acids such as EPA, DHA and ARA were found in newly-hatched and some enriched *Artemia* treatments. The EPA, DHA and ARA compositions in newly-hatched *Artemia* were 3.13%, 0.04% and 1.16% of total fatty acid, respectively. With enriched *Artemia*, EPA was detected in enriched *Artemia* with *Isochrysis* for 12h enrichment (0.08% of total fatty acid), with *Pavlova* for 24h (10.93%) and with cod liver oil for both 12h (0.73%) and 24h (0.76%). DHA was only found in 12h enriched *Artemia* with *Nanochloropsis* and cod liver oil. Only 24h enriched *Artemia* with *Pavlova* contained ARA (2.81% of total fatty acid).

The results in this study showed that the levels of the essential fatty acids were low and not identified in many treatments (Table 5). It should be noted that high content of un-identified fatty acid compositions was observed in this study.

Table 5: Relative fatty acid composition (% of total fatty acid) of newly- hatched and Artemia after 12h and 24h enrichment with Nanochloropsis, Isochrysis, Pavlova and Cod liver oil. ΣSFA is sum of saturated fatty acid, ΣMUFA is sum of mono unsaturated fatty acid, ΣPUFA is sum of poly unsaturated fatty acid. ∑FA is sum of total fatty acids. No value (-) means that fatty acid was not identified.

Fatty acid composition (%)	**Newly-hatched Artemia**	**Nanochloropsis enriched *Artemia***		**Isochrysis enriched *Artemia***		**Pavlova enriched *Artemia***		**Cod liver Oil enriched *Artemia***	
		12h	24h	12h	24h	12h	24h	12h	24h
C12:0	0.03	_	_	_	_	_	_	0.01	_
C14:0	0.24	_	_	_	_	0.84	_	0.8	0.02
C16:0	6.95	9.83	9.2	7.22	11.05	10.54	8.44	12.83	8.59
C17:0	0.42	_	_				_	0.5	0.07
C18:0	6.06	_	_	4.28	5.02	5.62	5.09	8.41	5.03
C20:0	0.05	4.56	3.31		_	_	_	0.02	_
C22:0	2.34	_	_	0.32	_	_	2.07	0.14	0.22
C23:0	0.1	0.32	0.45	_	_	_	_	_	_
C24:0	0.34	_	_	_	_	_	0.58	_	0.07
∑SFA	**16.53**	**14.71**	**12.96**	**11.82**	**16.07**	**17**	**16.18**	**22.71**	**14**
C16:1n7	0.96	1.61	0.92			3.51	3.24	0.69	3.35
∑C18:1n9,n7,n5	25.69	33.88	_	20.14	31.26	25.73	21.33	29.33	25.45
∑C20:1n11,n9,n7	0.78	0.06	_	_	_	23.48	0.64	14.6	10.19
C22:1n9	0.42	4.48	5.34	_	_	_	_	0.12	0.02
C24:1n9	_	_	_	3.15	6.01	_	_	_	_
∑MUFA	**27.85**	**40.03**	**6.26**	**23.29**	**37.27**	**52.72**	**25.21**	**44.74**	**39.01**
C16:2n4	0.42	_	_	_	_	0.23	_	3.07	0.26
C18:3n3	20.11	0.07	_	_	_	_	_	0.01	_
C18:4n3	4.7	20.03	18.52	17.68	19.95	_	_	0.28	1.52
C20:4n3	_	0.8	2.59	_	_	_	_	_	_
C20:5n3	3.19	_	_	0.08	_	_	10.93	0.73	0.76
C22:5n3	0.06	_	_	0.12	_	_	_	0.03	0.38
C22:6n3	0.04	0.12	_	_	_	_	_	0.01	_
C20:2n6	0.04	3.36	3.1	11.26	7.89	4.64	2.28	2.53	_
C18:2n6	3.99	_	_	_	_	4.55	3.29	8.31	4.77
C20:3n6	0.03	_	_	_	_	_	_	0.01	3.15
C20:4n6	1.16	_	_	_	_	_	2.81	_	_
∑PUFA	**33.74**	**24.38**	**24.21**	**29.14**	**27.84**	**9.42**	**19.31**	**14.98**	**10.84**
∑ (n-3)	28.1	21.02	21.11	17.88	19.95	0	10.93	1.06	2.66
∑ (n-6)	5.22	3.36	3.1	11.26	7.89	9.19	8.38	10.85	7.92
(n-3)/(n-6)	5.38	6.26	6.81	1.59	2.53	_	1.3	0.1	0.34
DHA/EPA	1.25	_	_	_	_	_	_	1.37	_
EPA/ARA	2.75	_	_	_	_	_	3.89	_	_
∑FA	**78.16**	**79.13**	**78**	**64.25**	**81.18**	**79.15**	**60.69**	**82.42**	**63.86**
Unknown	**21.84**	**20.87**	**22**	**35.75**	**18.82**	**20.85**	**39.31**	**17.58**	**36.14**

5 DISCUSSION

5.1 Survival and energy content of *Artemia*

High survival rate of *Artemia salina* (from 66% to 95%) in this study indicated that all enrichment feeds including *Nanochloropsis*, *Isochrysis*, *Pavlova* and Cod liver oil can be used successfully in the rearing of *Artemia* for short term feeding (form 12h to 24h enrichment) (Figure 9). The survival rate of *Artemia* was highest in cod liver oil (95%), followed by *Nanochloropsis* and *Isochrysis* However, there was not significantly different ($p > 0.05$) in survival rate among the enrichment feeds for both two enrichment time, except for 24h enriched *Artemia* with *Pavlova* (66%). This may be caused by over-feeding in *Pavlova* enriched *Artemia* tanks. Excess amount of feed may result in the collapse of *Artemia* tanks because of oxygen deplete and water degradation (Dhont & Stappen, 2003).

In the present study, the energy content of both cyst and newly hatched *Artemia salina* was not significantly different (15 kJ/g, $p < 0.05$), which is lower than values reported in previous studies of brine shrimp *Artemia* (Caudell & Conover, 2006; Paffenhöfer, 1967; Paul & Michael, 1994). Two earlier results was showed higher energy content of newly hatched *Artemia salina* nauplii (24 kJ/g) (Paffenhöfer, 1967; Paul & Michael, 1994). Caudell and Conover (2006) reported that the energy content of *Artemia* cyst was 23 kJ/g. The lower energy content in *Artemia* cyst in this study can be due to cyst quality that effected by storage time or to different breeding conditions (Paffenhöfer, 1967). Long term storage may result in a decrease of energy content and hatchability of cysts (Paul & Michael, 1994).

The energy content of *Artemia salina* enriched with all feeds (*Nanochloropsis*, *Isochrysis*, *Pavlova* and Cod liver oil) was not influenced by enrichment time (12h and 24h) (Figure 10). There was no significantly different in energy content ($p > 0.05$) among cyst, newly hatched *Artemia* and enriched *Artemia*. This indicates that the energy content of *Artemia* was not lost during de-capsulation and enrichment processes.

Difference in energy contents of *Artemia* fed *Nanochloropsis*, *Isochrysis*, *Pavlova* and Cod liver oil was detected with 24h enrichment treatment ($p < 0.05$). The higher energy content was found with cod liver oil treatment (19 kJ) compared with the other feeds treatments (from 7.7 kJ to 11.6 kJ). The reason could be explained by the higher energy content of cod liver oil compared with *Nanochloropsis*, *Isochrysis* and *Pavlova*.

5.2 Fatty acid composition

In this study, the dominant fatty acids in newly-hatched *Artemia* were 16:0, 16:1, 18:1, 18:3n-3 and lower levels were 18:4n-3 and 20:5n-3 (EPA). The results in this study are in agreement with previous reports on *Artemia* (Paul & Michael, 1994; Thinh, 1999). Newly-hatched *Artemia salina* had a relative high content of EPA (3.19% of total fatty acid, Table 5) and a low DHA and ARA content, that similar to reports by other researchers (Figueiredo & Woesik, 2009; Han, 2001).

Fatty acid content, particularly essential fatty acid, of live feeds such as *Artemia* is critical for larval cultivation (Sorgeloos & Dhert, 2001). Therefore, enrichment is essential to improve essential fatty acid levels. The composition of fatty acid in enriched *Artemia* varies as a function of the enrichment protocol (Figueiredo & Woesik, 2009; Han, 2001; Thinh, 1999). However, in this study, the fatty acid composition of enriched *Artemia* did not reflect the enrichment diets.

The results also did not allow a clear conclusion for the effect of enrichment time on fatty acid composition, especially PUFA composition, of enriched *Artemia* with *Nanochloropsis*, *Isochrysis* and *Pavlova* and cod liver oil. The reason could be explained that several fatty acid compositions were not identified in this study (Table 5).

Maine fish requires high (n-3) PUFA level and high levels of DHA and EPA. For finfish species, they tend to need high DHA and ARA levels and low EPA level, leading to high DHA:EPA and ARA:EPA ratios (Hauville & Main, 2014; Ogata & Emata, 2004; Yanes-Roca & Rhody, 2009). While pompano nutrient requirement, they may require high ARA level in diets because egg of Florida pompano species have a high ARA:EPA ratio (Hauville & Main, 2014). Therefore, the diets which have high levels of (n-3) PUFA, especially DHA and ARA levels, could be suitable for pompano.

From the results obtained in this study, high DHA and ARA levels were detected in enriched *Artemia* with *Nanochloropsis*, *Pavlova* that may be used for popamno cultivation. However, because of high un-identified fatty acid compositions, the result of fatty acid composition must be re-analyzed to have accurate data and then suggest suitable enrichment methods for marine fish species.

6 CONCLUSION AND RECOMMENDATION

All enrichment feeds including *Nanochloropsis*, *Isochrysis* and *Pavlova* and cod liver oil can be used successfully in the rearing of *Artemia* for short term feeding (form 12h to 24h enrichment) because of high survival rate of *Artemia* (from 66% to 99%) and energy content which was maintained throughout de-capsulation and enrichment processes. However, the results of fatty acid composition did not allow a clear conclusion for the effect of enrichment on fatty acid composition of *Artemia* because of high un-identified fatty acid compositions in this study.

Therefore, fatty acid composition must be re-analyzed with higher amount of *Artemia* or by another analysis method for small samples of live feeds. Next experiment should be conducted with fish, such as popamno, to examine the effect of enriched *Artemia* with different feeds on survival and growth of pompano larvae. Besides, suitable amount of *Artemia* used for fatty acid analysis must be studied and determined in next experiment.

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