PROPENSITY OF BIO-ENRICHMENT PROTOCOL IN MARINE ORNAMENTALFISH LARVICULTURE

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By

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Dedicated

to

my beloved uncle

A. Chinnathambi

Declaration

I do hereby declare that the thesis entitled "Propensity of bio-enrichment protocol in marine ornamental fish larviculture" is an authentic record of research work carried out by me under the guidance and supervision of Dr. Kajal Chakraborty, Senior Scientist, Marine Biotechnology Division, Central Marine Fisheries Research Institute, Cochin-682018 and the same has not previously formed the basis for the award of any degree or diploma.

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G. IYYAPPARAJANARASIMAPALLAVAN

Place: Cochin
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Certificate

*This is to certify that this thesis entitled "**Propensity of bio-enrichment protocol in marine ornamental fish larviculture**" is an authentic record of research work carried out by G.Iyyapparajanarasimapallavan (Reg.No.MU/ACC/Ph.D./CR.33/2011-12/A3,Dated 08.11.2011) under my guidance and supervision in Central Marine Fisheries Research Institute; in partial fulfillment of the requirements for the Ph.D. degree under the Department of Biosciences of the Mangalore University. I further certify that this thesis or part thereof has not previously formed the basis for the award of any degree, diploma, and associateship of any other University or Institution.*

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Abstract

The Marine ornamentals are the most flourishing international business activity, valued at 8 billion US$ with an average annual growth rate of 9% across the world. Even though marine ornamental fish culture for trade is being practised globally, the early mortality of these altricial fish larvae in captivity are the major hitch of this sector that occurs due to lack of proper feed or feeding technology. This research is focused on the propensity of bio-enrichment protocol in marine ornamental fish larviculture using clownfish larvae (Amphiprion ocellaris) as a model species. Specifically, it was designed to optimize the feeding protocol of A. ocellaris using live feed enriched with DHA rich oil emulsion and to study their effects on larval growth and survival under captivity.

The larvae of A. ocellaris were fed with six different diets including the indigenously produced DHA rich marine pelagic fish oil emulsion, which were delivered through rotifers Brachionus plicatilis. Locally available low-value oil sardine (Sardinella longiceps) and fish roe (egg) were screened for long chain n-3 PUFAs and were used as raw material to prepare the PUFA by chemical and/or enzymatic process. This study has been contemplated for the development of n-3 PUFA enriched formulation based on different physicochemical properties associated with the olefinic bonds in fatty acids and/or acyl chain length. The long chain $C_{20\text{-}22}$n-3 PUFAs such as EPA and DHA present in the sardine oil were concentrated by saponification reaction followed by their enrichment by amide fractionation and chromatographic methods (the silica gel complexed with a d-block element). The unique substrate specificity of microbial triacylglycerol acyl hydrolases has also been utilized to further enrich the $C_{20\text{-}22}$ LC PUFAs in triglycerides.

The microalgal species were isolated from different locations of marine and backwater niches of Kerala, and their culture conditions in laboratory were optimized. The nutritional properties with respect to the fatty acid compositions of the purified microalgae at their exponential growth phase were obtained. The live feed rotifers were cultured in mass by using the shortlisted microalgae, and were further enriched with indigenously produced DHA rich fish oil emulsion to enhance the nutritional threshold of live feed. The optimum time period to enrich the live feed by the oil emulsion was described. The marine ornamental finfish A. ocellaris larvae was fed with the DHA enriched live feed rotifer in different experimental combinations, and the fatty acid compositions of the larvae were recorded to understand their effect on the growth and development of the fish larvae. The rotifers (Brachionus plicatilis) were mass cultured in the presence of DHA rich marine microalgae Isochrysis galbana and further enriched with DHA rich fish oil emulsion. The fatty acid profile along with the growth and survival of the enriched live fed were compared with those fed with different commercial diets including a lyophilized algal powder.

The refined sardine oil was found to contain long-chain polyunsaturated fatty acids (LC-PUFAs), particularly eicosapentaenoic acid (EPA; 17.80 % TFA) and docosahexaenoic acid (DHA; 7.67 TFA). It is of note that among the long chain polyunsaturated fatty acids, DHA contributed a maximum value of 20.34 % TFA by using the amide-fatty acid ratio of 3:1 at 2 ^{0}C. It is therefore this particular fraction was used for further purification of C_{22} PUFAs. An extracellular lipase with specific activity of 386 LU/mg derived from Bacillus subtilis isolated from marine macroalga, Turbinaraia conoidesm was used to prepare C22 n-3 polyunsaturated fatty acid concentrates from the ester fraction. The total DHA of fatty acyl ester fraction increased with time up to 3 h by enzyme-catalyzed hydrolysis (35.27% TFA), beyond which it slowly decreased (9.81% TFA after 9 h). The DHA enriched fatty acid concentrate was enriched by biochemical and microbiological procedures were used to formulate enrichment emulsions, which contained grossly, 90 % DHA enriched fatty acid concentrate and 10 % phospholipids fraction extracted from fish roe (seer fish). The aggregate content of DHA in fish roe phospholipidic fraction was recorded as 24.3% DHA (greater than 35%) along with 5.2% EPA. Thus $C_{20\text{-}22}$n-3 polyunsaturated fatty acid concentrate prepared from the inexpensively available marine sources, such as, sardine oil can be the potential substitute of the imported PUFA supplements for use in mariculture.

Among different n-3 LC-PUFAs, the fatty acid DHA has been considered to be indispensable for the growth and survival of the marine fish larvae at the initial stage of their feeding. The fatty acid composition of DHA in the microalgae was found to be present in considerable amount (7-9% TFA) in Isochrysis galbana and Isochrysis sp strain RA_{10} and RA_{10}, followed by Chlorella salina and Pavlova lutheri, which shared about 6% TFA of the aggregate content of fatty acids. Among the different experimental microalgae, Chaetoceros calcitrans was found to contain the greater content of aggregate PUFA as compared to other microalgae species. The present study also attempted to develop an indigenous microalgal powder which is readily available product to culture rotifer or to feed finfish and shellfish larvae. Chaetoceros calcitrans was shortlisted to develop a microalgal formulation based on its characteristic physical and chemical features. In future the same product can be used by adding other essential nutrients as a cost effective alternative to the imported products, for example, Algamac. The aggregate content of PUFAs of rotifers enriched with Isochrysis galbana was of its maximum at the 12 h (20 %TFA) of enrichment period, and thereafter a diminishment in their content was noted. However, the microalga I. galbana used not only as an enrichment media per say, but to impart better growth and development traits of the live feeds.

The DHA rich oil emulsion, with DHA content of 39% TFA and EPA content of 19% TFA was used to enrich rotifers. A maximum content of polyunsaturated fatty acids (~ 40%) were apparent after a period of 6 h, while the oil emulsion was used at the dose of 1 mL/L. The C_{22}n-3 fatty acid (DHA) in the

rotifers exhibited a significant increase (~ 9 %) after the enrichment period of 6 h, while the oil emulsion was used at the identical dose (1 mL/L). The ratio of DHA: EPA in rotifers after enrichment duration of 6 h was 2.76: 1 which was reported as ideal for marine fish. The rotifers enriched with the PUFA enriched oil emulsion for a period of six hours has yielded greater quantities of the desired fatty acids ($C_{20\text{-}22}$n-3 fatty acid, EPA, 20:5n-3 and DHA, 22:6n-3).

Concurrently, different enrichment media, for example, Algamac 2000 (Biomarine Inc., Aquafauna, USA), S.presso (Selco S.presso ®, INVE Aquaculture), an indigenously powdered microalgal derived from Chaetoceros calcitrans and marine microalgae (Isochrysis galbana) were utilized to compare their efficacy with the $C_{20\text{-}22}$ PUFA rich oil emulsion. However, the PUFA enriched oil emulsion developed in the present study has yielded the rotifer with greater content of these fatty acids (~40 %) than those recorded with other enrichment formulation. A greater content of DHA (~ 20% TFA) in the egg (5^{th} day) of the ornamental fish larvae signified the importance of this C_{22} long chain fatty acid for the larval growth and development. It is therefore desirable to feed the larvae of ornamental fish with an external source, preferably a live feed (rotifer) with a greater content of DHA for the optimum growth and development in captivity. Thus, rotifers enriched with the indigenously developed $C_{20\text{-}22}$ long chain n-3 PUFA enriched oil emulsion will increase the DHA content of ornamental fish larvae grown in captivity with a maximum survival percentage of 93% that states the PUFA enriched product may be a cheaper alternative to the imported PUFA supplements. The indigenous n-3 polyunsaturatated fatty acids emulsion developed from the locally available low-value fish may also serve as a cheaper alternative to the imported fatty acid emulsions for use in mariculture and larval nutrition.

Hence our research has made an innovative attempt to optimise the bio-enrichment protocol in the marine ornamental fish larvae by using various formulations such as indigenously produced $C_{20\text{-}22}$ long chain polyunsaturated fatty acid particularly docosahexaenoic acid (DHA, 22:6n-3), enriched marine microalgae with optimized enrichment time period and lyophilised marine microalgal powder rich in n-3 polyunsaturated fatty acids. These $C_{20\text{-}22}$ long chain polyunsaturated fatty acid enriched formulations might prove to be the effective alternatives to the costly enrichment products available in the market.

Abbreviations

ALA	–	Alpha-linolenic acid
AMER	–	AlgaMac enriched rotifer
ANOVA	–	Analysis of variance
APER	–	Algal powder enriched rotifer
ARA	–	Arachidonic acid
BF_3	–	Boron trifluride
CH_3OH	–	Chloroform
CMFRI	–	Central Marine Fisheries Research Institute
DGLA	–	Dihomo-gama-linolenic acid
DHA	–	Docosahexaenoic acid
DMRT	–	Ducan's multiple range test
DPA	–	Docosapentaenoic acid
EA	–	Eicosadienoic acid
EDTA	–	Ethylenediamine tetra acetic acid
EFA	–	Essential fatty acid
EPA	–	Eicosapentaenoic acid
FA	–	Fatty acid
FAME	–	Fatty acid methyl esters
FID	–	Flame ionised detector
FRP	–	Fibreglass Reinforced Plastic

GF/C	–	Glass Microfiber filter
GLA	–	Gamma-linolenic acid
GLC	–	Glass Liquid Chromatography
HUFAs	–	Highly unsaturated fatty acids
Ig	–	*Isochrysis galbana*
IGER	–	*Isochrysis galbana* enriched rotifer
KNO_3	–	Potassium Nitrate
KOH	–	Potassium Hydroxide
LA	–	Linoleic acid
LC	–	Long chain
LNA	–	Lenolenic acid
L-type	–	Large type
MeOH	–	Methanol
MUFA	–	Monounsaturated fatty acid
Na_2O_3Si	–	Sodium silicate
Na_2SO_4	–	Sodium sulphate
Na_3PO_4	–	Sodium phosphate
NaCl	–	Sodium chloride
NaOH	–	Sodium hydroxide
OEER	–	Oil emulsion enriched rotifer
PE	–	Petroleum ether
pH	–	Potential hydrogen

PSI	–	Pound-force per square inch
PUFAs	–	Polyunsaturated fatty acids
RPM	–	Rotation per minute
SDA	–	Stearidonic acid
SFA	–	Saturated fatty acid
SPER	–	S.presso enriched rotifer
S-type	–	Small type
TBHQ	–	tertiary Butylhydroquinone
TFA	–	Total fatty acids
UNER	–	Unenriched rotifer

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CHAPTER 1

Introduction

Chapter 1

Introduction

Marine ornamental fishes are one of the most popular aquatic organisms due to their beauty and their adaptability to live in confinement. The marine ornamental fish trade has a significant role in the economy of the developed and developing countries both as a foreign exchange earner and as a source of employment. It was estimated that nearly 20-30 million reef fishes includes approximately 1,400 fish species are collected mainly for the aquarium trade (Bruckner, 2005). Most of the world's supply is from Asia and Europe. In Asian region, the major suppliers in the ornamental fish trade are from Singapore (34 %), Malaysia (15 %), Indonesia (11 %), and Japan (10 %). It is of note that India contributes a significant share of about 2 % of the global ornamental market share (Ponniah, 2007). The world ornamental fish trade is about 4 to 15 billion US $, while India's contribution through export is only about 0.5 million US $ (Sakthivel, 2002). Every year, millions of marine organisms are being exploited for their ornamental values especially from the coral reefs and their associated habitats (Rhyne *et al.*, 2012). In India, the ornamental fish trade has been reported predominantly from fresh water habitat contributing about 90 %, of which 98 % are cultured and 2 % are captured from wild. The rest 10 % of total ornamental fish trade are from marine habitat, of which 98 % are captured and 2 % from cultured (Ponniah *et al.*, 2007). The United States of America alone imports ornamental fishes worth greater than 500 million US $ per year. In Holland, 20 % of the houses maintain ornamental fishes, 14 % in UK, 8 % in USA, 5 % in Germany, and 4 % in Belgium (Sakthivel, 2002). All over the world, the ornamental aquarium keeping is considered as the second popular hobby, next to photography (Saroj, 2008).

The export of marine ornamentals has grown as a major global enterprise, which was fuelled by certain high profile exposure movies like Pixar's 'Finding Nemo'. Thus, the marine ornamental fishery is considered as a potential economic source for many people residing in the coastal areas and provides a good source of income. The global trade of the ornamental fish species, their associated aquarium and pond accessories is in excess of U. S. $\$7 \times 10^9$ each year. Even though majority of the trend in the trade of freshwater ornamental fishes are from intensive culture, considerable counts are still removed from their natural habitat. It is of note that, almost 99% of the marine ornamental fishes in the trade are wild-caught. Whilst habitat destruction, pollution and overfishing for food are the main threats to the fish populations in the wild, the ornamental fish trade may have adverse effects as a result of the introduction of non-native organisms, and by the direct depletion of wild stocks. Further, studies are needed, if the collection of fish from the wild for the ornamental fish trade, and how it is affecting wild populations (Andrews, 1990).

World's coral reefs cover less than 1 % of the marine environment, and therefore, they are universally deliberated bring the most biologically rich and productive ecosystems. This ecosystem supports over 4000 fish species, about 800 species of reef-building corals and several thousands of other reef invertebrates, such as, cnidarians, sponges, mollusks, crustaceans, polychaetes and echinoderms (Olivotto *et al*.,2011). Targeted marine ornamental fishing activity supplies the demands for marine aquarium trade. This played an important role in the decline of coral reef ecosystem (Dee *et al*., 2014). Instead, the prospective effects of collection, stock status and sustainable harvest levels of most ornamental species remain largely unidentified. In addition, unwise traders continue to support the use of destructive fishing techniques, *viz.* dynamite fishing, and use of cyanide, to anaesthetize the highly priced fish species (Saila *et al*., 1993). These practices in the coral reef ecosystem are well known to harm targeted as well as non-targeted species. The top ten traded species of coral reef

aquarium fish according to the Global Marine Aquarium Database records from 1997 to 2002 are *Amphiprion ocellaris, Chrysiptera cyanea, Dascyllus aruanus, Amphiprion percula, Chromis viridis, Abudefduf* sp, *Dascyllus trimaculatus, Paracanthurus hepatus, Dascyllus albisella,* and *Chrysiptera hemicyanea.* The aforementioned species have been listed according to import volume from highest to lowest. The family Pomacentridae, particularly clownfish genus *Amphiprion*, represents the most important group of captive-bred marine species (Lupatsch *et al.,* 2013). Among all species, *Amphiprion* sp is the best known to aquarium traders due to its colour pattern, interesting behaviour, and robustness (Dee *et al*.,2014). From 1997 to 2002, *Amphiprion ocellaris* was the most common species of marine ornamental fish and made up 15.6 % export worldwide and over 25 % into the European countries (Wabnitz *et al*., 2003).

Though the commercial culture of *A. ocellaris* entertained or cultured worldwide, its larval production and development has many difficulties like many other marine fish larvae. The present work addresses the larviculture nutrition issues of this clown fish. The farming of marine ornamental fishes faces several challenges including growth, survival and physical development, especially at earlier stages during embryo development, hatching and transition from endogenous to exogenous feeding of larvae (Olivotto *et al.,* 2011). Marine fish larvae have rudimentary digestive system thereby making their uptake and utilization of essential nutrients difficult. The most common perception is that feeding larvae with easily digestible live feed is vital to the ornamental larval nutrition, than the formulated feed since most of the larvae are altricial (born helpless) in nature. Live feed enrichment is a well-known practice in the aquariculture. However, it is of note that the common live preys rotifers and *Artemia* nauplii are naturally deficient in polyunsaturated fatty acids (Sargent *et al.*, 1999).

Most fish hatcheries follow a protocol of hatching in green water, followed by feeding with rotifers and *Artemia* nauplii. Life cycle of most of the larvae is spent in the open ocean and its nutritional requirements are easily satisfied by the enormous diversity and variability of planktons. The common nutritional source of wild marine fish larvae are copepods (zooplankton). Furthermore, zooplankton-fed larvae showed higher survival rate and free from deficiencies like skeletal deformation, less pigmentation and eye migration errors than those fed with cultured live prey like rotifers and *Artemia* (Baensch and Tamaru, 2009; Drillet *et al.*, 2011; Olivotto *et al.*, 2011). Due to the difficulty in continuous cultivation of copepods, most of the marine fish species are reared with live prey like rotifers, *Brachionus plicatilis* and brine shrimp, *Artemia* nauplii. These are the most favoured diets in aquaculture, especially in earlier larval stages (Gopakumar *et al.*, 2008; Olivotto *et al.*, 2011; Romero and Yufera, 2012).

The main advantage of rotifer is their ability to produce in high-densities, homogeneously distributed, having a lesser gut transit time and appropriate to the fish larval mouth size. The rotifer, *Brachionus plicatilis* is well-known as an indispensable as a live food for mass larval rearing of many aquatic organisms (Maruyama *et al.,* 1997). Though rotifers fill the digestive tract of larvae, its inappropriate nutritional quality is the greatest concern. It is deficient in indispensable nutrients for larvae, for example, long chain polyunsaturated fatty acids, amino acids, vitamins, and minerals (Avella *et al*., 2007; Maehre *et al*., 2013; Rasdi and Qin, 2014; Romero and Yúfera, 2012; Srivastava *et al*., 2006). Thus, the difference in nutritional quality in live feed is one of the major factors in the rearing success of fish larvae. The production of high quality fish larvae is the greatest constraint in many commercial hatcheries (Maehre *et al.*, 2013; Rasdi and Qin, 2014). The nutritional value of rotifers for larval fish depends on the rotifers food source, primarily highly polyunsaturated

fatty acids (HUFA), which are essential for the survival and growth of fish larvae (Watanabe, 1993).

Greater nutritional value of rotifers were of major importance for survival and growth of the fish larvae, and several cultivation techniques, including feeding with different algae, baker's yeast and artificial diets, which are used to improve their nutritional value. The nutritional quality of the diet offered to captive marine fishes are important during the first few weeks of larval life (Das *et al*., 2012). It is a well-known fact that lipid nutrition is particularly important for marine fish (Bell and Sargent, 2003; Carla *et al*., 2012; Copeman ., 2002).Since deficiencies in these molecules can cause a general decrease of larval health, growth, feed efficiency, and anaemia leading to high mortality (Faulk and Holt, 2005; Sargent *et al*., 1999). The dietary requirements of HUFAs from the *n*-3 family for marine fish larvae has long been known (Sargent *et al*., 1999) and in recent decades, a great amount of research has been directed towards the development of food enrichments (with particular emphasis on lipids) to improve larval fish survival and growth (Sargent *et al*., 1999).

Lipids in general and specifically *n*-3HUFA have an essential role in the larval diet (Rezek *et al*., 2010). Rotifers were found to synthesize some *n*-3HUFA by *de novo* synthesis, but the storage is less and deficient to meet the possible demand of fish larvae (Lubzens *et al*., 1986). The essential fatty acids must be provided to the rotifers through their food, which in most cases were provided by microalgae or through commercial enrichment media or oil emulsion. Enrichment techniques currently in use are the French technique - microencapsulated oils containing high concentration of *n*-3 HUFA's; Japanese technique - baker's yeast and yeast in emulsion; Belgiun technique - emulsified marine oils rich in *n*-3HUFA's; and the British technique - live micro algae (Gopakumar, 2004). Enrichment of rotifers by re-feeding with these products and tailored boosters are common practices to

improve the nutritional value of the live feeds before being transferred to the rearing tanks (Romero and Yufera, 2012; Maehre *et al.*, 2013). The major concern of ornamental fish larviculture is their early mortality in captive conditions (Green, 2003; Hamre *et al.*, 2013). The major reason of the early mortality is attributed to the nutritional deficiency, particularly C_{20-22} long chain PUFAs. It is of note that the feed used for ornamental fish mariculture are largely imported and expensive, which had often been reflected upon the unit production price of the ornamental fishes. The commercial products were used as feed for fish larviculture are Reed Mariculture (Instant algae *Isochrysis* 1800, Instant algae *Nanno* 3600); Aquafauna Biomarine (AlgaMac-2000, AlgaMac-3050); Bern Aqua-Invivo NSA (Caviar, Nori, Redpepper); Aller Aqua (AllerParvo EX, AllerFutura EX); Inve Aquaculture (O.range, S. Presso, and DHA Protein Selco); Cargill (AquaXcel, AquaXcel Warm Water, and AquaXcel Marine); Skretting Marine Hatchery feeds (Ori-One and Ori-Green) (Hatchery feed guide, 2015). These products are being used as enrichment media for better growth and survival of fish larvae (Rasdi and Qin, 2014)are expensive. One of the cheapest ways to enrich rotifers is by using oil emulsions. Home-made emulsions can be prepared with egg lecithin and fish oils (Watanabe *et al.,* 1982). The first emulsion was made from (*n*-3)HUFA rich fish oils (i.e. cuttlefish oil, pollack liver oil, cod liver oil, menhaden oil, etc.) and emulsified with egg yolk and seawater (Watanabe *et al.,* 1982, 1983). Recently, more purified oils containing specifically high levels of the EFA (*20:5 n*-3)have been used. Since the stability and storage possibility of these products is relatively low, they are usually made fresh and used immediately.

In the present study, our focus was to develop the indigenous alternatives to the imported enrichment products for culturing the high value ornamental fish species in an attempt to reduce the unit production cost. To satisfy the demand and conservation of the coral reef ecosystems, captive breeding is necessary. Even though marine ornamental fish

culture for trade is being practised globally, all cues to prevent the early mortality of marine fish larvae are still unidentified (Green, 2003; Hamre *et al.*, 2013). An import substitutes as alternatives to expensive imported products is a national need for augmenting indigenous production and trade of marine ornamentals.

The present study was therefore designed to with the following objectives:

1) To prepare an oil emulsion enriched with long chain $C_{20\text{-}22}n$-3 PUFAs, particularly DHA from marine sources.

2) To screen different microalgal species for greater content of *n*-3 long chain PUFAs, and to construct a database of nutritionally valuable microalgae for use in marine fish start nutrition.

3) To optimize the production of biomass from the short listed microalgal species under laboratory culture conditions in lieu of use in aquaculture.

4) To enrich live feed (rotifer *Brachionus plicatilis*) by utilizing the oil emulsion with greater DHA percentage, and to compare the enrichment efficiency with other available enrichment media.

5) To optimize the feeding protocol of clownfish larvae *Amphiprion ocellaris* by using live feed enriched with DHA rich oil emulsion, and their effects on larval growth and survival under captivity.

CHAPTER 2

Review of Literature

Chapter 2

Review of Literature

This chapter deals with the importance of marine ornamental fishes in global trade, their culture techniques in captivity, and utilization of bio-enriched live feed with polyunsaturated fatty acid rich media for the marine ornamental fish species.

2.1. Marine Ornamental Trade

Earliest 2000-1000 B.C., the aquaculture rehearsal had been initiated in China mainly on common carp (*Cyprinus carpio*). However no scientific record is available to substantiate this fact, except few written works (Herminio, 1988). During 500-473 B.C., authors named by Fan Lai wrote a book on Classic of Fish Culture, the first proof written in Chinese facsimile translated in English. This custom of culturing fish started and during 618-906 B.C., the Chinese people involved in fish culture as a source of food and livelihood made to culture other species namely silver carp, big-head carp, the grass carp, mud carp, etc. Likewise then, during 1900-1700, the seed production in captivity had been initiated with Asiatic carps, including the Indian major carps in fish and penaeid shrimp species, and giant freshwater prawns. During the ancient period, the sub-continent of India started building reservoir to store water and construction of pond in the temple in an attempt to culture fish in captivity. Initially, these practices of culturing fish are not for human consumption as food, but keeping as ornamentals. The first report of the marine ornamental fishery under captivity dates back during 1980s Murty (1996); Tomey, (1985, 1986).

The report during 1988 by Mintel and Anon stated that more than 20 million British and American homes maintained ornamental fish, and the share of marine ornamentals had

been lesser than 1 %. During the 1970s, the trend of marine ornamental fish keeping registered a sharp increase, and there were reports of extensive trade between many countries around the world Conroy (1975). Among the total number of 124 million fishes imported to USA, about 4 million species belonged to marine Henley (1984). The increased global importance of marine ornamentals led to their techniques to culture these valuable species in captivity Andrews (1990). Chapman *et al*. (1997) reported that even though the marketing value of the ornamental fish trade increased and reached approximately US$1,000 million, there are no optimized method to culture these species in captivity. It was expected that by the end of 1990s, nearly 25 species cultured for commercial purpose were from captive bred, although a substantial quantities of approximately 98 % were collected from wild Moe, (1999). It was estimated that only 1 – 10 % of the marine ornamental species in trade were captive bred, although a total of 6% were exported worldwide. The most demanding marine ornamental fish species in the marketis *Amphiprion ocellaris* Wabnitz *et al*. (2003); Wood (2001); Green (2003); Moe (2003); Oliver (2003); FAO (2006).

Chou *et al*. (2002) and Lecchini *et al*. (2006) mentioned that the marine ornamental trade from the southeast Asian countries were particularly at risk, with 88 % of all reefs were at medium to very high threat from anthropogenic impacts including overfishing, destructive fishing methods like breaking corals or using chemicals, coral mining, marine pollution, boat anchoring and tourism related activities Jones (2008). Townsend (2011) stated that about 27 million tropical marine ornamental fishes are traded each year. The trade depends only on wild capture, but the trend in recent years increasingly concentrating on supplementation by aquaculture-produced ornamental species. Rhyne *et al*. (2012) stated that marine aquarium trade and their associated organisms showed an increase in USA and other countries. Cohen *et al*. (2013) concluded that there is a lack of captive breeding techniques (larviculture protocols) to develop advanced ornamental mariculture.

2.2. Larvae Nutrition

The marine aquaculture began for centuries back in Asia with milkfish culture, based on the capture of fry from the wild. For this rearing method, live feed in the hatchery was not required. In the 1800s, hatcheries were constructed in Europe and North America for providing fertilized eggs, developed embryos and larvae for distribution back into the ocean. In the 1890s, the countries like Britain, France, Canada and USA had fish hatcheries devoted to the propagation of commercially important species, such as, cod, haddock, turbot, winter flounder, and lobster. These species maintained until the pro-larva stage, and after this stage; the larvae were released back to the ocean. This is because of the lack of knowledge of the initial feed (live feed) to grow them in hatcheries during the post-larval stage. The high fecundity of many marine fishes implies that an extreme high rate of mortality is experienced during a particular time period during the development process, which is referred to as the critical period, which often is defined a relatively short period in early development May (1973).

The term "Critical period" was first applied to fish by two early French fish culturists, Domergue and Bietrix (1897), who used it to describe the time of complete yolk absorption, when they had observed high mortality among marine fish larvae in laboratory rearing attempts. Fish culturists used the term critical period to refer to stages when mortality among captive larvae is high Liao *et al*. (1970). The Norwegian fishery biologists Johan Hjort put the most general understood definition of the term "critical period". In a pioneering study, Hjort established that the year class strength of Norwegian herring and cod stocks varied widely and that the strength of a year-class was determined early in its history Hjort, (1914, and 1926). He indicated that the critical growth period is synonymous to the very earliest larval and young fry stages as also reported by other related studies Hjort (1914). Hjort

(1914) suggested that survival during larval stages affected in two ways one is the lack of food availability at the time, which can cause catastrophic mortality and the other by currents that could transport larvae to the areas unfavourable to their further development, resulting in high mortality. Hjort highlighted the first of the above mechanisms as most critical, and cited the experience of fish culturists as evidence that the time of yolk exhaustion was particularly sensitive period.

The important challenge in aquaculture is larval nutrition, and till now very little is known about the nutritional requirements of marine ornamental fish larvae Holt (2011). In the past decades, most research work were carried out on first feeding of fish larval nutrition Lin *et al*. (1982); Watanabe *et al*. (1983); Kanazawa *et al*. (1985); Watanabe (1985, 1993); Enright *et al*. (1986); Bell *et al*. (1986); Minkoff (1987); Hirayama *et al*. (1989); Sargent *et al*. (1989, 1993, 1997); Volkman, (1989); Rainuzzo *et al*. (1994); Natalia *et al*. (1995); Feuga (1996); Izquierdo (1996); Pickova *et al*. (1997); McEvoy *et al*. (1998); Pulz and Scheibenbogen (1998); Borowitzka (1999); Lin *et al*. (2002); Iwamoto (2004); Gopakumar (2004); Spolaore *et al*. (2006); Avella *et al*. (2007); Patil *et al*. (2007); Khairy and El-Sayed (2012); Olivotto *et al*. (2012, 2011a, 2011b); Hamre *et al*. (2013a, 2013b); Maehre *et al*. (2013). These studies have contributed to the knowledge necessary to estimate the nutritional requirements and the importance of marine microalgae used in larval fish. Watanabe (1985) demonstrated that the provision of the large numbers of juveniles is beset by the problems of mortalities in the larval phase. The content of essential fatty acids (EFA) in the live feeds is the principle determinant of their dietary values. Mortalities could also be reduced by enhancing egg quality through brood stock nutrition.

Blaxter & Hempel (1963) reported that unless exogenous feeding is initiated at, or closely following the period of yolk absorption, their condition rapidly declines. First feeding

success has an immediate impact on the potential number of larvae surviving to metamorphosis in the hatchery and depends on the type of species, size and density of prey. Kanazawa *et al*. (1985) and Enright *et al*. (1986) mentioned that the main zoological groups produced by aquaculture, such as, marine fish, shrimp and mollusks, exhibit reduced ability to biosynthesize highly unsaturated fatty acids (HUFAs) by desaturation and chain elongation, and are thus essential substances that must be supplied by external food sources. Watanabe *et al*. (1978) demonstrated that the chemical composition of marine eggs and the early larval stages showed the presence of greater quantities of *n*-3 HUFAs among other major components of the lipids of several marine species. Nhu (2004) demonstrated that the L-strain rotifers can be cultured in bakers' yeast and microalgae, which revealed that rotifers fed on microalgae showed better viability, larger size and low ciliate contamination compared to those fed on yeast. Hirata (1979) was the first to suggest that the rotifers, *Brachionus plicatilis*, previously considered a pest in culture ponds, could be used as a first food for larvae of both fresh water and marine fish species. Kimata (1983); Tocher and Sargent (1984); Watanabe (1985); and Izquierdo *et al*. (1989) demonstrated that docosahexaenoic acid is the major limiting component for fish larviculture and these glycerophospholipids are available in marine fish roe. Milinaire *et al*. (1983); Franicevic *et al*. (1987); Koven *et al*. (1989); and Izquierdo *et al*. (1992) mentioned that the EFA requirements differ not only between different species but also between growth stages. They found that among various essential fatty acids, C_{22} docosahexaenoic acid was superior to the C_{20} fatty acid eicosapentaenoic acid. Watanabe (1993) pointed out that*n*-3 highly unsaturated fatty acid (HUFA), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are essential fatty acids for marine fish larvae, and enrichment of this fatty acid in rotifer has reduced the mass mortality, in fish larvae.

Leger *et al*. (1986) concluded that among different principal factors affecting the nutritional value of live prey of marine fish, these particularly the long chain C_{20-22} fatty acids play predominant role. A large amount of effort has lately been devoted to study the nutritional requirements of marine larval fish and especially the research effort related to the essential fatty acid requirements of fish larvae. Rodriguez *et al*. (1993) discussed the effect of *n*-3 HUFA levels in rotifers on their survival, growth, activity, and fatty acid composition of gilthead bream larvae. They found that a good correlation was found between the growth and *n*-3 HUFA levels in rotifers. Larval survival also significantly improved by the elevation of the *n*-3 HUFA levels in rotifers. Watanabe (1993) demonstrated that the reduced range of food available for the larvae might lead to nutritional imbalances or nutritional deficiencies. Izquierdo (1996) demonstrated that the feeding of marine fish larva is often limited to the administration of few live prey organisms, such as, rotifers and *Artemia*. The reduction in the range of food available for the cultured larvae may occasionally lead to nutritional imbalances or deficiencies. Larval growth, survival, and activity were found to be better when these long chain C_{20-22} fatty acids were fed to the live prays, which thereafter were utilized to increase the nutritional composition of the marine fish larvae.

Sargent *et al*. (1997) discussed the advantages and drawbacks of using single cell eukaryotic organisms or purified oils, as primary sources of these essential nutrients in larval production systems. It is important to understand the PUFA requirements of marine fish larvae and the optimal dietary ratio of polyunsaturated fatty acids (PUFA) for marine fish larvae. Danilowicz and Brown (1992); Ogawa and Brown (2001); and Johnston *et al*. (2003) considered the importance of culturing the coral reef ornamental fishes of marine origin for the aquarium trade, and in order to reduce overexploitation in the wild. They suggested that even though great efforts were made to rear marine ornamental fish in captivity, advancement is necessary for their successful larviculture. Tlusty (2002); and Wabnitz *et al*. (2003) pointed

out that among different marine ornamentals, greater than 90% were from wild-caught fisheries, because this appears to be cheaper than rearing fish. High fishing pressure due to marine ornamental trade can have a considerable impact on populations of the target species. Whitehead *et al*. (1986) mentioned that due to continuous collection of targeted marine ornamental species in Australia, there was decreased abundance of a particular species in the localised region. Hawkins *et al*. (2000) demonstrated that the overexploitation of the marine ornamental fishes resulted in depletion of many species in their wild habitat resulting in serious ecological imbalance. Tissot and Hallacher (2003) pointed out that in Hawaii that the density of aquarium fish species at a control site was higher than at collection sites. Shuman *et al*. (2005) showed that in the Philippines, both anemone and anemone fish density and size were significantly lower in exploited areas than in protected areas.

Sobhee (2004) showed that the socioeconomics of ornamental fishery are important to understand, because they are significantly linked with environmental degradation. A study in Mauritius showed that environmental degradation caused by overexploitation of ornamental fisheries was linked with low educational level of the local fisher communities. Barbier (2010) demonstrated that environmental degradation in the marine coral ecosystem and depletion in the wild stock of the ornamental fish species in the developing countries is closely related to poverty. Even though the so-called "poverty-environment trap" does not fully describe all facets of this relationship correctly, it is clear that communities without access to other income than natural resource use of the commons, are prone to a vicious cycle of overexploitation and further degradation the environment.

2.3. Feeding

Though the commercial culture of *A. ocellaris* is entertained worldwide, its larval production and development has many difficulties like many other marine fish larvae. The

farming of marine ornamental fishes faces several challenges including growth, survival and physical development, especially at earlier stages during embryo development, hatching and transition from endogenous to exogenous feeding of larvae Olivotto *et al.*, 2011). Marine fish larvae have rudimentary digestive system so their uptake and utilization of essential nutrients are very difficult. The most common perception is that feeding larvae with live feed is easily digestible and become vital than the formulated feed since most of the larvae are altricial (born helpless) in nature. The nutritional quality of the diet offered to captive marine fishes are important during the first few weeks of larval life (Das *et al*., 2012). It is a well-known fact that lipid nutrition is particularly important for marine fish (Bell and Sargent, 2003; Carla *et al*., 2012; Copeman *et al*., 2002) since deficiencies in these molecules can cause a general decrease of larval health, growth, feed efficiency, anaemia leading to high mortality (Faulk and Holt, 2005; Sargent *et al*., 1999). The dietary requirements of HUFAs from the *n*-3 family for marine fish larvae has long been known (Sargent *et al*., 1999) and in recent decades, a great amount of research has been directed towards the development of food enrichments (with particular emphasis on lipids) to improve larval fish survival and growth (Sargent *et al*., 1999).

Shields *et al*. (1999); Villalta *et al*. (2005) demonstrated that in the wild, marine fish larvae mainly feed on copepod nauplii and copepodites, which are naturally rich in essential fatty acids (EFA). They mentioned that the nutritional enrichment of EFA to the fish larvae were provided through the most widely used live prey of rotifers and *Artemia* nauplii. Holt (2003), suggesting that the use of unenriched live prey during the initial period of the fish larvae is not preferable advised. This is because those live prey species usually donot provide an adequate fatty acid combinations. So, they concluded with the statement that the need of proper enrichment of live prey were compulsory before being fed to the larvae. Carla *et al*. (2012), Andersen *et al*. (2000), Ibabe *et al*. (2004), Batista-Pinto *et al*. (2009), and Grimaldi

(2007) demonstrated that the highly unsaturated fatty acids (HUFAs) are able to act directly on the genome, via specific nuclear receptor, such as, the peroxisome proliferator activated receptors (PPAR -α, -β, and γ). Burdick *et al*. (2006) pointed out that the nuclear receptors are involved in several biological processes, such as, skeletal development during ontogenosis, adipogenesis, lipid homeostasis, lipid metabolism regulation, lipid transport, lipid and glucose oxidation, peroxisomal biogenesis, immune functions, cell proliferation, and epithelial cell differentiation. Olivotto *et al*. (2003, 2005, and 2006) demonstrated that the importance of essential fatty acids in larval diets of ornamental species, such as, the yellow tail damselfish (*Chrysiptera parasema*), cleaner goby (*Gobiosoma evelynae*) and sunrise dotty-back (*Pseudochromis flavivertex*) were subjected with a HUFA enriched diets. They noticed in an improvement of larval survival and growth. McPherron *et al*. (1997) demonstrated that the fish growth was controlled by the polypeptides called insulin, were termed as growth factors I and II (IGFI, IGFII). Acosta *et al*. (2005) pointed out that the other growth factor called as myostatin (MSTN), a member of the transforming growth factor-β (TGF-β) family, was a negative factor that inhibits myoblast proliferation and noticed in mice. They concluded that these growth factors IGFs and MSTN might modulate the gene expression and control larval growth by an enriched diet.

Avella *et al*. (2007) suggested that the formulating specific diets had been one of the main goals to enhance the captive production of tropical marine species for the aquarium trade. Fatty acids are most important limiting factor to improve the culture of one of the preferred ornamental fish species, the false percula clownfish. Ali *et al*. (2007) suggested that the weight growth of larvae showed a significant difference for the rotifers and *Artemia* feeding phases. The maximum survival rates noticed in cod liver oil enriched rotifer fed to the larvae. Umur*et al*. (2008) showed that the yolk sac was completely consumed within 5 - 7 days at 25 °C. Clownfish larvae was found to readily accept live feed rotifers after hatching

and a complete dietary shift from rotifer to *Artemia* that wasaccomplished at 10 days after hatch (DAH). Kotani *et al*. (2010) mentioned that the nutritional enrichment for rotifer is necessary for the stable production in larviculture. Although manufacturers recommend feeding amount for enrichment diets, the effects of these recommended amounts on nutritional enrichment of rotifer are unknown. Sargent *et al*. (1989) and Olivotto *et al*. (2011b) mentioned that the *n*-3 essential fatty acids, such as, eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3), were extremely important in larval diets for fish larval growth and survival. Sargent *et al*. (2002) suggested that during the development of marine finfish larvae, it is more important to ingest essential fatty acids (EFA) in the diet. This is because marine fish are unable to synthesize these fatty acids from their precursors 18:3*n*-3 and 18:2*n*-6. Sargent *et al*. (1999) and Luis*et al*. (2010) suggested that the major restricted access in fish farming is the natural deficiency in essential fatty acids (EFA) of live prey. The positive results were noticed by feeding EFA enriched live prey diets to the larvae.

Rainuzzo *et al*. (1997) suggested that the lipids in brood stock nutrition considered an important for the quality of the larvae and the PUFA are of importance for the essential structure of living system and for the regulation of life process. Baker *et al*. (1998) showed that the enrichment of live food for marine fish larvae with highly unsaturated fatty acid (HUFA) improved the survival, growth and stress resistance in many species. He concluded that the growth and metamorphosis were directly correlated with levels of the DHA in the diet. Gapasin *et al*. (1998) studied the effect of essential fatty acid and vitamin C enriched live food on growth, survival, resistance to salinity stress and incidence of deformity in milkfish larvae reared in tanks. They demonstrated that the effect of HUFA enrichment in enhancing milkfish larval growth and resistance to salinity stress but not overall survival. Copeman *et al*. (2002) studied the role of dietary ratios of docosahexaenoic acid,

eicosapentaenoic acid and arachidonic acid on early growth, survival, lipid composition, and pigmentation of yellowtail flounder. He suggested that the strong relationship observed between the DHA/EPA ratio in the diet and larval survival. He concluded that the yellow tail larvae required a high level of dietary DHA for maximum growth and survival. Cavalin and Weirich (2009) studied the larval performance of Florida pompano (*Trachinotus carolinus*) fed rotifer (*Brachionus plicatilis*) under captivity. The rotifer enriched with selected commercial diets and compared based on growth, survival, and fatty acid content of larval Florida pompano. They observed that similar oil emulsions such as Super Selco affect the amino acid composition of the prey.

Castillo *et al*. (2009) studied to evaluate the changes in lipid and essential fatty acid contents of the rotifer *Brachionus plicatilis* fed with freeze dried cells of tropical thraustochytrid *Schizochytrium mangrovei*. They suggested that the proper amount of enrichment product and the duration of enrichment in boosting the DHA contents of rotifers would effectively ensure a reliable production of nutritionally superior at the minimal cost. Hache and Plante (2011) observed a reduction of total PUFAs and an increase of total SFAs and MUFAs in enriched rotifers reduced the growth and survival of marine fishes. They concluded that the PUFA enrichment diets were necessary for the better growth and survival of marine fishes since they cannot able to synthesize several long chain fatty acids naturally. Takaoka *et al*. (2011) demonstrated that the pathogenic bacteria *Vibrio anguillarium, Edwardsiella trada, Aeromonas salmonicida* and *Pasteurella piscida* causes heavy losses on farms and suggested that somepathogens invaded fish larvae and juveniles *via* rotifer feeding. The treatment of rotifer cultures with antibiotics proved the effective in reducing infectious disease. He revealed that the *Crataegi fructus*, *Artemisia capillaries* and *Cnidium officianle* and their mixture in rotifer was useful to promote growth and resistance against *Vibrio anguillarium* in red sea bream larvae. Olivotto *et al*. (2011) observed the live prey

enrichment, with particular emphasis on HUFAs, as limiting factor in false percula clownfish larval development and metamorphosis. The HUFA administration may improve larval development through the presence of better-structured central position in the metabolism, with respect to controls.

Khairy (2012) studied the growth, development stages and survival rates of *Sparus aurata* larvae fed with rotifers and *Artemia* enriched with microalgae *Tetraselmis chuii,* theirresult concluded that the Rotifer (*Brachionus plicatilis*) and *Artemia* (*Artemia salina*) enriched with microalgae *Tetraselmis chuii* grown on was effective for good growth and survival rate of *Sparus aurata* larvae. Kaplan *et al*. (1986) pointed out the cultivating the alga in outdoor cultures stayed monoalgal and free predators as long as the temperature did not fall below 19 $^{\circ}$C. The cultures maintained at 27 $^{\circ}$C low light adapted cultures were sensitive to photodynamic damage. Not all algae, however, conferred good growth results on marine fish larvae and found that in beneficial algae such as Chlorella species. Howell (1979) observed that the fish larvae grew and developed well when the larvae fed by rotifer enriched with microalgae (*Paviova lutheri, Isochrysis galbana* or *Phaeodactylum tricornutum*). Watanabe *et al*. (1983b) pointed out that the microalgae *Tetraselmis tetrathele* and *Isochrysis galbana* contained greater quantity of unsaturated fatty acids (HUFA) especially, 20:5*n*-3 and 22:6*n*-3. The larvae fed with on rotifers enriched with microalgae (*Dunalliela tertiolecta*) showed stunted growth and high mortalities. Sargent *et al*. (1999) pointed out that the ideal marine fish larval diet is one containing about 10% of the dry weight as *n*-3 HUFA-rich, marine phospholipids with less than 5% triacylglycerols, as exemplified by the lipid compositions of marine fish egg yolk. The marine fish larvae increased these levels by consuming their selective prey themselves from the wild. Such diets provide 22:6*n*-3, 20:5*n*-3 and 20:4*n*-6 in the desired levels and ratios, which simultaneously satisfied the known requirements for phospholipids, inositol, and choline.

Park *et al*. (2006) pointed out that the docosahexaenoic acid (DHA) affected the growth and survival of marine finfish larvae. From their study, a positive effect noticed in the growth and survival of cod larvae fed with rotifer enriched by high DHA proportions and they demonstrated that Atlantic cod larvae require a high ratio of dietary DHA to EPA. Coutteau and Sorgeloos (1997) suggested that the manipulation of dietary lipids, fatty acids, and vitamins in zooplankton cultures live prey organisms is required for larval growth. The live prey particularly rotifer and *Artemia*, can be bio-encapsulated with a variety of enrichment diets to manipulate their content in certain nutrients, including *n*-3 highly unsaturated fatty acids (HUFA) and the vitamins C, A and E. Stefanov *et al*. (1997) and Navarro *et al.* (1999) studied the lipid conversions during *Artemia* enrichment. *Artemia nauplii* were enriched for 24 h with radiolabeled fatty acid ethyl esters and then starved for a subsequent period of 24 h. The proportions of radioactivity recovered in free fatty acids and phospholipids increased during the starvation period, which indicated the mobilisation of fatty acids from triacylglycerols for use in catabolism and in the formation of bio-membrane lipids. From these results, the distribution pattern of radioactivity from [U– ^{14}C] 22:6 (*n*-3) in the fatty acids of the nauplii demonstrates that *Artemia* are capable of converting 22:6 (*n*-3) to 20:5 (*n*-3).

Penglase *et al*. (2011) used rotifers as the first feeding diet for the larvae of many commercially marine fish species. However, the nutritional requirements of marine fish larvae appeared to fulfil by their wild feed, copepods. The copepod fed fish larvae still have higher growth rates and survival than rotifer fed larvae. This may occur because of the less investigated mineral differences that exist between rotifers and copepods. Gopakumar *et al.* (2008) pointed out that since 1980s the major expansion of marine finfish and shellfish aquaculture increased globally due to the improvement of innovative techniques for mass production with advancement in nutritional bio-enrichment protocols of live feed that could

act as a key role to the bottleneck problem of larviculture. Gopakumar *et al*. (2009a) concluded with the experiment that the ornamental fish larvae were unable to accept rotifers as starter feed, which resulted in total mortality of the larvae. The absence of any mortality from this stage onwards indicated that if the larvae could fed with suitable feed initially, the larviculture of the marine ornamentals could be accomplished easily with conventional live feeds. Gopakumar (2009b) demonstrated that the live prey during the initial feeding period played an important role for the better growth and survival of the marine fish larvae and suggested that the negative impact of growth and survival of fish larvae were noticed by feeding them with rotifer. The successful results were obtained by feeding with egg bearing copepods and their nauplii during the initial starter feeding trial, which could be considered as an appropriate live feed with the required amount of essential fatty acids.

Ruyet *et al*. (1993) worked on replacement of acceptance of live prey with formulated diets in marine larviculture. They pointed out that the total replacement of live prey is still impossible in marine fish. They concluded that better results could be obtained when formulated diets were used in combination with live prey from first feeding. Maehre *et al*.(2013) examined the biochemical composition of intensively reared rotifers after enrichment with three commonly used enrichment media, Multigain, Ori-Green and DHA-enriched freshwater algae Chlorella. From their study, they demonstrated that the enrichment media currently in use were not effective for enhancing the nutritional quality of rotifers. Mahmoudzadeh *et al*. (2009) showed by an experiment, which was designed to sustain *Coregonus lavaretus* production, and examined the efficacy of a live organism (*Brachionus plicatilis*), which was an artificial diet (48% crude protein), and a mixed feed for the first 3 month of rearing fry. Their results showed that fry utilized live organisms more efficiently than the artificial and mixed diets.

Villalta *et al*. (2008) examined the effect of dietary eicosapentaenoic acid (EPA, 20:5*n*-3) on growth, survival, pigmentation and fatty acid composition of Senegal sole larvae. They concluded that Senegal sole larvae had a very low EPA requirement during the live feeding period. Mandal *et al*. (2010) tried partial replacement of live feed with formulated feed in *Bettasplendens* to evaluate their growth, survival, and reproductive performance. From their study, they concluded that the live feed can be successfully replaced to the extent of 25% by formulated feed without any detrimental effect on the growth, survival and spawning performance of *B. splendens.* Ferreira *et al*. (1999) demonstrated that the overproduction of gilthead seabream *Sparus aurata* L. egg in commercial hatcheries was not successful. They investigated the potential use of fresh and freeze-dried gilthead seabream eggs for enrichment of live prey. From their data they concluded that there was a need to have an overall perspective of the nutritional requirements of the organisms in culture Zhang *et al*. (2009) worked on the effects of the timing of initial feeding on the growth and survival of spotted mandarin fish (*Siniperca scherzeri*) larvae. They suggested that the first feeding of *S. scherzeri* larvae should be initiated at 2 days after hatching for achieving good growth and survival. Wold *et al*. (2008) studied the development of the gut epithelium in cod *Gadus morhua* during the larval period in intensive rearing systems. Cod larvae fed with enriched rotifers from mouth opening. On 17 days post-hatch one group of larvae fed *Artemia* nauplii while another group was fed with both rotifers and a formulated diet (co-fed). At the end of the experiment (30 DPH) the larvae receiving live feed were almost three times larger than the co-fed larvae. From this work, they suggested that the gut tissue was flexible and could withstand periods of suboptimal nutrition at this stage.

Callan *et al*. (2012) conducted experiment to determine the effects of dietary highly unsaturated fatty acids (HUFA) on flame angelfish (*Centropyge loriculus*) reproduction, and egg and larval quality. Their data revealed that flame angelfish egg quality could respond

rapidly (within weeks) to maternal dietary changes. From their study, they suggested that dietary HUFA composition could significantly affect brood stock reproductive performance as well as subsequent performance of eggs and larvae. Garcia *et al*. (2008) conducted a feeding experiment to evaluate the effect of rotifers (*Brachionus plicatilis*) enriched different diets (Algamac 2000, Aqua grows and a combination of *Pavlova* sp paste and Algamac 2000) on early growth, survival and lipid class composition of Atlantic cod larvae *(Gadus morhua*). The results revealed that the larvae fed by rotifer enriched with *Pavlova* sp paste and Algamac 2000 was significantly heavier than the other treatments. They suggested that the high larval concentrations of docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (AA) and *n*-6 docosapentaenoic acid (*n*-6 DPA) could be linked to better larval growth and survival.

Tamaru *et al*. (2011) examined the use of live and preserved algal (T-Iso and *Nannochlropsis* sp) products and their utilities in supporting growth and survival of the feather duster worm, *Sabellastarte spectabilis*. From their work they pointed out that the live and preserved T-Iso resulted in the highest survival compared to other treatments. Rezek *et al*. (2010b) determined the effects of the dietary docosahexaenoic acid to arachidonic acid (ARA) ratio on the survival, growth, hypersaline stress resistance, and tissue composition of black sea bass larvae raised from first feeding to metamorphic stages. Their results suggested that black sea bass larvae fed prey containing 10% DHA with increasing ARA within the range of 0.6% showed improved growth and survival from first feeding through metamorphic stages. Ma and Qin (2012) compared the efficacy of four products that are commonly used in hatcheries for nutritional enhancement of rotifer *Brachionus plicatilis* as the starter food for yellowtail kingfish *Seriola lalandi* larvae. Their study indicated that fresh algae could be replaced with S.presso, and Algamac 3050 was not as good as other formula for rotifer enrichment in rearing yellowtail kingfish larvae in this system. Gago *et al*. (2010) evaluated

the potential of eggs and endotrophic larvae of captive *Paracentrotus lividus* as alternative live prey for marine fish larvae first feeding. They concluded that, in spite of the alternative live prey readily consumed by all tested fish larvae, they could not compete with rotifers in marine fish larvae first feeding. Villegas *et al*. (1990) pointed out that the food value of *Brachionus plicatilis* fed marine microalgae *Tetraselmis tetrahele*, *Isochrysis galbana* and marine *Chlorella* sp as live food for milkfish, *Chanos chanos* fry production. Their results demonstrated that the *T. tetrahele* and *I. galbana* improved the food value of *Brachionus plicatilis* as live food for the milkfish fry.

Rimmer *et al*. (1994) showed that the larval and juvenile barramundi, *Lates calcarifer* reared intensively on test diets comprising nutritionally supplemented and unsupplemented rotifers, *Brachionus plicatilis* and brine shrimp, *Artemia salina* had good nutritional threshold. Both growth and survival of barramundi larvae fed on nutritionally supplemented brine shrimp were superior to those of larvae fed on untreated brine shrimp. However, with the rotifer supplements, only a minor increase in growth rate was noticed and there was no apparent beneficial effect on survival, and thus therefore was of little practical use in barramundi hatcheries. Barnabe and Guissi (1994) demonstrated that the use of rearing of sea bass *D. labrax* using five different feeding regimes, including two combining immobilized live prey and microparticles. The author further demonstrated the use of inert rotifers and microparticles towards the survival rates analogous to those obtained with live prey. Estevez and Kanazawa (1995) showed that the emulsions with different content of *n*-3 PUFA and vitamin A used to enrich *Artemia* and examine the effect of these nutrients on pigmentation success in turbot, *Scophthalmus maximus* (L.). The better pigmentation obtained by using an overdose of vitamin A, but coincided with a high incidence of skeletal deformations and higher growth and pigmentation rates achieved by increasing the quantity of *n*-3 PUFA oil in the emulsion. The use of *n*-3PUFA-deficient diet caused the highest occurrence of albinism

as well as a cessation of metamorphosis. Takii *et al*. (1995) showed that the hatching rates and hatching times of red sea bream, *Pagrus major*, reared in culture media prepared by the eggs. Their results suggested that hatching enzyme secretion from the hatching glands was promoted to perceive the chemical stimuli in the water soluble fraction of rotifer extract via the embryo's chemoreceptors, from the heart-beat initiation stage to immediately before hatching, thus shortening the hatching time.

Berntson *et al*. (1997) worked on effects of broodstock diets on fatty acid composition, survival and growth rates in the larvae of European flat oyster, *Ostrea edulis.* They suggested that a fast assay for essential PUFA like 22:6 *n*-3 might significantly improve management of commercial bivalve hatcheries. Baker *et al*. (1998) studied the effects of rotifer and *Artemia* fatty acid enrichment on survival, growth, and pigmentation of summer flounder *Paralichthys dentatus* larvae. They demonstrated that the enrichment of live food for marine fish larvae with highly unsaturated fatty acids (HUFA) shown to improve survival, growth, and stress resistance in many species and to decrease pigmentation abnormalities in flatfish. Theilacker and Mcmaster (1971) suggested that the laboratory-cultured rotifers, fed in high concentrations, appear to be a nutritious food source for anchovy larvae. The larval anchovy early growth rate on a diet other than that of wild plankton equalled and exceeded that of larvae fed on wild plankton. Houde (1973) suggested that the growth and survival of fish larvae were enhanced when a phytoplankton bloom has been maintained in the rearing system. Larvae grow and survive better in large culture tanks than in smaller ones. Lubzens *et al*. (1986) pointed out that the synthesis of fatty acids in the rotifers, *Brachionus plicatilis* depend on that oftheir food. In order to supply large amounts of PUFA to marine fish larvae, rotifer should be enriched with PUFAs rich source.

According to Ketchum (1939) the nutrient requirements of algae may be absolute, normal, minimum, or optimum. Although many media were devised for culturing different algae, the experiment by Ketchum (1939) proved that no single medium can be said to be the best one. A clear understanding of the nutritional requirement of members of various classes of microalgae is therefore a prerequisite for determining the technique of culturing and maintaining the algae for a long period. Gopinathan (1986) demonstrated in their experiment that no available culture medium was uniformly effective in all the cases, unless supplemented with either trace elements or vitamins, according to the specific requirement of the alga. Stottrup and Attramadal (1992) demonstrated that the turbot larvae fed with three different rotifer enrichment diets, Dry Selco, Protein Selco, and ICES low-HUFA (an enrichment emulsion containing low amounts of highly unsaturated fatty acids-HUFA) had no significant effect on larval growth and survival. Chang *et al*. (2006) suggested that weaning of *Cynoglossus semilaevis* from early development would appear to be feasible and larval co-feeding with live and inert diets improves growth and survival. Madhu *et al*. (2012) developed the broodstock development, breeding, embryonic development and larviculture of *Premnas biaculeatus* under different feeding and environmental conditions for the first time. The higher survival rate (78.9%) was observed in larvae fed with enriched *Brachionus rotundiformis* (0 to 20 days) followed by co-feeding with newly hatched *Artemia nauplii* (from 9 to 20 days) of post-hatch whereas lowest survival rate (30.2%) was observed in groups fed exclusively on the larger non-enriched rotifer *Brachionus plicatilis* and *Artemia* nauplii.

Ignatius *et al*. (2001) reported two types of combination of live feed (rotifer and *Artemia*; rotiter and copepods) that were tried to rear clownfish (*Amphiprion sebae*) larvae. Higher survival and growth observed in the combination of rotifer and copepod, which signified the suitability of copepods to *Artemia.* Dhaneesh *et al*. (2012) suggested that the

first feeding of the clownfish *Amphiprion percula* larvae could be initiated before 12 hour, for better growth and survival. Madduppa *et al*. (2014) showed that the clown anemonefish 'Nemo' *Amphiprion ocellaris* was the most popular fish species in the global marine ornamental trade. Castell *et al*. (2003) showed the effect of different HUFA enrichment emulsions on the nutritional value of rotifers (*Brachionus plicatilis*) fed to larval haddock (*Melanogrammus aeglefinus*), suggesting that all treatments provided the minimal essential fatty acid requirements for haddock. Divya *et al*. (2011) showed that the clownfish larvae in the first feeding period required small sized food and those algae might play an important role during this period and rotifers are an adequate starter diet for the first larval stage of the clownfish larvae.

Cassiano *et al*. (2012) suggested that porkfish is an ideal candidate species for existing aquaculture technologies. Duray *et al*. (1997) documented that the larval growth and survival rate were improved when larvae were fed with *Brachionus* (less than 90 μm) during the first 2 weeks. Survival was even better among larvae fed *Brachionus* until the day 35. Zhukova and Aizdaicher (1995) analyzed the fatty acid composition of 15 marine microalgal species, and demonstrated that each class of microalgae is characterized by a specific fatty acid profile. Yufera *et al*. (2000) showed the use of an inert food to replace live prey during the early stages of gilthead seabream *Sparus aurata* larvae. They concluded that the microcapsule was able to efficiently support the growth and development of the larvae, at least during the first 2 weeks of life, although the larvae still need to feed on the rotifers during first 2-4 days of exogenous feeding. Najdek *et al*. (2002) demonstrated that the aggregates of the inert feed modified during their stay in the water column. Among the phytoplankton, diatoms appeared to have a dominant role in the 'life' of all types of aggregates.

King *et al*. (2002) suggested that the use of algae stored up to four weeks for feeding rotifers was an intended practice, since the food source for marine larviculture seems viable more than four weeks, and the percentage of total unsaturated fatty acids were found to be decrease thereafter. Gatenby *et al*. (2003) demonstrated that the lipid contents of two green algae, *Neochloris oleoabundans, Bracteacoccus grandis*, and one diatom, *Phaeodactylum tricornutum*, were different at different growth stages, and were identified as the potential diets for rearing captive freshwater mussels. From these results, they suggested that there was no effect of growth stage or species on total fatty acids. Wen and Chen (2003) studied the heterotrophic production of eicosapentaenoic acid by microalgae, and suggested that the EPA production potential of microalgae depends on the characteristics of the specific algal species and the cultivation strategies developed. López Elías *et al*. (2003) suggested that the outdoor biomass production and composition of microalgae were related to the type of culture containers and consequent differences in light penetration.

Chuan *et al*. (2003) suggested that better larval performance and an exponential increase in the production yield of ornamental fish larvae was achieved by using of freshwater rotifer *B. calyciflorus* in the early larvae stages. Ikawa (2004) mentioned the importance of algal polyunsaturated fatty acids and their effects on plankton ecology. Roncarati *et al*. (2004) showed the production of microalgae using controlled intensive culture methods, such as, indoor batch culture, whereas the carbon dioxide concentration improved the quality of biomass composition. Pratoomyot *et al*.(2005) suggested that among the ten microalgal species, *Nitzschia* cf. *ovalis* and *Thalassiosira* sp would serve as good nutritional sources of HUFAs for aquaculture animals. Shei *et al*. (2012) suggested that barber goby (*Elacatinus figar*) should be fed with the *n*-3 HUFAs enriched rotifer in order to maximize juvenile production.

Patil *et al*. (2005) showed that the microalgae might have superior lipid stability compared to traditional PUFAs because it microencapsulated by the algae cell wall. Asha and Muthiah (2006) suggested that *Chaetoceros calcitrans* itself or in combination with *Isochrysis galbana* is the effective feed for the growth and development of the larvae of sea cucumber *Holothuria spinijera*. Patil *et al*. (2007) pointed out that *Isochrysis galbana* and *Nannochloropsis oceanica* are the best candidate microalgae for use in the aquaculture feed as live feed and in formulations. Mahmoudzadeh *et al*. (2009) showed that the rotifer *Brachionus plicatilis* was a good live feed to rear the fry of *Coregonus lavaretus*. They pointed out that the fry utilized live organisms are more efficient than the fry fed with artificial and mixed diets.

CHAPTER 3

Long chain *n*-3 polyunsaturated fatty acid enriched oil emulsion from sardine oil

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Long chain n-3 polyunsaturated fatty acid enriched oil emulsion from sardine oil

3.1. Background of the study

Fatty acids are carboxylic acids with long hydrocarbon chains (usually C_{12-22}). Dietary fats are used to build every cell in the body and cell membranes are made of a variety of individual fatty acids. The essential fatty acids from marine fish have protective mechanisms againstcoronary heart disease, which became apparent in the investigations of the health status of Greenland Eskimos who consumed diets very high in fat from seals, whales, and fish, and yet had a low rate of coronary heart diseaseevents. This paradox was explained by the fact that Eskimos consumed contained large quantities of the very-long-chainand highly polyunsaturated fatty acids with C_{20-22} carbons and 5-6 olefinic bonds, which are abundant in marine fish, and are scarce or absent in land animals and plants. Fatty acids with ≥ 2 double bonds are termed as polyunsaturated fatty acids (PUFAs). PUFAs are broadly divided into two major families', n-3 and n-6 PUFAs. However, long chain C_{20-22} n-3 fatty acids are found to be abundantly available in marine sources particularly fish and phytoplanktons.

These fatty acids affect many physiological processes including cognitive function, visual acuity, immunosuppressive, and anti-thrombic activities along with having major role on glucose and lipid metabolism. The n-3 and n-6 long-chain polyunsaturated fatty acids (LC-PUFAs), viz., eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3) are essential fatty acids in the diet of a majority of marine finfish and crustaceans, especially for the larvae and broodstock because they cannot synthesize it de novo from precursor molecules. Diets deficient in these PUFAs particularly DHA have been found to

have a negative effect on ovarian development, fecundity, and egg quality. The important natural sources of n3 LC-PUFAs are marine fish oils such as sardine, mackerel, cod, shark, and menhaden, which contain PUFA levels of about 30%. For this reason, marine fish oils are preferentially used as raw material to prepare *n*3 PUFA concentrates. However, they contain relatively high levels of triacylglycerols and sterols, which are too heterogeneous, rendering it difficult to isolate individual fatty acids efficiently with a single separation method. Additionally, it was reported that *n*3 PUFAs were moderately absorbed by the intestine as triglycerides and most promptly absorbed when free fatty acids (FFA) were given orally (Bottino *et al*., 1967; Henderson *et al*., 1993). Therefore, it is convenient to prepare *n*3 concentrates as FFA after chemical hydrolysis of marine oils.

The technologies available for purifying individual PUFAs and PUFA concentrates from fish oil are based on differences in physicochemical properties associated with the number of double bonds in the molecule or the chain length like urea complexation method (Marschner, 1995). Recently, argentation silica gel column chromatography has been employed to obtain high purity EPA methyl ester from hydrolysates of fish oil that contained other polyunsaturated fatty acid esters including linolenic acid (18:3*n*3) and oleic acid (18:1*n*9) (Guerrero and Belarbi, 1983; Haagsma *et al*., 1982). Partial success in purification of linolenic and oleic acid has been achieved by using a mixture of acetonitrile and propionitrile (1:2) at -60 ^{0}C (Arudi *et al*., 1983). Argentation silica gel chromatography of urea inclusion adducts from cod liver oil yielded highly pure DHA in the process (100% purity, 64% yield), while for EPA, the recovery in the combined process was 29.6%, and the final purity was 90.6%.

The recovery in the urea inclusion method was strongly enhanced by application of orbital agitation during the crystallization process, in which EPA yield increased from 60-

70% without agitation to 90-97% at 800 rpm (Guerrero and Belarbi, 2001). EPA and DHA have been concentrated from shark liver (*Isurus oxyrinchus*) in one single step, in which fish liver oil was simultaneously extracted, saponified, and concentrated. Additionally, the PUFA concentrate was winterized to crystallize the remaining saturated fatty acids, resulting in a further increase in the concentration of DHA and EPA (Guerrero *et al*., 2007). The polyunsaturated fatty acids EPA and AA have been purified from the red microalga *Porphyridium cruentum* by the urea inclusion method followed by silica gel column chromatography of the urea concentrate. Total AA and EPA recoveries reached 39.5% and 50.8%, respectively, for purity and approximately 97% for both fatty acids (Guerrero *et al*., 2000).

Solvent winterization of seed oil and free fatty acids (FFAs) was employed to obtain ç-linolenic acid (GLA) concentrates from seed oils of two Boraginaceae species, *Echium fastuosum* and *Borago officinalis*. Different solutions of seed oils and FFA from these two oils at 10%, 20%, and 40% (w/w) were crystallized at 4, -24, and -70 ^{0}C, respectively, using *n*-hexane, acetone, diethyl ether, isobutyl alcohol, and ethanol as solvents. Best results were obtained for *B. officinalis* FFAs in *n*-hexane, reaching a maximum GLA concentration of 58.8% (Martinez *et al*., 2004). Also, there are reports for the production of EPA or DHA concentrates by a combination of techniques such as molecular distillation, fractional distillation, liquid chromatography, and supercritical fluid extraction (Guerrero *et al*., 2000). PUFAs are widely available in a large variety of marine organisms such as microalgae, polychaetes (Vazhappilly and Chen, 1998; Mansour *et al*., 2005), finfish, and shellfish, but sardine oil is easily available and inexpensive and contains a considerable amount of PUFAs (33.26%) essential for larval and broodstock nutrition in marine aquaculture. The present chapter highlights a method for purification of sardine fatty acids with the goal to get a PUFA concentrate with high DHA.

3.1.1. Why long chain n-3 polyunsaturated fatty acids are important?

Research on exploring sources long-chain C_{20-22} PUFAs (LC-PUFAs), such as, eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3) for use in nutrition have received considerable attention (Figure 3.1.). These PUFAs, which are usually low in abundance in fish and their larvae, are regarded as essential and must be supplied in diet. The importance of PUFAs in fish nutrition has been extensively investigated during the past 20 years. DHA is one of the important PUFAs, which maintains structural and functional integrity in larval cell membranes in addition to the neural development and function, while arachidonic acid (AA, 20:4*n*-6) and EPA are involved in, respectively, the production and modulation of eicosanoids. DHA is a vital component of the phospholipids of cellular membranes, and is necessary for their proper functioning. The *n*-3 fatty acids are necessary from conception through pregnancy and infancy and, undoubtedly, throughout life. An imbalance in *n*-3/*n*-6 ratio can accentuate *n*-3 fatty acid deficiency state, as shown by earlier studies. The ratio may have increased in industrialized societies because of increased consumption of vegetable oils rich in *n*-6 fatty acids, i.e., linoleic acid (18:2*n*-6), and reduced consumption of foods rich in *n*-3 fatty acids. Another important feature of *n*-3 fatty acids is their role in the prevention and modulation of certain fish diseases that are common. The long chained (LC) PUFAs are also recognized to have beneficial therapeutic, physiological, and nutritional effects on fish health. The present work aims at concentrating LC-PUFAs for use as health food supplement and for mariculture purposes.

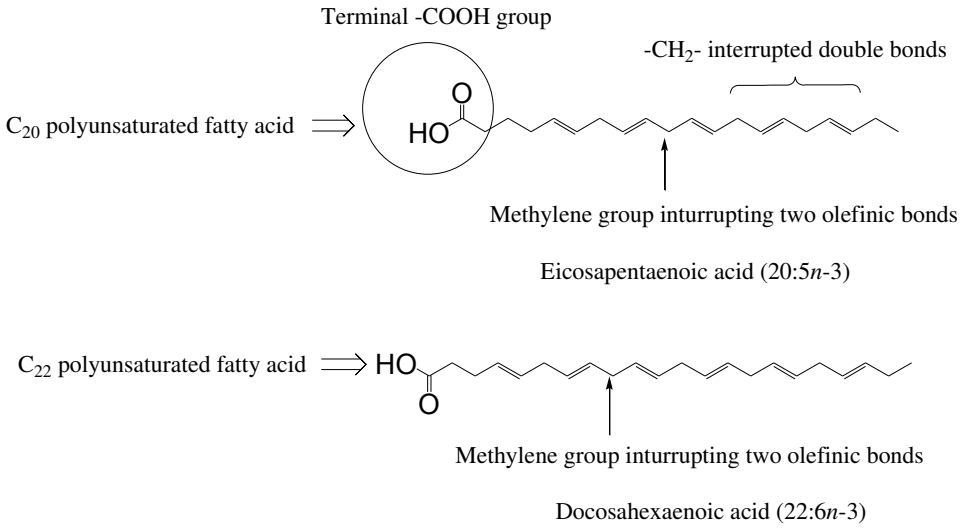


Figure 3.1. $C_{20\text{-}22}$ polyunsaturated fatty acids (eicosapentaenoic and docosahexaenoic acids)

3.1.2. Imported products (PUFA supplement)

Seven Seas are a UK healthcare company at the forefront of nutritional science and production technology for PUFA rich Cod Liver Oil by Ocean Gold™ technology. As a rich natural source of the most effective type of *n*-3 fatty acids (EPA and DHA) sales of Cod Liver Oil have paralleled the phenomenal growth of Omega-3 popularity. A value-added PUFA concentrate named "fish oil-1000 natural omega 3®" containing 100 capsule manufactured by Healtheries of New Zealand Ltd. priced about Rs. 1150/- per pack, and is currently being marketed in India by Perma Healthcare, Bangalore. "EPAX 1050 TG®", a marine omega 3 formula produced from selected marine oils, and marketed by EPAX AS, Norway is a product containing 17% EPA. This product is also being sold at very high prices and imported to India for use as fish feed supplements. OMEGA XL® marketed by DeColores Marketing is a bottle containing 60 capsules of refined combination of omega-3's costing about $50 per pack, and is being imported to India for nutraceutical purpose. Great Health Works manufactures a concentrate of PUFA from Blue Grenadier Fish that is also a costly PUFA supplement. DSM Nutritional Products, Switzerland manufactures and sells

PUFA concentrate under the trade name ROPUFA® produced from refined vegetable and marine oils. These products also currently being imported to meet the domestic demand of PUFA concentrate formulation for fish feed supplements and nutraceutical purpose.

3.1.3. Need to have an indigenous product: Status and scope of indigenous n-3 polyunsaturatated fatty acids (PUFA) supplement

Locally available low-value fishes were screened for n-3 PUFAs, concentrating these essential fatty acids therefrom by chemical and/or enzymatic process. This study carried out contemplated to the development of indigenous n-3 PUFA enriched formulation(s) comprising fatty acid concentrate and individual or combination of additives with potential antioxidant properties to form a stabilized and concentrated form of long chained n-3 PUFAs. The PUFA enriched product may be a cheaper alternative to the imported PUFA supplements.

Their most important application of these LC-C_{20-22} PUFAs are in mariculture is larval nutrition. It is believed that the optimal formulations for first-feeding larvae should simulate the yolk composition and to some extent reflect the nutrient requirements and metabolic capacities of pre-feeding fish. Dietary long chain PUFAs play an important role as vital sources of essential fatty acids, needed for normal growth and survival. Larvae of many marine fishes are believed to require highly unsaturated fatty acids of the *n*-3 series, such as, DHA due to the absence of essential enzymes required for biosynthesis of C_{20-22} LC-PUFAs from their short chain analogues (Figure 3.2.). Some investigations have shown that DHA is superior to EPA for larval fish suggesting a different physiological function.

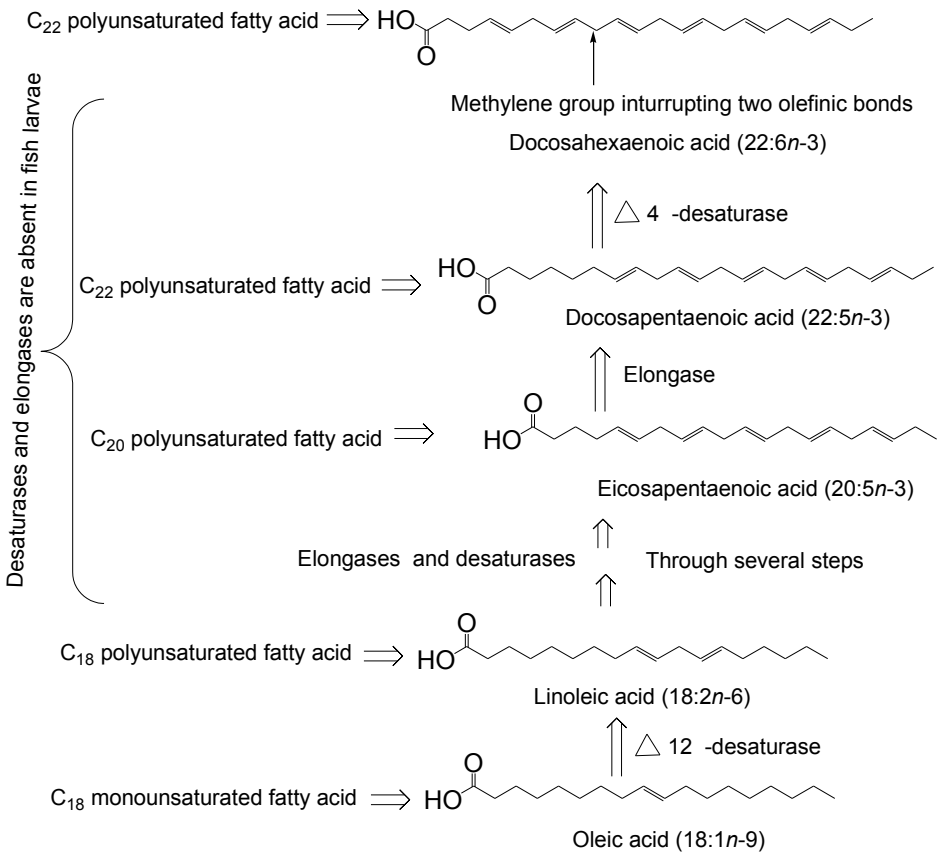


Figure 3.2. Biosynthetic pathways of the long chain polyunsaturated fatty acids (LC-PUFA). The desaturases and elongases catalyzing the formation of long chain C_{20-22} fatty acids are absent in fish larvae, and therefore required to be supplemented by external source for larval growth and development

The *n*-3 C_{20-22} LC-PUFAs are also essential fatty acids in the diet of marine fish larvae and broodstock because they cannot synthesize these *de novo* in sufficient quantity from the precursor molecules, such as, short-chain fatty acids. Therefore they need greater concentrations of these long chain fatty acids for their biological processes. The important natural sources of *n*-3 LC-PUFAs are various marine organisms, such as, mackerel, sardine, shark, microalgae, polychaetes, etc. Among these, sardine oil is inexpensively available and contains a considerable amount of PUFAs, particularly 20:5*n*-3. It is therefore that sardine oil was used as preferred raw material to formulate the *n*-3 C_{20-22} LC-PUFA concentrates (Figure

3.3.). The PUFA concentrate from sardine oil was prepared based on different physicochemical properties associated with the olefinic bonds in fatty acids and/or acyl chain length. The objective of this study is to purify EPA and DHA from sardine oil by saponification of fish oil to derive free fatty acids, enrichment of PUFA content from the mixed fatty acid concentrate by amide fractionation, and chromatography by utilizing the silica gel complexed with a d-block element.

The unique substrate specificity of microbial triacylglycerol acyl hydrolases has also been utilized for the enhancement of PUFA content in triglycerides to further enrich the C_{20-22} LC PUFAs. Triacylglycerol acyl hydrolases specifically hydrolyse carboxyl esters of triglycerides into free fatty acids and partial acylglycerols. The unique characteristics of this group of enzymes, such as, positional and stereospecificity were utilized to selectively concentrate targeted fatty acids in triglycerides that can be readily absorbed into plasma triglycerides.

Figure 3.3. Oil sardine used to prepare polyunsaturated fatty acid concentrate

3.1.4. PUFA enrichment by different physicochemical procedures

Marine fish larvae and broodstock lack the adequate titre of the essential enzymes (like elongase and desaturase) required to synthesise polyunsaturated fatty acids (PUFAs) de novo in sufficient quantity from precursor molecules like shortchain fatty acids. Therefore they require greater concentrations of PUFAs for their growth, reproduction and survival

(Cahu *et al*., 1994). Diets deficient in these PUFAs have been found to have a negative effect on ovarian development, fecundity and egg quality (Harrison, 1990). Since these physiological functions drew attention to these essential fatty acids, the production of PUFA-rich fish oil as a food material is a growing research area. For commercial exploitation of a high value added products such as PUFA concentrates, the acids must first be separated from their triglycerides and purified to a high degree based on differences in physico-chemical properties associated with the number of double bonds in the molecule or acyl chain length. Most of the existing chemical purification methods are based on hydrolysis of oils to free fatty acids; these methods are non-selective to different fatty acids. Lipases (triacylglycerol acyl hydrolases) are one of the most important classes of hydrolytic enzymes that specifically hydrolyse carboxyl esters of triglycerides into free fatty acids and partial acylglycerols.

Lipases have been used as animal feed supplement to increase bioavailability of PUFAs (Akoh *et al*., 1996; Huang & Akoh, 1994; Kosugi & Azuma, 1994). Chemical hydrolysis may partially destroy the natural all cis-PUFAs if the process is inadequately carried out due to the high temperatures involved (Heimermann *et al*., 1973). The mild conditions used in enzymatic reactions offer a promising alternative to chemical hydrolysis, avoiding the formation of undesirable oxidation products, polymers, and isomeric conversion of natural all cis-PUFAs to deleterious trans-PUFAs. The unique characteristics of lipases, i.e., substrate, positional (acyl side-chain and olefinic double bonds) and stereospecificity can be utilised to selectively concentrate targeted fatty acids in triglycerides that can be readily absorbed into plasma triglycerides (Jaeger & Reetz, 1998). Many attempts have been carried out in trans- or interesterification reactions to modify and enrich the content of PUFAs in triglycerides by using various lipases as biocatalysts (Hoshino *et al*., 1990; Sridhar & Lakshminarayana, 1992). Lipases occur widely in animals, plants and microorganisms (Jaeger & Eggert, 2002). Among microbial lipases bacterial lipases are the most widely used

class of enzymes in biotechnological applications because of their higher stability (Chakraborty & Paulraj, 2008a; Ternstrom, Lindberg, & Molin, 1993). The unique substrate specificity of microbial lipases has been utilised for the enhancement of PUFA content in fish oils by several groups (Harris, 1989; Matori *et al*., 1991). Isolates of *Pseudomonas fluorescens* have been found to produce enzymes active on lipolytic substrates under alkaline conditions (Kojima & Shimizu, 2003). Lipase genes from *Pseudomonas* have been cloned and expressed in *Escherichia coli,* due to their potential industrial applications (Kojima *et al*., 2003). *Pseusomonas fluorescens* SIK W1 was found to produce extremely heat-stable lipase that has high lipolytic activity for short- to medium-chain triacylglycerols (Chung *et al*., 1991).

An extracellular alkaline metallolipase with molecular weight of 74.8 k Da derived from cultures of *Bacillus licheniformis* MTCC 6824 was found to enrich D5 olefinic double bond fatty acids, viz., EPA and AA (Chakraborty & Paulraj, 2008b). A thermophilic and alkalophilic lipase from *Bacillus coagulans* BTS-3 was purified and biochemically characterised (Kumar *et al*., 2005). A lipase produced by recombinant *B. licheniformis* was found to be stable at alkaline pH of 12.0 (Nthangeni *et al*., 2001). These results are in contrast to thermotolerant lipases from *Bacillus thermoacetenulatus* and *Bacillus thermoleovorans*, which display maximum activity at pH 8.0 (Lee *et al*., 1999; Rua *et al*., 1997). *B. coagulans* NCIMB 9365 has been reported to possess an intracellular carboxylesterase (Molinari *et al*., 1996). The substrate specificity of lipase has been utilised for the recovery of EPA (D5) and DHA (D4) from marine oils and c-linolenic acid (D6) from borage seed oil (Morioka *et al*., 1987). DHA-rich triglycerides were prepared from fish oil with lipases obtained from *Candida cylindracea* and *Chromobacterium viscosum* (Tanaka, Hirano, & Funada, 1992, 1994). Substrates containing D2–D7 isomers of 18:1 were resistant to pancreatic lipase-

catalysed hydrolysis, resulting in higher concentrations of oleic acid, and the discrimination was the greatest for the D5 isomer (Heimermann *et al*., 1973).

One important challenge in mariculture is larval nutrition. It is believed that the optimal formulations for first-feeding larvae should simulate the yolk composition and to some extent reflect the nutrient requirements and metabolic capacities of pre-feeding fish (Heming and Buddington, 1988). Knowledge of the nutritional requirement of larvae after the transition from endogenous to exogenous feeding must be provided in order to compose a satisfactory diet for the first-feeding larvae. The dietary lipids play an important role as sources of essential fatty acids, needed for normal growth and survival. Larvae of many marine fishes are believed to require highly unsaturated fatty acids of the *n*-3 series such as docosahexaenoic acid (DHA; 22:6*n*-3) (Sargent *et al*., 1989). Some investigations have shown that DHA is superior to EPA as EFA for larval fish suggesting a different physiological function (Watanabe, 1993).

3.2. Materials and methods

3.2.1. Chemicals and Reagents

All solvents used for sample preparation were of analytical grade, and the solvents used for MS analyses were of LC grade (E-Merck, Darmstadt, Germany). Analytical grade solvents were redistilled in an all-glass system. The solvents were nitrogen degassed prior to use. Doubly distilled water was used throughout this work, while all reagents used were of analytical grade and purchased from E-Merck. Standards of fatty acid methyl ester (Supelco^{TM} 37 component FAME mix, catalog no. 47885-U) and boron trifluoride/methanol (14% BF3 in methanol, w/v) were procured from Sigma-Aldrich Chemical Co. Inc. (St. Louis, MO). Crude sardine oil (*Sardinella longiceps*) was obtained from a local fishmeal plant located in Cochin, India, bleached with 4% activated charcoal, and stabilized with

butylated hydroxyquinone (TBHQ, 0.01% w/v). The oil was stored under nitrogen at -20 ^{0}C, in a 2 L sealed dark amber glass container, until used. All glassware was rinsed with $CHCl_3$-CH_3OH (2: l v/v) and dried under N_2.

3.2.2. Saponification and Extraction of Fatty Acid from Sardine Oil

Sardine oil (50 g) was saponified by refluxing with 100 mL of alkaline solution [prepared by dissolving 250 g of NaOH and 2.5 g of Na_2-EDTA in a 1.5 L solution of water/methanol (1:1) at 60 ^{0}C] under continuous stirring for 30-45 min under an inert atmosphere of N2 following the procedure adopted by Haagsma with modification (Haagsma *et al*., 1982). The hydrolyzed mixture was diluted with water (50 mL), and the unsaponifiable matter was extracted successively with diethyl ether (50 mL x 3) and *n*-hexane (5 x 75 mL) and discarded. The pH value of the lower hydroalcoholic phase containing the saponified fatty acids was adjusted to 1.0 with HCl-H_2O (1:1 v/v, 15 mL), and the resultant free fatty acids were recovered by extraction with *n*-hexane (6 x 75 mL), washed with water to neutral pH, and dried over anhydrous $MgSO_4$. The residual solids were removed with a Buchner funnel under suction, and the solvent was evaporated to recover the free fatty acids, dried by passing N_2, weighed (34 g), and converted to methyl esters for further fractionation and gas-liquid chromatographic (GLC) and gas chromatographic-mass spectroscopic (GC/MS) analysis.

3.2.3. Formation of the Fatty Acid-Urea Inclusion Complex

The urea-fatty acid complexation was accomplished following the procedures (Marschner, 1955; Haagsma *et al*., 1982) with modification. In the first step of crystallization, urea was added to methanol in the ratio of 1:3 (w/v). The mixture was heated at elevated temperature (65-75 ^{0}C) with constant stirring until a clear, homogeneous solution was formed, and the fatty acids derived from saponification were applied in incremental portions

into the resulting urea solution, the urea/fatty acid ratios being 2:1, 3:1, and 4:1 (w/w). The solution was cooled to room temperature at a cooling rate of 0.5 ^{0}C /min and subsequently crystallized at three different sets of temperatures (2, 4, and 6 ^{0}C) for 24 h. The crystals were thereafter kept at -20 ^{0}C for 24 h. After thawing, the urea crystals (urea complex fraction) were separated from the mother liquor (non-urea complex fraction) by vacuum filtration. The filtrate containing the unsaturated fatty acids was evaporated using a rotary vacuum evaporator to remove the residual methanol. The solid residue thus obtained was diluted with water (200 mL) and acidified with dilute HCl (6 M, 30 mL) to pH 4-5 under stirring to remove traces of residual urea and methanol. The liberated fatty acids after acidification with dilute HCl were extracted with different solvents (*n*-hexane, chloroform, dichloromethane), and hexane was found as the best with higher recovery percentage of fatty acids. The fatty acids were extracted with *n*-hexane three times (3 x 100 mL) in a separatory funnel to cause the phase separation of urea and concentrated PUFAs. The upper layer of *n*-hexane containing the PUFAs was separated and dried over anhydrous $MgSO_4$. The resulting PUFA concentrate was dissolved in methanol, added with TBHQ (0.01% w/v) as an antioxidant to increase the stability of the fatty acids, and kept under a blanket of N_2 at -20 ^{0}C for 48 h.

3.2.4. Acid-Catalyzed Transesterification of Fatty Acid to Methyl Esters (FAMEs)

The extraction of total lipid from the crude sardine oil was performed according to the method of Bligh and Dyer (*13*) by using $CHCl_3/CH_3OH/H_2O$ (2:4:1, v/v/v). The lipid extract thus obtained was saponified with 0.5 N KOH in CH_3OH. After removal of the nonsaponifiable material with *n*-hexane and acidification with 1(N) HCl, the saponifiable materials were extracted with petroleum ether : diethyl ether (1:1, v/v), and transesterified into their methyl esters by reaction (30 min under reflux) with a methylating mixture (14% BF_3/MeOH, 5 mL) in a boiling water bath under an inert atmosphere of N_2. The FAME thus

obtained was cooled to ambient temperature and was distilled water (20 mL) was added. The solution was extracted with *n*-hexane (10 mL X 6); the upper *n*-hexane layer was removed and concentrated under an inert atmosphere of nitrogen. Similarly, the fatty acids obtained by urea-aided crystallization at various combinations of temperature (2-6^{0}C), and urea/fatty acid ratios (2:1, 3:1, and 4:1), were transesterified for analysis by GC/GC-MS. The PUFA concentrates obtained by urea fractionation were directly methylated, and the FAMEs were extracted with *n*-hexane, concentrated under inert nitrogen atmosphere, dissolved in *n*-hexane (30 mL), for further characterization.

3.2.5. Silver Ion Thin-Layer Chromatography ($AgNO_3$ TLC)

The recovered fatty acid methyl esters from urea fractionation and column chromatography were resolved by TLC (5 cm x 20 cm), precoated with silica gel, and impregnated with $AgNO_3$. Silica gel G (15.0 g) was mixed with a 10% (w/v) solution of $AgNO_3$ (40 mL) in methanol/ water (9:1 v/v) and spread in a uniform thickness (0.25 mm). Plates were drained, air-dried, activated at 110-120 ^{0}C in dim light for 30 min, and stored in a light-tight desiccator container. The methyl esters were applied to the plate as a narrow band. The plates were developed twice in *n*-hexane/diethyl ether/acetic acid (94:5:1 v/v/v) to separate individual bands. The bands were stained with 2, 7-dichlorofluorescein in alcohol (0.1% w/v) and examined under UV light.

3.2.6. Gas-Liquid Chromatography Analysis of FAMEs

FAMEs were analyzed by an Perkin-Elmer AutoSystem XL gas chromatograph (Perkin-Elmer) equipped with an Elite-5 (cross-bond 5% diphenyl polsiloxane-95% dimethyl polsiloxane) capillary column (30 m x 0.53 mm i.d., 0.50 µm film thickness; Supelco, Bellfonte, PA) using a flame ionization detector (FID). The oven temperature ramp was 110 ^{0}C for 1.0 min, followed by an increase of 45 ^{0}C /min to 250 ^{0}C, where it was held for 1.0

min, followed by an increase of 30 ^{0}C /min to 250 ^{0}C, where it was held for 1.0 min, followed by an increase of 25 ^{0}C /min to 285 ^{0}C, where it was held for 2.0 min, until all peaks had appeared. Ultrahigh purity He (>99% purity) was used as the carrier gas at a flow rate of 1 mL/min. The injector temperature was maintained at 285 ^{0}C. The injection volume was 1 µL. The detector temperature was 290 ^{0}C. FAMEs were identified by comparison of retention times with the known standards (37-component FAME mix; Supelco).

3.2.7. Preparation of DHA concentrates by *Bacillus subtilis* lipase-catalysed hydrolysis of fatty acyl esters

The free fatty acids were esterified by using a mixture of ethyl alcohol and 0.1 (N) H2SO4 to result in the ethyl ester of the fatty acids. An extracellular lipase derived from *Bacillus subtilis*, isolated from marine macroalga, *Turbinaraia conoides*, was used to prepare C22 *n*-3 polyunsaturated fatty acid concentrates from the ester fraction. An extracellular lipase from 48 h broth culture of *Bacillus subtilis* was purified to homogeneity by a combination of ammonium sulphate precipitation (70% saturation) and chromatographic separation on anion exchanger Amberlite IRA 410 (Cl^-form) and Sephadex G-100 gel exclusion chromatography. To the reaction mixture of fatty acyl ester (100 ml, 0.01% w/w tert-butylhydroquinone) in PIPES–NaOH buffer (5 ml of 0.1 M solution at pH 7.0) stabilised with Triton X-100 (0.5% v/v), and $CaCl_2$ (0.4 ml of 100 mM), purified lipase (500 LU) was added in a 250 ml-screw cap round-bottomed flask, to initiate the hydrolysis. The reaction flask was flushed with N_2 to replace air and to prevent oxidation, and placed into an incubator at 45 ± 1 ^{0}C with stirring (500 rpm). Samples (0.5 ml) from the reaction mixture were withdrawn periodically (3–9 h), and methanolic KOH (0.5 N, 25 ml) solution was added to the mixture to neutralise the free fatty acids released during hydrolysis, and the triglycerides were extracted thrice with n-hexane (3 X 100 ml). Hydrochloric acid (2 N HCl, 10 ml) was added to the mixture to neutralise the alkali, and the fatty acids in the aqueous layer were

extracted with n-hexane (2 X 100 ml). The lower aqueous layer was discarded, and the upper n-hexane layer containing fatty acyl esters was further extracted with distilled water (3 X 50 ml), to remove free fatty acids. The n-hexane layer was vacuum concentrated at 40 ^{o}C, using a rotary evaporator. The concentrated fatty acyl esters were maintained under N2 at -20 ^{0}C until further use. The hydrolysis products of the reaction catalysed by lipase were monitored by thin-layer chromatography (TLC), using silica gel as adsorbent, and eluted with chloroform/acetone/acetic acid (95:4:1, v/v/v). The fatty acyl esters were visualised by exposure to iodine vapour. Free fatty acids obtained from fatty acyl esters after saponification (Metcalf *et al*., 1966) were derivatised to their methyl esters and N-acylpyrrolidides, using an established procedure for gas chromatographic (GC) and gas chromatographic–mass spectroscopic (GC–MS) analyses (Andersson, 1978).

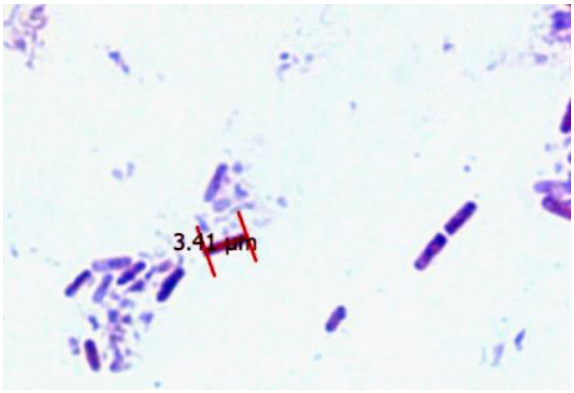


Figure 3.4. *Bacillus subtilis* used to isolate bacterial lipase at 100x

3.2.8. Separation of phospholipid fraction from the seerfish roe and preparation of DHA rich oil emulsion

The total lipids of seerfish roe were separated into different lipid classes by silicic acid column chromatography. The column was successively eluted using chloroform, acetone and methanol to recover the phospholipids (Christie, 2003). The lipid fractions were qualitatively analyzed by TLC for identifying triglycerides, glycolipids and phospholipid components (Jacin and Mishkin, 1965). The solvent system chloroform: methanol: water

(70:25:5, v/v/v) was used for separating the phospholipids. The eluted spots were identified by developing with ammonium molybdate - perchloric acid (Mangold, 1961). Fatty acid methyl esters (FAMEs) of the total lipid and the individual lipid classes were prepared by transesterification using 2% sulphuric acid in methanol. The FAMEs were extracted into acetone and thoroughly washed with water to make them free of acid and dried over anhydrous sodium sulphate. All the analyses were conducted in triplicate, standard deviation (SD) was computed and the data is presented as mean of triplicate values ± SD. The DHA enriched fatty acid concentrate was enriched through biochemical and microbiological procedures were used to formulate enrichment emulsions which contained grossly, 90% DHA enriched fatty acid concentrate and 10% phospholipids fraction extracted from fish roe (seer fish). Other than the stability and emulsification ability imparted by lecithin, tocopherol acetate (Vitamin E) at 0.5% was also included as a stabilizer. An experiment was conducted to understand the effect of emulsifier to stabilize the DHA concentrate. The emulsifier used was Tween 20 (or Polysorbate 20), which was able to contain the stability of the preparation for an extended time period. Polysorbate 20 is a polysorbate surfactant whose stability and relative non-toxicity allows it to be used as an emulsifier in stabilizing the oil. The oil-water emulsions contained 100 µL/mL of oil and 1 mg/mL of emulsifier. Emulsifier (Tween 20) was weighed and mixed with deionized distilled water. The Tween 20/water mixture was sonicated until clear in an ice/water bath by using a probe type sonicator (High intensity ultrasonic processor, 600 W). The aqueous phase was added to the oil phase and sonicated in an ice/water bath for about 4 min to form emulsions.

To avoid the complications in interpreting the results by using oil mixtures, we have chosen single-component oil systems to elucidate the emulsion stability. The stability of the emulsion was quantified in terms of the emulsion droplet size and/or its change of kinetic stability with time. The size and droplet number measurements were performed by using

microscopic observations. Emulsion was diluted by 300 times in water for these measurements. The measurements were repeated 3 times at least for a single emulsion system. Relative number of droplet particles was obtained in different viewing zones. In this paper, the average particle size will represent the size distribution function of an emulsion system.

3.3. Results and discussion

3.3.1. Saponification and Extraction of Fatty Acids

The refined sardine oil was found to contain long-chain polyunsaturated fatty acids (LC-PUFAs), particularly eicosapentaenoic acid (20:5 n-3 or EPA; 17.80 ± 1.57% of total fatty acids, TFA) and docosahexaenoic acid (22:6 n-3 or DHA; 7.67 ± 1.50% of TFA) along with other *n*-3 and *n*-6 PUFAs like linolenic acid (LA or 18:3 *n*-3; 4.47 ± 0.84% TFA), linolenic acid (18:2 *n*-6; 0.71 ± 0.23% TFA), and docosapentaenoic acid (DPA or 22:5 n-3; 1.14 ± 0.08% TFA) (Table 2.1). The *n*-6 fatty acids have a minor share of the total fatty acid content of sardine oil (0.81% TFA). The PUFAs containing C18–C22 acyl chain length contributed a major share of the total fatty acids of the sardine oil (>30% TFA). Among the saturated fatty acids (SFAs), 14:0 was found to be predominant (7.04 ± 0.22% TFA), while 16:1 *n*-7 contributed the major share (31.56 ± 2.59% TFA) among monounsaturated fatty acids (MUFAs) (Table 2.1). When fatty acids are required in free form for further analyses, lipids were hydrolyzed in alkaline medium for extracting the unsaponifiable material. Sardine oil (100 g) was saponified with NaOH/Na_2EDTA to yield free fatty acids (34 g). Na_2EDTA appeared to form complex with traces of metal ions (Cu, Fe), which catalyze oxidation of unsaturated fatty acids during saponification, and subsequently removed by extraction with water thus hindering the interferences of metal ions during the course of further purification process. Relatively large volumes of n-hexane (5 X 100 mL) were added to the aliquot of the

salt of fatty acid mixtures for better phase separation, thus removing the unsaponificable materials. After acidification of the lower phase, the remaining n-hexane was separated, and the fatty acids were quantitatively extracted. Among saturated fatty acids (SFAs), 14:0 was found to be predominant (7.04% TFA), while 16:1n7 contributed the major share among all individual fatty acids in the crude sardine oil (>31% TFA). EPA and DHA were found to be the major n3 PUFAs contributing to 17.8% and 7.67% of TFA, respectively. The n6 fatty acids have minor share in the total fatty acid content of sardine oil. Solvent extraction resulted in marginal increase of unsaturation (0.85%) in the fatty acid profile; the PUFA exhibited an increase of 6.49%, while MUFA and SFA reduced by 2.96% and 6.91%, respectively. The n3 fatty acids exhibited an increase of 5.73% in the solvent extract of fatty acids.

3.3.2. PUFA Enrichment by Urea Fractionation

The free fatty acids (34 g) derived from sardine oil were subjected to urea crystallization using methanol as solvent at three different temperatures (2, 4, and 6^0C), and urea-fatty acid ratios (2:1, 3:1, and 4:1) to obtain PUFAs of high purity. Urea occludes straight-chain compounds such as long-chain saturated and monounsaturated fatty acids in a hexagonal crystalline structure, and excludes methylene-interrupted polyunsaturated fatty acids (having –C=C-C-C=C- moiety) due to the irregularities in their molecules caused by the bends at each double bond (Bligh and Dyer, 1959). The interfering SFAs and most of the MUFAs were removed in the form of urea inclusion compound. Further, as oxidized products do not form urea adducts, the peroxidation of *n*-3 PUFAs could be avoided during the extraction of free acids from fish oil triglycerides.

Urea inclusion method allows handling of large quantities of material in a more efficient way than fractional crystallization or selective solvent extraction (Bligh and Dyer,

1959). The fatty acid profiles of crude sardine oil, solvent extracted sardine oil, and n3 PUFA concentrates obtained by urea inclusion are presented in Table 3.1. Urea fractionation resulted in total reduction of SFAs (>95%) (14:0, 16:0, and 17:0), moderate reduction of MUFAs (>65%) The combination of three different temperatures of crystallization, both of which are strongly related, and urea/fatty acid ratio (U/FA) ratio were tested for optimizing higher DHA recovery, and a comparison of the results was made. At 6°C, the ΣPUFA was found to be 53.6% at the U/FA ratio of 4:1, while at 4° C the corresponding value was considerably higher (78.35%). The U/FA ratio of 4:1 (w/w) was found to be optimal for getting high-purity EPA (47.8%) whilst that at 3:1 (at 2 degree C) yielded higher content of DHA (>20 %) when crystallized at 2° C. Linolenic acid (18:3n3) was found to be concentrated at 4° C, and U/FA ratio of 4:1 with 8.33%. As a consequence of these results, DHA obtained from sardine oil at 2° C temperature of urea-crystallization by using a U/FA ratio of 3:1 selected for subsequent purification of C22 fatty acid DHA. The purity of 16:1n7, the predominant MUFA, was reduced by 62.4% at the U/FA ratio of 4:1, and a temperature of 4° C.

At higher temperature (6°C), the urea fractionation could not resolve the SFAs and MUFAs effectively (at U/FA = 4:1), resulting in lower concentration of PUFAs in the urea concentrate (1.81 %). It is likely that at lower temperature (2° C), the reaction kinetics to form urea-inclusion complex with SFAs and MUFAs was relatively lower resulting in higher DHA in the extract. On the contrary, at higher temperature (>2° C), the urea-fatty acid complex dissociates; resulting in comparatively higher amount of SFA and MUFA in the fatty acid concentrates. Also, while the urea inclusion complexes were primarily formed with SFAs and MUFAs; the inclusion complexes contained some linoleic acid (16:2*n*6), while EPA was completely excluded. It is of note that among the long chain polyunsaturated fatty acids, the fatty acid 22:6n-3 contributed a maximum value of 20.34 % TFA by using the urea

fatty acid ratio of 3:1 at 2 ° C. It is therefore this particular fraction was chosen for further purification of C_{22} PUFAs. As evident from the TLC chromatogram, the uppermost band of saturated fatty acids, and the second band of monoenoic fatty acids were apparent. Tetraene, pentaene, and hexaene methyl esters appeared to be concentrated to > 90% in the lower band of the TLC plates.

3.3.3. Change in fatty acid composition as a function of lipase-catalysed hydrolysis of fatty acids from sardine oil

The free fatty acids were esterified by using a mixture of ethyl alcohol and 0.1 (N) H2SO4 to result in the ethyl ester of the fatty acids. An extracellular lipase derived from *Bacillus subtilis*, isolated from marine macroalga, *Turbinaraia conoides*, was used to prepare C22 n-3 polyunsaturated fatty acid concentrates from the ester fraction. The enzyme was purified 132-fold with specific activity of 386 LU/mg. The urea fractionated fatty acyl esters were hydrolyzed with lipase purified from the bacterium *Bacillus subtilis*, and the total fatty acid content of fatty acyl esters after lipase hydrolysis were analyzed. SFA levels showed a reduction to 0.05% after 3 h of hydrolysis (Table 2.2). The total MUFA content was found to be reduced to 34% after 3 h of lipase-catalysed hydrolysis. The decrease in the content of SFAs and MUFAs in the fatty acyl ester mixture with the progress of hydrolysis suggests that SFAs and MUFAs were more easily hydrolysed by the lipase than those in esterified fatty acids that contain DHA, resulting in the enrichment of the latter in the ester fraction (Fig. 1). An earlier report stated that substrates containing D2–D7 isomers of 18:1 were resistant to pancreatic lipase-catalysed hydrolysis resulting in higher concentration of oleic acid (18:1 *n*-9), and the discrimination was the greatest for the D5 isomer (Heimermann *et al*., 1973).

The variations of PUFA content of sardine oil triglycerides as a function of time during the lipase-catalysed hydrolysis are illustrated in Fig. 3.6. and Table 3.2. The total

DHA of fatty acyl ester fraction increases with time up to 3 h of lipase-catalysed hydrolysis (35.27% TFA), beyond which it slowly decreased (9.81% TFA after 9 h). The purified lipase was able to enrich DHA with 35.27% 22:6n-3 after 3 h of hydrolysis. Lower hydrophobic constants of *n*-3 fatty acids (22:6*n*-3log P = 6.96) resulted in higher hydrolytic resistance of the former toward lipase, leading to their enrichment in the fatty acyl ester fraction. Attempts have been made by various workers to modify and enrich the content of individual PUFAs by using various lipases as biocatalysts by trans- or inter-esterification reactions. Several groups have utilized the unique substrate specificity of microbial lipases for the enhancement of PUFA content in fish oils. An earlier report described a lipase purified from *P. fluorescens* HU380 used to concentrate EPA and DHA from oils (Kojima & Shimizu, 2003). Immobilised lipases IM60 from *Mucor miehei* and SP435 from *Candida antarctica* were used to modify fatty acid composition of vegetable oils by enrichment of *n*-3 PUFAs (Huang & Akoh, 1994).

The results also suggest that the esteritic bonds of C22 acyl chain lengthened n-3 PUFAs are resistant to hydrolysis by the lipase. However, after prolonged hydrolysis (>9 h), when only a few target fatty acid ester bonds (n-6 fatty acyl ester bonds and esters other than C22 n-3 fatty acids) are available in the enzyme hydrolysate that are susceptible to hydrolysis by a lipase, the microbial lipase can even cleave bonds that are resistant (or nearly resistant) to hydrolysis, i.e., DHA. It can be concluded that it might be possible to separate and concentrate C22 PUFAs with *n*-3 double bonds like DHA using lipase from seaweed associated *Bacillus subtilis*. There are reports of modification of borage oil (*Borago officinalis* L.) fatty acid composition to incorporate EPA (31% EPA), with an immobilised SP435 lipase from *C. antarctica* as biocatalyst. With the progress of incubation time, EPA incorporation was also increased up to 36 h (Akoh & Sista, 1995). In an earlier attempt to concentrate the content of DHA in a glyceride mixture, fish oil was hydrolysed with six kinds

of microbial lipase. When the hydrolysis with *C. cylindracea* lipase was 70% complete, the EPA content was recorded to be 70% of the original fish oil (Tanaka *et al*., 1992). DHA and EPA concentration was reported to be doubled by *Pseudomonas cepacia* and *Candida rugosa* lipase-assisted hydrolysis of Atlantic salmon (*Salmo salar* L.) viscera oil (Sun, Pigott, & Herwig, 2002).

Six commercial lipases (Novozyme 435 from *C. antarctica*, Lipozyme IM from *M. miehei*, PS-30 from *Pseudomonas* sp., AP-12 from *Aspergillus niger*, AY-30 from *C. rugosa,* and Novozyme-677BG from *Thermomyces lanuginosus*) were tested for their ability to incorporate DHA into evening primrose oil by Senanayake and Shahidi (2004). Among the enzymes examined, Novozyme 435 from *C. antarctica* was chosen over the other enzymes to catalyse the trans-esterification reaction owing to higherincorporation of DHA. For the time course reaction, incorporation of DHA increased up to 25.2% after 24 h (Senanayake & Shahidi, 2004). Synthesis of triglycerides by enzymatic esterification of PUFAs with glycerol, using microbial lipase Novozyme 435 (Novo Nordisk, A/S) from *C. antarctica*, was carried out to obtain a triglyceride yield of 93.5% from cod liver oil PUFA concentrate; the product was reported to contain 25.7% EPA and 44.7% DHA (Medina *et al*., 1999). DHA was enriched from the recovered free fatty acid fraction from sardine oil by selective enzymatic esterification using Lipozyme^{TM} lipases that allowed up to 80% DHA enrichment but gave no EPA enrichment (Schmitt-Rozieres, Deyris, & Comeau, 2000). An earlier study reported the hydrolysis and urea adduction of refined cod oil including 12.2% EPA and 6.9% DHA with HUlipase produced by *P. fluorescens* HU380 to furnish free fatty acids with 43.1% EPA and 7% DHA, respectively (Kojima*et al*., 2006).

Table 3.1. Fatty acid composition of crude and solvent extracted sardine oil, and PUFA concentrates obtained by the urea fractionation at three different temperatures and urea/fatty acid ratios

			Temperature for urea fatty acid crystallization (°C)								
			2 °C			4 °C			6 °C		
Fatty acids	Sardine oil	[a]SE	U/FA[b] =2:1	3:1	4:1	U/FA[b] =2:1	3:1	4:1	U/FA[b] =2:1	3:1	4:1
Saturated fatty acids											
12:0	0.06	0.05	0.07	0.99	0.49	0.00	0.00	0.00	0.00	0.00	0.00
14:0	7.04	6.58	0.97	0.00	0.00	4.29	1.08	0.00	6.60	0.73	0.00
16:0	0.45	0.50	0.55	0.00	0.00	3.68	0.41	0.00	0.00	0.00	0.00
17:0	0.28	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Σ SFA	7.82	7.28	1.60	0.99	0.49	7.97	1.49	0.00	6.60	0.73	0.00
Monounsaturated fatty acids											
16:1*n*7	31.56	30.19	30.86	21.04	12.73	16.91	12.80	11.35	28.90	30.01	18.05
18:1*n*9	16.86	16.79	17.44	16.20	8.35	12.22	6.55	1.25	16.92	16.38	13.64
17:1	0.59	0.62	0.00	0.00	0.00	3.95	4.11	0.17	3.00	0.09	0.00
20:1*n*11	0.30	0.24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Σ MUFA	49.30	47.84	48.31	37.24	21.08	33.08	23.45	12.78	48.83	46.49	31.69
Polyunsaturated fatty acids											
16:2*n*6	0.71	0.96	2.19	1.94	2.09	0.86	2.22	2.23	5.75	2.28	1.17
18:3*n*3	4.47	5.11	1.07	4.38	5.62	4.48	5.34	8.33	4.86	5.46	0.93
18:4*n*3	1.38	1.40	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
20:4*n*6	0.10	0.14	0.00	0.00	0.00	0.22	0.00	0.00	0.00	0.55	0.00
20:5*n*3	17.80	18.50	27.83	29.89	34.96	33.63	42.96	47.78	22.37	25.80	45.71
22:5*n*3	1.14	1.10	2.79	3.06	4.42	1.60	3.02	2.90	1.81	2.36	2.19
22:6*n*3	7.67	8.21	15.37	20.34	17.03	13.33	17.73	17.11	6.28	7.31	3.59
Σ PUFA	33.26	35.42	49.25	59.62	64.11	54.11	71.27	78.35	41.09	43.74	53.59
Others	9.61	9.46	0.84	2.15	14.32	4.84	3.79	8.86	3.48	9.04	14.72
Σ *n*3	32.46	34.32	47.06	57.67	62.02	53.03	69.05	76.12	35.33	40.92	52.42

Individual fatty acid is expressed as percentage of total identifiable fatty acids. [a]SE implies the solvent extract of sardine oil; ND = fatty acid identified on the GC trace, but not integrated by the instrument.

The crude sardine oil was clarified by a sequential process of degumming, decolorization, and deodorization, and was found to contain LC-PUFAs, particularly 20:5 *n*-3 or EPA (17.80 ± 1.57%) of total fatty acids, TFA) and 22:6 *n*-3 or DHA (7.67 ± 1.50% of TFA) along with other *n*-3 and *n*-6 PUFAs like linolenic acid (LA or 18:3 *n*-3; 4.47 ± 0.84% TFA), linolenic acid (18:2 *n*-6; 0.71 ± 0.23% TFA), and docosapentaenoic acid (DPA or 22:5 *n*-3; 1.14 ± 0.08% TFA) (Figure 3.5.). The n-6 fatty acids have a minor share of the total fatty acid content of sardine oil (0.81% TFA). The PUFAs containing C_{18}–C_{22} acyl chain length

contributed a major share of the total fatty acids of the sardine oil (>30% TFA). Among the saturated fatty acids (SFAs), 14:0 was found to be predominant (7.04 ± 0.22% TFA), while 16:1 *n*-7 contributed the major share (31.56 ± 2.59% TFA) among the monounsaturated fatty acids (MUFAs). When fatty acids are required in free form for further analyses, lipids were hydrolyzed in alkaline medium for extracting the unsaponifiable material. Sardine oil was saponified with NaOH/Na_2EDTA to yield free fatty acids. Na_2EDTA appeared to form complex with traces of metal ions (Cu, Fe), which catalyze oxidation of unsaturated fatty acids during saponification, and subsequently removed by extraction with water thus hindering the interferences of metal ions during the course of further purification process. Relatively large volumes of n-hexane were added to the aliquot of the salt of fatty acid mixtures for better phase separation, thus removing the unsaponificable materials. Among saturated fatty acids (SFAs), 14:0 was found to be predominant (7.04% TFA), while 16:1*n*-7 contributed the major share among all individual fatty acids in the crude sardine oil (>31% TFA). EPA and DHA were found to be the major *n*-3 PUFAs contributing to 17.8% and 7.67% of TFA, respectively. The *n*-6 fatty acids have minor share in the total fatty acid content of sardine oil. Solvent extraction resulted in marginal increase of unsaturation (0.85%) in the fatty acid profile; the PUFA exhibited an increase of 6.49%, while MUFA and SFA reduced by 2.96% and 6.91%, respectively. The *n*-3 fatty acids exhibited an increase of 5.73% in the solvent extract of fatty acids.

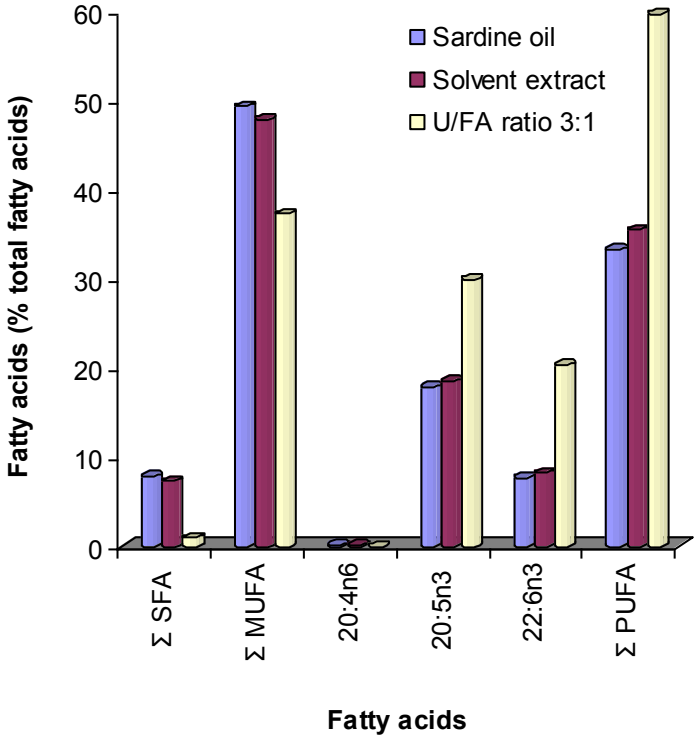


Figure 3.5. Fatty acid composition of crude and solvent extracted sardine oil, and PUFA concentrate obtained by the amide fractionation (U/FA: amide/fatty acid ratio).

The free fatty acids derived from sardine oil were subjected to amide fractionation using methanol as solvent at three different temperatures and urea-fatty acid ratios to obtain PUFAs of high purity. The interfering SFAs and most of the MUFAs were removed in the form of amide inclusion compound. Further, as oxidized products do not form amide adducts, the peroxidation of n-3 PUFAs could be avoided during the extraction of free acids from fish oil triglycerides. Amide fractionation resulted in total reduction of SFAs (>95%) (14:0, 16:0, and 17:0), moderate reduction of MUFAs (>65%). The U/FA ratio of 4:1 (w/w) was found to be optimal for getting high-purity EPA (47.8%) whilst that at 3:1 (at 2 degree C) yielded higher content of DHA (>20 %) when crystallized at 2°C. As a consequence of these results, DHA obtained from sardine oil at 2°C temperature of amide-crystallization by using a U/FA ratio of 3:1 selected for subsequent purification of C22 fatty acid DHA. It is likely that at

lower temperature (2°C), the reaction kinetics to form amide-inclusion complex with SFAs and MUFAs was relatively lower resulting in higher DHA in the extract. It is of note that among the long chain polyunsaturated fatty acids, the fatty acid 22:6n-3 contributed a maximum value of 20.34 % TFA by using the amide-fatty acid ratio of 3:1 at 2 ^{0}C. It is therefore this particular fraction was chosen for further purification of C_{22} PUFAs.

3.3.4. Change in fatty acid composition as a function of microbial triacylglycerol acyl hydrolase-catalyzed hydrolysis of fatty acids from sardine oil

The free fatty acids were esterified by using a mixture of ethyl alcohol and 0.1 (N) H_2SO_4 to result in the ethyl ester of the fatty acids. An extracellular triacylglycerol acyl hydrolase derived from *Bacillus subtilis*, isolated from marine macroalga, *Turbinaraia conoides*, was used to prepare C_{22}*n*-3 polyunsaturated fatty acid concentrates from the ester fraction. The enzyme was purified 132-fold with specific activity of 386 triacylglycerol acyl hydrolase units/mg. The urea fractionated fatty acyl esters were hydrolyzed with enzyme purified from the bacterium *Bacillus subtilis*, and the total fatty acid content of fatty acyl esters after lipase hydrolysis were analyzed. SFA levels showed a reduction to 0.05% after 3 h of hydrolysis. The decrease in the content of SFAs and MUFAs in the fatty acyl ester mixture with the progress of hydrolysis suggested that SFAs and MUFAs were more easily hydrolyzed by the lipase than those in esterified fatty acids that contain DHA, resulting in the enrichment of the latter in the ester fraction.

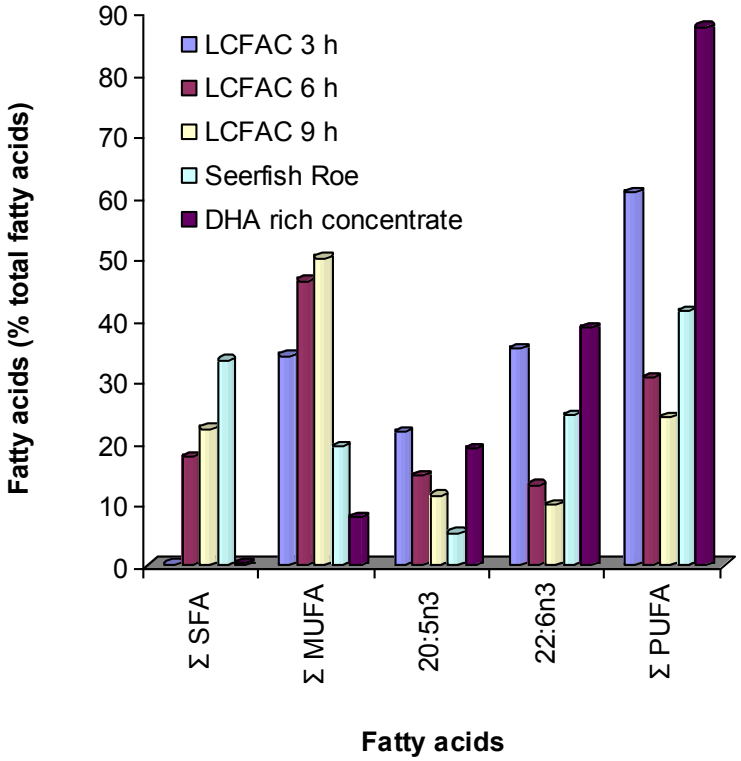


Figure 3.6. Fatty acid composition of urea concentrated fatty acids, triacylglycerol acyl hydrolase-catalyzed fatty acid concentrate, seerfish roe, and DHA rich concentrate

The variations of PUFA content of sardine oil triglycerides as a function of time during the microbial triacylglycerol acyl hydrolase-catalyzed hydrolysis were illustrated in Figure 3.6. The total DHA of fatty acyl ester fraction increased with time up to 3 h of enzyme-catalyzed hydrolysis (35.27% TFA), beyond which it slowly decreased (9.81% TFA after 9 h) (Figure 3.6.). The purified triacylglycerol acyl hydrolase was able to enrich DHA with 35.27% 22:6*n*-3 after 3 h of hydrolysis. The results also suggested that the esteritic bonds of C_{22} acyl chain lengthened *n*-3 PUFAs are resistant to hydrolysis by the lipase. However, after prolonged hydrolysis (>9 h), when only a few target fatty acid ester bonds (*n*-6 fatty acyl ester bonds and esters other than C_{22} n-3 fatty acids) were available in the enzyme hydrolysate that were susceptible to hydrolysis by the bacterial hydrolase, the microbial triacylglycerol acyl hydrolase can even cleave bonds that are resistant (or nearly resistant) to

hydrolysis, i.e., DHA. It can be concluded that it might be possible to separate and concentrate C_{22} PUFAs with n-3 double bonds like DHA using lipase from seaweed associated *Bacillus subtilis*.

Table 3.2. Fatty acid composition of urea concentrated fatty acids, lipase catalysed fatty acid concentrate, seerfish roe, and DHA rich concentrate

Fatty acids	**Fatty acid (% TFA)**					
	Urea concentrated fatty acids	**Lipase catalysed fatty acid concentrate**			**Seerfish Roe (*Scomberomoros guttatus*) phospholipids**	**DHA rich concentrate after adding fish roe**
Saturated fatty acids		**3 h**	**6 h**	**9 h**		
12:0	0.99	0.05	0.12	0.28	0.09	0.03
14:0	0.00	0.00	0.06	0.18	1.54	0.02
16:0	0.00	0.00	16.34	20.71	29.53	0.13
17:0	0.00	0.00	1.05	0.97	2.17	0.04
Σ SFA	**0.99**	**0.05**	**17.57**	**22.14**	**33.30**	**0.22**
Monounsaturated fatty acids						
16:1n7	21.04	18.89	21.42	22.64	9.09	3.19
18:1n9	16.20	15.11	22.45	25.03	8.54	4.25
17:1	0.00	0.00	1.05	1.18	0.04	0.12
20:1n11	0.00	0.00	1.28	1.07	1.55	0.10
Σ MUFA	**37.24**	**34.00**	**46.20**	**49.92**	**19.22**	**7.66**
Polyunsaturated fatty acids						
18:2n6	1.94	0.86	1.26	1.12	1.53	6.58
18:3n3	4.38	0.42	0.85	0.63	0.46	11.40
18:4n3	0.00	0.00	0.06	0.08	0.47	3.19
20:4n6	0.00	0.00	0.18	1.07	6.03	4.11
20:5n3	29.89	21.48	14.60	11.26	5.15	18.96
22:5n3	3.06	2.63	0.63	0.06	3.34	4.63
22:6n3	20.34	35.27	12.95	9.81	24.28	38.65
Σ PUFA	**59.62**	**60.66**	**30.53**	**24.03**	**41.26**	**87.52**

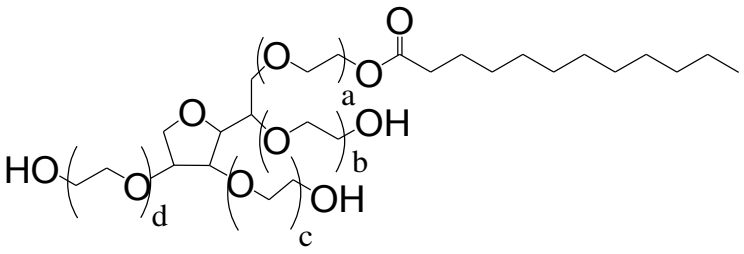
3.3.5. Separation of phospholipid fraction from the seerfish roe and preparation of DHA rich oil emulsion

The total lipids of seerfish roe were separated into different lipid classes by silicic acid column chromatography. The lipid fractions were qualitatively analyzed by TLC for identifying triglycerides, glycolipids and phospholipid components. Fatty acid methyl esters (FAMEs) of the total lipid and the individual lipid classes were prepared by

transesterification process. The FAMEs were extracted into a comparatively polar solvent system and thoroughly washed with water to make them free of acid. The DHA enriched fatty acid concentrate enriched through biochemical and microbiological procedures were used to formulate enrichment emulsions which contained grossly, 90 % DHA enriched fatty acid concentrate and 10 % phospholipids fraction extracted from fish roe (seer fish). The aggregate content of DHA in fish roe phospholipidic fraction was recorded as 24.3% DHA along with 5.2% EPA. The polyunsaturated fatty acid concentrate of the fish body oil after adding fish roe was found to contain greater than 35% DHA with significantly lesser content of saturated fatty acids (0.22%) and monounsaturated fatty acids (~7% of total fatty acids).

3.3.6. Use of emulsifiers to increase the stability of enriched PUFA emulsion

An emulsifier is a substance that stabilizes an emulsion by increasing its kinetic stability. An emulsion is a mixture of two or more liquids that are normally immiscible. Emulsion should be used when both the dispersed and the continuous phase are liquids. An experiment was conducted to understand the effect of emulsifier to stabilize the DHA concentrate. The emulsifier used was Tween 20 (or Polysorbate 20), which was able to contain the stability of the preparation for an extended time period. Polysorbate 20 is a polysorbate surfactant whose stability and relative non-toxicity allows it to be used as an emulsifier in stabilizing the oil. It is a polyoxyethylene derivative of sorbitan monolaurate, and is distinguished from the other members in the polysorbate range by the length of the polyoxyethylene chain and the fatty acid ester moiety (Figure 3.7.). The commercial product contains a range of chemical species. Longer chain TAGs such as polysorbate 20 is more hydrophobic and, therefore, have higher oil/water interfacial tension than shorter chain ones.



Tween 20: Cn (a, b, c, and d) = a + b + c + d = 20

Figure 3.7. Polyoxyethylenederivative of sorbitan monolaurate (or Polysorbate 20)

The notation Cn represents a triacylglycerol that has three identical acyl chains with n carbons per chain in this paper as shown below. Since the reduction of the oil/water interfacial tension by the emulsifier is considered the main driving force to form the emulsion, it is expected that oils that have different hydrophobicities will change the emulsion stability. The oil/water interfacial tension or the intrinsic viscosity of the oils has a correlation with the droplet size of the emulsions. An experiment was conducted to understand the effect of emulsifier to stabilize the DHA concentrate. The emulsifier used was Tween 20 (or Polysorbate 20) which was able to contain the stability of the preparation for an extended time period. Polysorbate 20 is a polysorbate surfactant whose stability and relative non-toxicity allows it to be used as an emulsifier in stabilizing the oil (Figure 3.10.). It is a polyoxyethylene derivative of sorbitan monolaurate, and is distinguished from the other members in the polysorbate range by the length of the polyoxyethylene chain and the fatty acid ester moiety. The commercial product contains a range of chemical species.

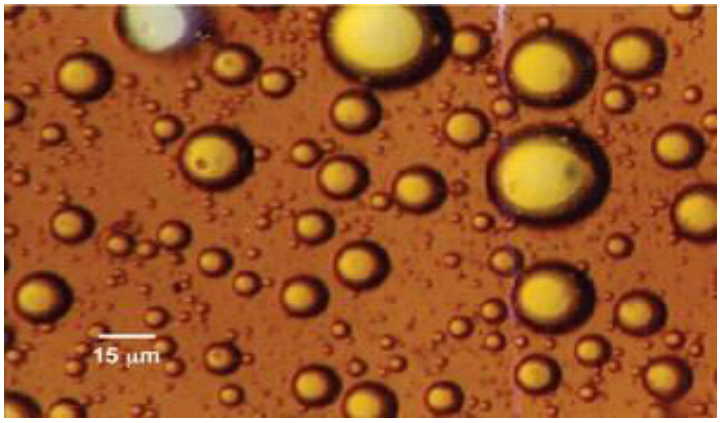


Figure 3.8. Photomicrograph of a water-in-oil emulsion by using Tween 20

As evident from the following figure, the stability of the PUFA concentrate (as such without Polysorbate 20) decreased after 15 min, whereas the same appended with Polysorbate 20 was able to maintain the stability for an extended period of time due to increased kinetics (increased Brownian movement).

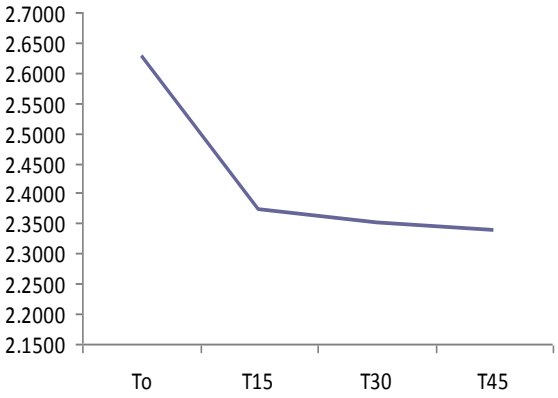


Figure 3.9. Emulsion stability of the DHA concentrate (without addition of emulsifier Polysorbate 20)

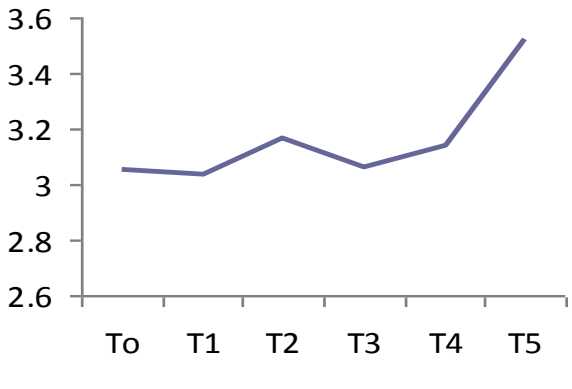


Figure 3.10. Emulsion stability of the DHA concentrate (with addition of emulsifier Polysorbate 20)

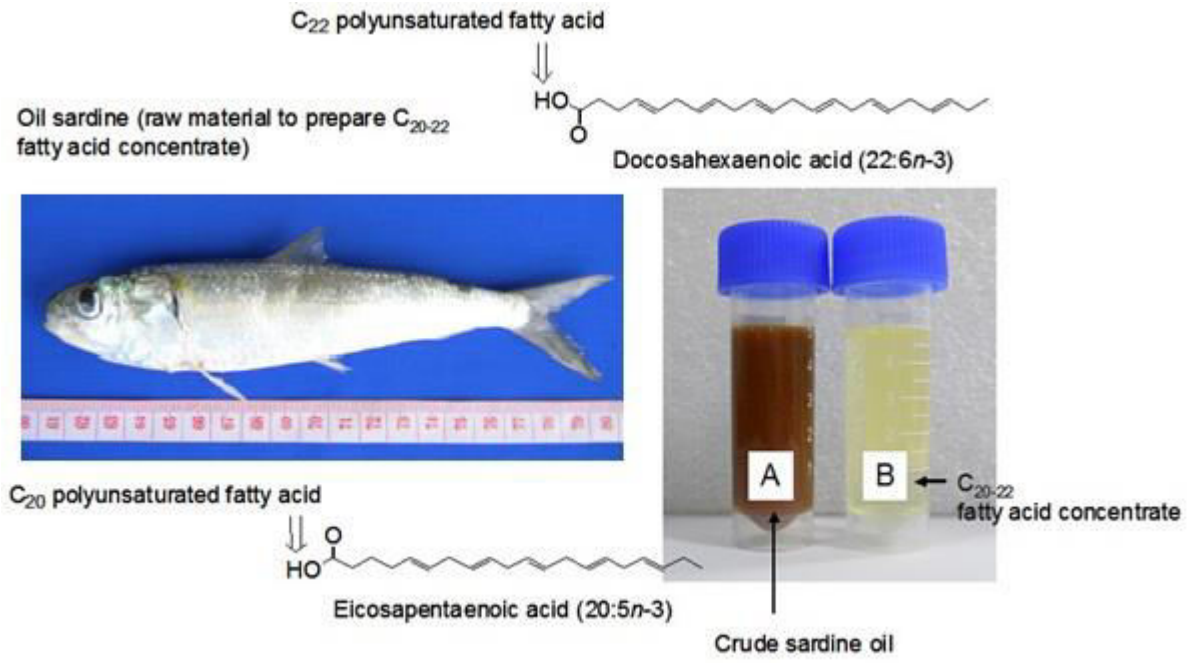


Figure 3.11. $C_{20\text{-}22}$*n*-3 polyunsaturated fatty acid concentrate prepared from sardine oil. (A) Crude sardine oil; (B) PUFA concentrate.

3.4. Conclusions

The $C_{20\text{-}22}$*n*-3 polyunsaturated fatty acid concentrate prepared from the inexpensively available marine sources, such as, sardine oil can be the potential substitute of the imported PUFA supplements for use in mariculture. The indigenous *n*-3 polyunsaturatated fatty acids emulsion developed from the locally available low-value fish may also serve as a cheaper alternative to the imported fatty acid emulsions for use in mariculture and larval nutrition.

CHAPTER 4

Screening of potential marine microalgae for use in aquaculture

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Screening of potential marine microalgae for use in aquaculture

4.1. Background of the study

Microalgae are microscopic organisms, classified as eukaryotic photosynthetic microorganisms, which are present in all water bodies around the globe. It has been stated that approximately 2, 00,000 to 8, 00,000 species exists, out of which 35,000 species were described (Cardozo *et al*., 2007). With recent research progress in algal biotechnology, this has become increasingly important as feedstock for industrial processing applications due to their fast growth and their novel products (Lie *et al*., 2009). Out of several microalgae strains isolated and cultured for the aquaculture, very few are capable of producing greater content of lipid (Gouveia and Oliveira, 2009). These primary producers in the marine ecosystem are important and being used for commercial rearing of shellfish and finfishes for many decades (Pratoomyot *et al*., 2005). They are directly or indirectly used as a constant food source from the initial green water technique to all growth stages (Gopakumar *et al*., 2009b; Vijayagopal *et al*., 2012).

In recent years, many studies on these microalgae have focused on various commercial applications (Spolaore *et al*., 2006). They are naturally rich in lipids, antioxidant, carotenoids and vitamins. Among these, lipids are bioencapsulated by the algal cell wall. Their ability to synthesize essential fatty acids and other nutrients required by finfish and shellfish larvae are used in aquaculture *viz*., microalgae as feed for zooplankton, larvae of bivalves and crustaceans, marine fish, and shrimps (Patil *et al*., 2007; Sargent *et al*., 1997). They contain polyunsaturated fatty acids (PUFAs) particularly *n*-3 PUFAs. Among them

eicosapentaenoic acid (EPA, *20:5n*-3) and docosahexaenoic acid (DHA, *22:6n*-3) are indispensable for the growth and survival of marine finfish and shellfish larvae.

The cultivated microalgae in captivity have long been known to be utilized in the hatchery for the production of many farmed finfish and shellfish (Robin and Ingrid, 2012). Microalgae are the only source of *n*-3 PUFAs naturally, and fish accumulates these long chain *n*-3 PUFAs by consuming microalgae (Guedes *et al*., 2011). Over the last four decades several hundred microalgae species have been tested as food. Probably less than twenty have gained widespread use in aquaculture. The strains *Isochrysis* sp*, Pavlova* sp*, Tetraselmis* sp*, Pseudoisochrysis* sp*, Chaetoceros* sp*, Skeletonema* sp and *Nannochloropsis* sp are being popularly used for successful bivalve and fish larvae culture (Brown, 2002). Microalgae play an important role in mariculture. These species are consumed directly by shellfishes, and indirectly through zooplankton like rotifer, copepod and *Artemia* etc. to finfishes (Palmtag *et al*., 2006). Since the diversity of microalgae is large, a number of microalgae have been intensively studied because of greater nutritional quality, in terms of fatty acid composition (Brown, 2002). In the present study, eighteen marine microalgal species have been screened for fatty acid composition for use in aquaculture.

4.2. Materials and Method

4.2.1. Microalgae culture

The microalgae belongs to the different genera were mentioned in **Table 4.1.** These species were isolated from different locations of marine and backwater niches in and around Ernakulam (9°59'18.22"N, 9°59'56.07"N, 9°55'45.83"N, 9°44'55.19"N, 9°45'4.73"N, 9°59'59.01"N Lat. and 76°16'23.83"E, 76°13'12.55"E, 76°18'0.76"E, 76°17'4.47"E, 76°16'59.54"E, 76°13'3.23"E Long.), Kerala, India. These species were identified and cultured by following the standard methodologies (Sournia, 1978; Gopinathan, 1982, 1996)

before being analyzed for their fatty acid composition (Metcalf *et al*., 1972; Chakraborty *et al*., 2007).

Table 4.1. List of marine microalgae used for study.

Sample code	Algal species	Code
1	*Nannochloropsis occulata*	RA_{01}
2	*Nannochloropsis* sp	RA_{02}
3	*Nannochloropsis* sp	RA_{05}
4	*Isochrysis* sp	RA_{06}
5	*Isochrysis galbana*	RA_{09}
6	*Isochrysis* sp	RA_{10}
7	*Dicrateria inornata*	RA_{14}
8	*Tetraselmis suecica*	RA_{17}
9	*Tetraselmis gracilis*	RA_{18}
10	*Tetraselmis* sp	RA_{19}
11	*Dunaliella* sp	RA_{20}
12	*Chlorella salina*	RA_{24}
13	*Chlorella* sp	RA_{26}
14	*Chaetoceros* sp	RA_{35}
15	*Chaetoceros calcitrans*	RA_{40}
16	*Pavlova lutheri*	RA_{45}
17	*Pavlova viridis*	RA_{46}
18	*Picochlorum* sp	RA_{47}

4.2.2 Stock and mass culture of microalgae

The starter culture of the marine microalgae was isolated and cultured followed by standardized method (Sournia, 1978, Gopinathan, 1982, 1996). The stock culture was inoculated into the 50 ml culture flasks filled with sterilized seawater enriched by Guillard and Ryther's F/2 media (Guillard, 1962). The content were incubated in the light intensity of 1500 lux with a photo period 12 hrs light/12 hrs dark cycle at a temperature of 25 ± 2 °C and an alkaline pH (7.8 - 8.1). The culture was shaken properly in clockwise and anti-clockwise direction twice per day to avoid the settling of the cell concentration. The culture was maintained and gradually increased to the mass culture in to 15 l transparent bucket. The culture was harvested when it reached maximum cell concentration of about 1.2 x 10^6 cells ml^{-1} (exponential growth phase). The fully grown culture was harvested by centrifugation

(9000 rpm, 4°C for 15 to 20 min, Thermo Sorvell Biofuge^{TM}, USA), and the microalgal pellets obtained in this process were analyzed for fatty acid profiling (Metcalf *et al*., 1972; Chakraborty *et al*., 2007).

Figure 4.1. Microalgae culture facility

Figure 4.2. Mass culture of Microalgae

Figure 4.3. Micralgae samples for centrifuge

Figure 4.4. Centrifuged microalgae for fatty acid analysis

4.2.3. Preparation of microalgal powder

The shortlisted microalga was cultured in mass under outdoor condition. The outdoor culture has been undertaken in 250 l capacity circular fibre reinforced plastic (FRP) tanks using (KNO_3: 12g, EDTA (Na): 6g, Na_3PO_4: 6g, Na_2O_3Si: 6 g/ton) (Gopinathan, 1996, Gopinathan and Gireesh, 2006). The whole medium was aerated properly to distribute the microalgae cells and salts in a uniform manner. The maximum growth rate with cell concentration was observed on 5^{th} or 6^{th} day. Thereafter, the culture was filtered through a 20 µ bolting cloth with minimum water flow rate. The cloth containing the filtrate was shaken and the filtered sample often transferred into another container to avoid clotting. The final semisolid material was then filtered with the vacuum filtration system by using the Whatman filter (GF/C) to drain the residual water content. The harvested sample was thereafter lyophilized, and stored at 4°C before being analyzed for fatty acid composition.

Figure 4.5. Vacuum filtration unit

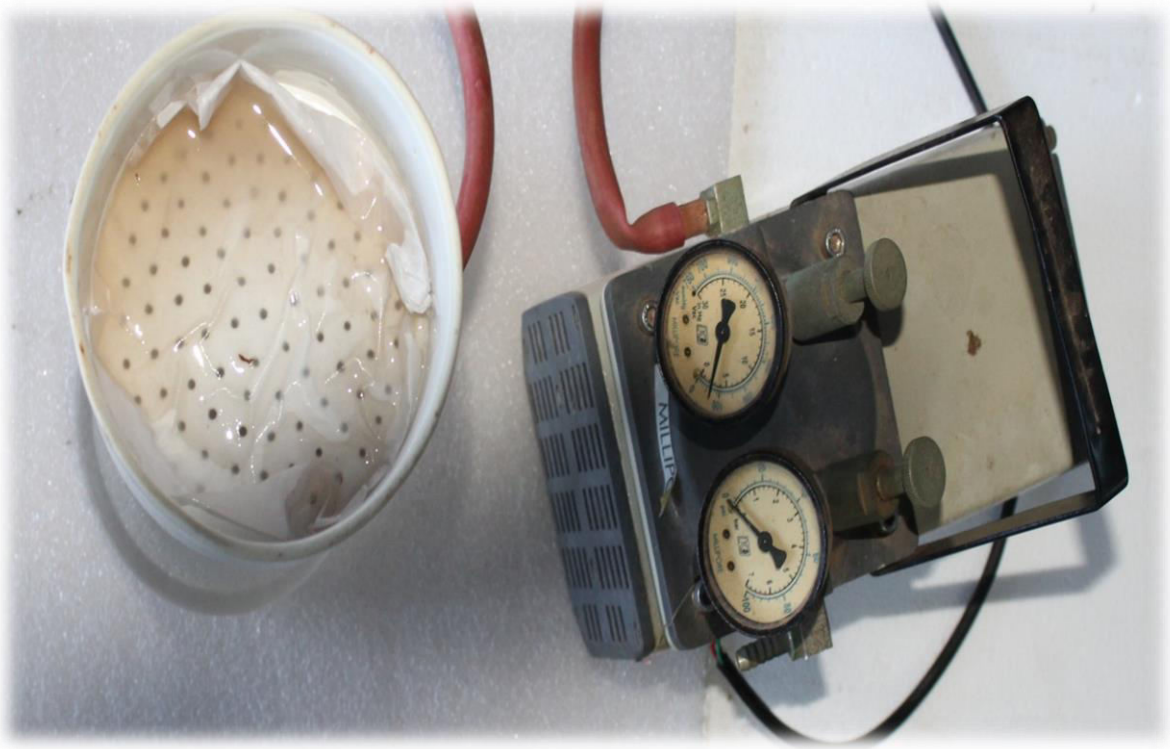


Figure 4.6. Filtration of marine microalgal speciesby vacuum filtration unit

Figure 4.7. Filtered marine microalgal species by using Whatman filter paper

Figure 4.8. Lyophilizer used to prepare microalgal powder

Figure 4.9. Lyophilized Marine microalgal powder

4.2.4. Fatty acid analysis of microalgae

4.2.4.1. Estimation of total lipids

The lipid content was estimated following method of Bligh and Dyer (1959) with suitable modifications. In brief, the minced samples (5 g wet weight) were extracted overnight (4ºC) in $CHCl_3$: MeOH (E-Merck, Germany) (60 ml, 2:1 v/v) containing TBHQ (0.02% w/v). The supernatant obtained after filtration was transferred into a separating funnel to which saturated saline (NaCl, 20 ml) were successively added for phase separation. The aqueous layer was discarded, and the organic portion (lower layer) was dried with anhydrous Na_2SO_4 and filtered (Whatman filter paper 1). The organic solvent was evaporated under vacuum using a rotary vacuum evaporator (IKA, Germany), and the total lipid was recovered. The total lipid contents were determined by gravimetric analysis, and stored at under N_2 at -20°C until further use for fatty acid analyses.

4.2.4.2 Fatty acid composition analyzes by gas liquid chromatography (GLC)

The fatty acid composition of the total lipids was determined as described elsewhere with suitable modification (Metcalf *et al*., 1972; Chakraborty *et al*., 2007). In brief, the triglycerides were saponified with alkaline reagent (3 mL, 0.5 N KOH/MeOH) (Metcalf *et al*., 1966). The saponificable materials were thereafter reacted with a methylating mixture (14% BF_3/CH_3OH) to *trans*-esterify the saponificable material yielding fatty acid methyl esters (FAME) that was later extracted with *n*-hexane/H_2O (1:2, v/v). After removal of the aqueous layer, the *n*-hexane layer was passed through Na_2SO_4, concentrated *in vacuo*, reconstituted in petroleum ether (40-60^{0}C), and stored at -20°C until required for analyses. The GC analyses were accomplished on a Perkin Elmer Gas chromatograph (HP 5890 Series II, Perkin Elmer, USA) equipped with a SP 2560 (crossbond 5% diphenyl 95% dimethyl polsiloxane) capillary column (100 m X 0.25 mm i.d., 0.50 µm film thickness, Supelco,

Bellfonte, PA) using a flame ionization detector (FID) equipped with a split/splitless injector, which was used in the split (1:15) mode. The oven temperature ramp program: 140°C for 1 min, rising at 30°C / min to 250°C, where it was held for 1.0 min, followed by an increase of 25°C /min to 285°C, where it was held for 2.0 min, until all peaks had appeared. The injector and detector were held at 285 and 290°C, respectively. Nitrogen (ultra-high purity >99.99%) was used as carrier gas at 25 cm/s flow rate. Hydrogen was used as the carrier gas at a head pressure of 20 psi. The injection volume was 0.02 µl. FAMEs were identified by comparison of retention times with known standards (37 component FAME Mix, Supelco). Results were expressed as percent weight of total fatty acids.

4.2.5. Statistical analysis

A suite of statistical analyses were carried out using statistical packages for triplicate values, the results have been expressed as mean $\pm$ standard deviation. All the elucidate graph for the fatty acids analysed in respective microalgae were analyzed through the Microcal Origin (Version 8).

4.3. Results and discussion

4.3.1. Fatty acid composition of the different microalgae

The fatty acid database study was undertaken for screening with two objectives (1) to shortlist the microalgae rich in long chain *n*-3 PUFAs, particularly DHA for use in live feed (rotifer) culture, and (2) to produce microalgal powder from the selected marine microalgal species rich in long chain total PUFA for use in aquaculture. Of the 18 marine microalgae strains screened, three each were from the genus *Nannochloropsis* (*Nannochloropsis occulata*, *Nannochloropsis* sp, *Nannochloropsis* sp), *Isochrysis* (*Isochrysis galbana*, *Isochrysis* sp RA_{06}, RA_{10}), and *Tetraselmis* (*Tetraselmis suecica*, *Tetraselmis gracilis*, *Tetraselmis* sp); two each were from the genus *Chlorella* (*Chlorella salina*, and *Chlorella*

sp), *Pavlova* (*Pavlova lutheri*, and *Pavlova viridis*), and *Chaetoceros* (*Chaetoceros calcitrans*, *Chaetoceros* sp); whilst a single species was shortlisted from the genus *Dicrateria* (*Dicrateria inornata*); *Dunaliella* (*Dunaliella* sp); and *Picochlorum* (*Picochlorum* sp).

The fatty acid composition with respect to a total of 28 different fatty acid components was analyzed. A total of eight each fatty acids belonged to SFA (14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0, and 24:0), and MUFA (14:1, 15:1, 16:1, 18:1*n-7*, 18:1, 20:1, 22:1, and 24:1), whilst ten fatty acids formed the PUFA series (18:2*n*-6, 18:3*n*-6, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 183*n*-3, 18:4*n*-3, 20:5*n*-3, 22:5*n*-3, 22:6*n*-3). The detailed fatty acid compositions were shown in Table 4.2.

Among the fatty acid composition, the share of the saturated fatty acids were predominant with above 50 % total fatty acids (%TFA) in two microalgae species *Chaetoceros* sp and *Dunaliella* sp with (Table 4.2.). It was demonstrated that the seven microalgae were found to contain greater than 40 % SFAs. These species included *Chaetoceros calcitrans* (41.08% TFA), *Nannochloropsis salina* (44.10% TFA), *Nannochloropsis* sp (44.4% TFA), *Tetraselmis gracilis* (44.8% TFA), *Tetraselmis suecica* (45% TFA), *Tetraselmis* sp (46.6% TFA), and *Picochlorum* sp (46.7 %TFA). A lesser content of SFA was apparent in *Dicrateria inornata* (21.7% TFA), *Isochrysis galbana* (27.9% TFA), and *Pavlova lutheri* (28.7% TFA). Among the different fatty acids, palmitic acid (16:0) constituted major share of SFAs.

The composition of the monounsaturated fatty acid of the eighteen microalgae were analyzed and recorded under the Table 4.2. *Nannochloropsis oculata* recorded more noteworthy content of PUFAs (37.8 %TFA), followed by those in *Pavlova viridis* holding (36.1% TFA), *Nannochloropsis salina* (35.8% TFA), *Nannochloropsis* sp (32.3% TFA), *Picohlorum* sp (32.2% TFA), *Tetraselmis suecica* (30.7% TFA), and *Tetraselmis* sp (30.4%

TFA), in descending order. The aggregate content of MUFA was found to be greater than 20% TFA in *Dunaliella* sp (20.7% TFA), *Isochrysis galbana* (24.4% TFA), *Pavlova lutheri* (24.6% TFA), *Isochrysis* sp (RA_{06}) (25.9% TFA), *Dicrateria inornata* (26.4% TFA), *Chlorella salina* (26.9% TFA), *Isochrysis* sp (27.1% TFA), and *Tetraselmis gracilis* (28.9% TFA).Among various species of microalgae, *Chaetoceros* sp was found to contain lesser than 10% of the total MUFA content (7-8% TFA). Palmitolic acid (16:1*n*-7) and oleic acid (18:1*n*-9) contributed the predominant share among various MUFAs.

The most important form of fatty acid necessary to be considered in aquaculture farming has been the polyunsaturated fatty acids (Sargent *et al*., 1997). This group of fatty acids were found to be predominant in *Chaetoceros calcitrans* (49.6% TFA), *Chlorella* sp (45.2% TFA), *Isochrysis gabana* (43.4% TFA), *Pavlova lutheri* (41.2% TFA), *Chaetoceros* sp (39.6% TFA), *Dicrateria inornata* (38.9% TFA), *Isochrysis* sp (RA_{10}) (38.4% TFA), in descending order.

Among various classes of PUFAs, the *n*-3 PUFA series were reported to play a significant role in the growth and development of the finfish and shellfish larvae. The aggregate *n*-3 LC-PUFAs in the present study was predominant with *Isochrysis galbana* (34.1% TFA), followed by *Dicrateria inornata* (30% TFA), *Pavlova lutheri* (23.6% TFA), *Chlorella* sp (22.7% TFA), and *Isochrysis* sp (20.7% TFA), in descending order.

Among different *n*-3 LC-PUFAs, the fatty acid DHA has been considered to be indispensable for the growth and survival of the marine fish larvae at the initial stage of their feeding. The fatty acid composition of DHA in the microalgae was found to be present in considerable amount (7-9% TFA) in *Isochrysis galbana* and *Isochrysis* sp strains RA_{10} and RA_{10}, followed by *Chlorella salina* and *Pavlova lutheri*, which shared about 6% TFA of the aggregate content of fatty acids (Table 4.2).

The proper growth and survival of the marine fish larvae nutrition is based on the feed intake during the initial period of their life cycle. During this period the live feed like rotifers, copepods, and *Artemia* are to be fed with the formulation required by the marine fish larvae (Coutinho *et al*., 2006). Microalgae are the only source to provide the essential nutritious composition for live feeds and marine larvae (Benemann, 1992). For normal growth and survival of marine fish larvae, the *n*-3 PUFAs, particularly eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) were found to be essential (Watanabe, 1993). It has been considered that the most required essential fatty acid value to marine species is DHA (Glencross, 2009).

In the present study the fatty acid analysis of the potential marine microalgae, which were collected from various places around southwestern coast of India, for use in aquaculture was carried out. A total of eighteen species of microalgae were isolated before being analyzed for their fatty acid composition (Metcalf *et al*., 1972; Chakraborty *et al*., 2007). The eighteen microalgae belonged to the classes referred to as Eustigmatophyceae, Prymnesiophyceae, Prasinopyceae, Chlorophyceae, Bacillariophyceae, and Trebouxiophyceae. Among different Eustigmatophyceae, the microalgae belonging to the genus *Nannochloropsis* has been considered to be the most common species used in mariculture for live feed (Watanabe *et al*., 1983). The genus *Nannochloropsis* considered in the present study was found to contain greater content of PUFAs, particularly eicosapentaenoic acid (Volkman *et al*., 1989). Likewise EPA content was found to greater in *Nannochloropsis* (~12% TFA). The study conducted by Tonon *et al*. (2002) demonstrated that the EPA content of the *Nannochloropsis occulata* was near to 8% TFA, which was lesser than those obtained in the present study. The predominant fatty acids were found to be 16:0, 16:1, and 20:5*n*-3 in the microalgae belonging to the class Eustigmatophyceae, as also supported by previous studies (Kobayashi *et al*., 2008; Zhukova and Aizdaicher, 1995). DHA

was found to be predominant fatty acids in the microalgae belonging to the class Prymnosiophyceae. For example, the microalga, *Isochrysis galbana* was found to contain 9.8% TFA DHA among different fatty acids considered in the present study. However, the share of the content of this long chain C_{22} fatty acid was found to be lesser (~2.4-2.7 %TFA) in a different strain of *Isochrysis galbana* (Reiriz *et al*., 1989; Reiriz and Labarta, 1996). Among different classes of fatty acids, the share of the *n*-3 PUFA was significant (~34% TFA). Similar results were reported by Lie *et al*. (1997) depicting the presence of *n*-3 PUFAs as predominant class of fatty acids in *Isochrysis galbana*.

Among different class of microalgae considered in the present study, those belonging to Prasinophyceae (*Tetraselmissuecica*, *Tetraselmis gracilis*, and *Tetraselmis* sp) were found to contain ~20-24% PUFA among different fatty acids. These results shared close similarly with those published by Guzman *et al*. (2010) and Patil *et al*. (2007a) on *Tetraselmis* spp, who reported that the total content of PUFA was about 19-23%. Among PUFAs the major fatty acid found to be EPA (9-11% TFA) in the present study, whereas previous reports (Guzman *et al*., 2010; Patil *et al*., 2007; Pratoomyot*et al*., 2005) demonstrated 18:3*n*-3 as the dominant fatty acid in PUFAs. The share of PUFAs among different fatty acids was found to be significantly greater in the microalgae belonging to the class Bacillariophyceae (~50% TFA). The content of the total PUFA in the same microalgal class (Bacillariophyceae) as reported in the previous studies shared similar results (~47% TFA) (Renaud *et al*., 1999, 2002; López Elías *et al*., 2003; Prartono *et al*., 2013). The microalgae *Chlorella* sp belonging to the class Trebouxiophyceae was found to be contain considerable PUFAs (45% TFA), although earlier studies reported the presence of PUFAs in meager quantities (~1% TFA) (Widianingsih *et al*., 2013). However, the PUFAs found in this class of microalgae are of short chain C_{18} series, particularly 18:3*n*-3, which contibuted the predominant share

amongother fatty acids (22% TFA). Likewise, Costard *et al*., (2012) found 18:3*n*-3 as the major fatty acid among the polyunsaturated fatty acids.

It has been reported that under captivity, the long chain PUFAs present in the marine microalgae transferred to the fish through bio-enrichment (Gil and Roque, 2003). It is therefore apparent that the fatty acid profile, particularly those of long chain C_{20-22} PUFAs of the newly isolated microalgal species should be known for their use in mariculture. It is therefore that the potential marine microalgae considered in the present study were shortlisted based on the greater content C_{20-22} PUFAs with reference to EPA and DHA.

The SFAs were found to be present in lesser quantities in *Dicrateria inornata, Isochrysis galbana, Pavlova lutheri, Isochrysis* sp (RA_{10}), and *Chlorella salina*. The greater content of PUFA were found in *Chaetoceros calcitrans, Chlorella* sp, *Isochrysis galbana, Pavlova lutheri*, and *Chaetoceros* sp. The aggregate content of *n*-3 LC-PUFAs was found to be predominant (~34% TFA) in *Isochrysis galbana*, *Dicrateria inornata*, *Pavlova lutheri*, *Chlorella* sp, and *Isochrysis* sp (RA_{10}). DHA was found to be dominant (~10% TFA) in *Isochrysis galbana*, *Isochrysis* sp (RA_{10}), *Isochrysis* sp (RA_{06}), *Chlorella salina*, and *Pavlova lutheri*.

From the present background, it has been considered that *Isochrysis galbana* might possess the vital qualities, such as, greater content of DHA (~10% TFA) and *n*-3 LC-PUFAs (~34% TFA) to suit for mariculture purposes (Figures 4.12. and 4.13.). The results obtained in the earlier studies on the fatty acid profile of *Isochrysis galbana* (Martínez-fernández *et al.*, 2006; Rohani-Ghadikolaei *et al.*, 2012) were in accordance with the present study. The long chain C_{20-22} PUFAs in microalgae could be transferred to the cultured finfish and shellfish larvae through bio-encapsulation techniques using live feeds, such as, rotifer.

Table 4.2. Fatty acid composition of marine microalgae (% total fatty acids)

Fatty acids (%)	**RA_{01}**	**RA_{02}**	**RA_{05}**	**RA_{06}**	**RA_{09}**	**RA_{10}**
14:0	5.21±0.06	6.17±0.01	5.64±0.08	12.26±0.04	6.40±0.30	13.67±0.22
15:0	1.22±0.20	1.50±0.01	1.41±0.17	3.11±0.01	0.73±0.21	3.17±0.03
16:0	21.62±0.18	33.58±0.06	34.57±0.26	14.20±0.27	14.32±0.12	10.73±0.12
17:0	0.00±0.00	0.28±0.01	0.22±0.03	2.15±0.02	0.00±0.00	2.30±0.30
18:0	8.52±0.24	1.19±0.05	1.11±0.04	0.52±0.02	5.86±0.08	0.58±0.05
20:0	0.00±0.00	0.57±0.01	0.58±0.04	1.27±0.02	0.00±0.00	1.27±0.06
22:0	0.00±0.00	0.75±0.02	0.82±0.03	0.57±0.02	0.00±0.00	0.55±0.03
24:0	0.52±0.03	0.07±0.02	0.03±0.01	0.30±0.02	0.60±0.03	0.25±0.03
ΣSFA	**37.09±0.30**	**44.10±0.05**	**44.40±0.34**	**34.38±0.26**	**27.92±0.46**	**32.51±0.44**
14:1	0.00±0.00	0.09±0.02	0.04±0.01	0.23±0.02	0.00±0.00	0.27±0.02
15:1	0.00±0.00	1.65±0.02	1.81±0.04	3.16±0.03	0.00±0.00	3.07±0.11
16:1	16.57±0.33	21.55±0.06	20.02±0.17	7.09±0.06	3.42±0.24	6.36±0.10
18:1*n*-7	0.00±0.00	0.27±0.01	0.13±0.04	0.32±0.02	0.00±0.00	0.28±0.03
18:1	19.95±0.36	8.83±0.06	6.91±0.13	15.19±0.07	20.65±0.19	14.79±0.35
20:1	0.00±0.00	0.09±0.01	0.04±0.02	0.27±0.01	0.00±0.00	0.27±0.04
22:1	0.00±0.00	3.25±0.01	3.18±0.04	0.27±0.02	0.00±0.00	0.31±0.07
24:1	1.25±0.03	0.08±0.02	0.20±0.04	0.55±0.02	0.37±0.07	0.52±0.06
ΣMUFA	**37.77±0.06**	**35.81±0.05**	**32.32±0.38**	**27.07±0.12**	**24.44±0.49**	**25.88±0.33**
18:2*n*-6	4.80±0.11	2.92±0.03	3.31±0.03	8.81±0.08	8.31±0.04	8.87±0.04
18:3*n*-6	0.36±0.04	0.60±0.02	0.47±0.02	5.27±0.02	0.19±0.02	5.51±0.03
18:3*n*-3	0.67±0.04	0.49±0.02	0.55±0.04	0.27±0.02	5.83±0.07	0.20±0.02
18:4*n*-3	0.14±0.03	0.00±0.00	0.36±0.04	0.00±0.00	15.29±0.02	11.61±0.04
20:2*n*-6	0.84±0.03	0.39±0.03	0.19±0.04	11.65±0.03	0.06±0.03	0.38±0.05
20:3*n*-6	0.58±0.06	0.23±0.02	0.34±0.04	0.30±0.02	0.25±0.05	0.74±0.08
20:4*n*-6	2.24±0.05	0.48±0.01	0.19±0.04	0.82±0.02	0.49±0.05	2.16±0.07
20:5*n*-3	9.71±0.04	11.82±0.03	11.27±0.06	2.15±0.01	2.67±0.04	0.54±0.03
22:5*n*-3	0.18±0.04	0.00±0.00	0.57±0.05	0.00±0.00	0.48±0.05	1.18±0.05
22:6*n*-3	0.64±0.02	0.53±0.01	0.51±0.07	7.06±0.02	9.79±0.03	7.18±0.03
ΣPUFA	**20.16±0.29**	**17.47±0.09**	**17.75±0.21**	**36.33±0.08**	**43.35±0.26**	**38.35±0.13**
n-3	11.34±0.82	12.85±0.17	13.26±0.45	9.48±0.16	34.05±0.54	20.70±0.60
n-6	8.82±0.45	4.62±0.14	4.49±0.45	26.85±0.40	9.30±0.56	17.65±0.60
n-3/*n*-6	1.29±0.04	2.78±0.10	2.96±0.15	0.35±0.05	3.66±0.06	1.17±0.05

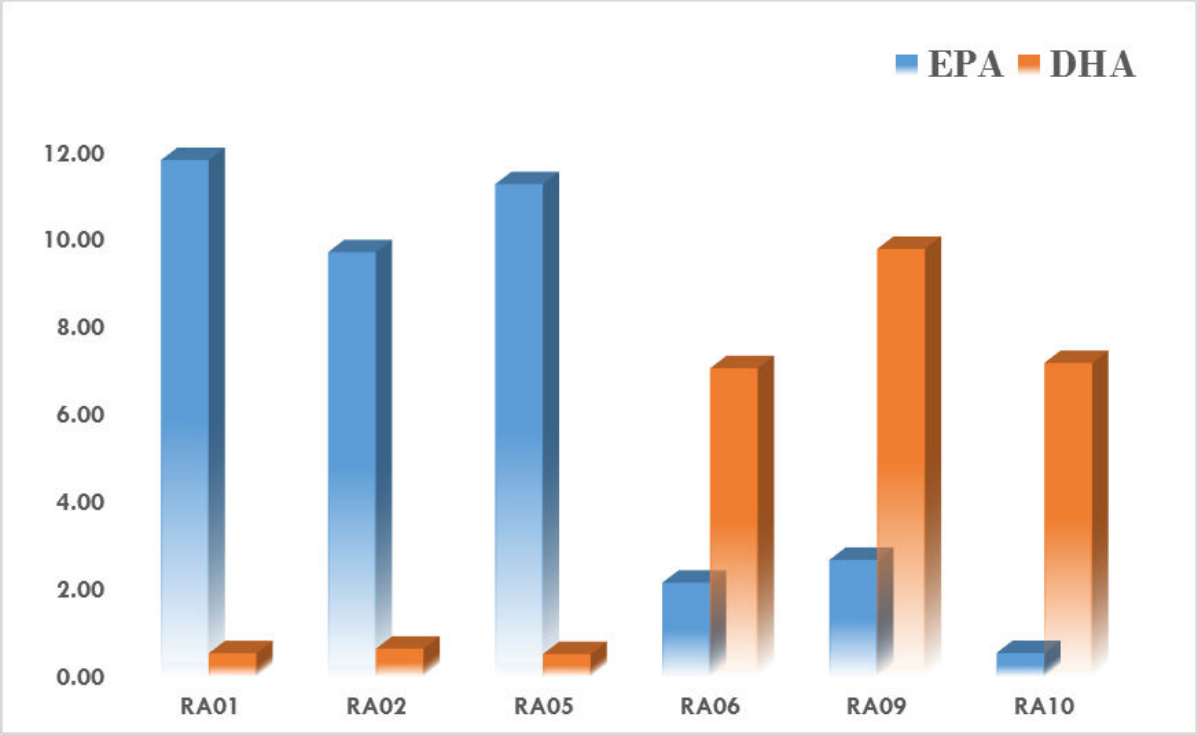


Figure 4.10. Differential content of aggregate EPA & DHA in the experimental microalgae (RA01, 02, 05, 06, 09, and 10)

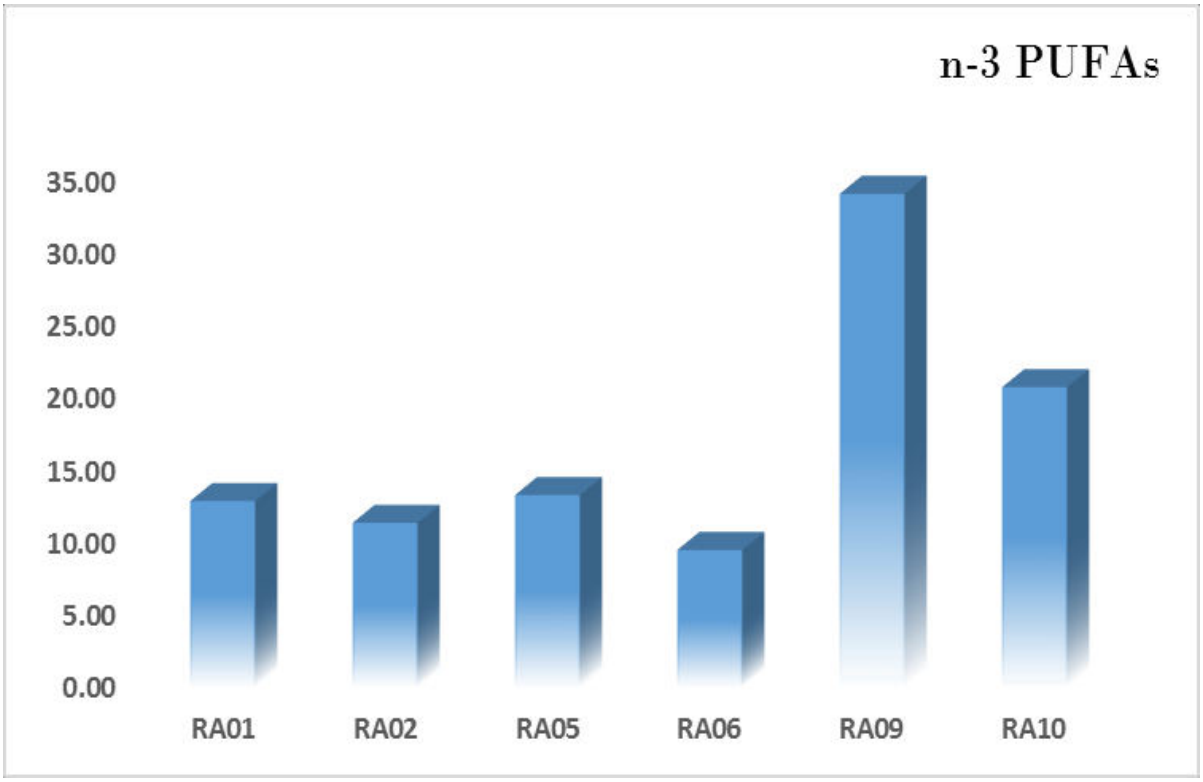


Figure 4.11. Differential content of aggregate *n*-3 PUFAs in the experimental microalgae (RA01, 02, 05, 06, 09, and 10)

Fatty acids (%)	**RA_{18}**	**RA_{19}**	**RA_{17}**	**RA_{35}**	**RA_{40}**	**RA_{26}**
14:0	9.14±0.02	7.22±0.05	6.17±0.02	16.74±0.07	15.53±0.22	0.45±0.02
15:0	0.37±0.02	1.04±0.01	0.27±0.01	1.29±0.02	1.33±0.11	0.14±0.01
16:0	27.73±0.23	29.40±0.10	31.65±0.13	21.30±0.21	11.69±0.05	23.44±0.31
17:0	1.07±0.02	1.17±0.02	0.95±0.03	0.50±0.02	0.34±0.04	0.11±0.02
18:0	4.29±0.04	5.63±0.04	3.93±0.05	8.23±0.04	8.43±0.08	8.63±0.05
20:0	1.56±0.02	0.85±0.02	1.48±0.01	0.66±0.02	0.71±0.03	0.24±0.02
22:0	0.23±0.02	0.43±0.01	0.25±0.02	1.94±0.03	2.13±0.04	0.18±0.01
24:0	0.44±0.03	0.82±0.02	0.33±0.01	0.84±0.03	0.91±0.07	0.19±0.03
ΣSFA	**44.84±0.17**	**46.56±0.06**	**45.04±0.22**	**51.50±0.26**	**41.08±0.22**	**33.38±0.32**
14:1	1.17±0.03	0.75±0.02	1.26±0.03	0.38±0.03	0.47±0.05	0.13±0.01
15:1	0.14±0.04	0.17±0.02	0.17±0.02	0.35±0.03	0.24±0.02	0.36±0.02
16:1	8.22±0.08	10.20±0.06	7.28±0.10	3.43±0.03	3.76±0.14	0.15±0.03
18:1*n*-7	0.12±0.01	0.27±0.02	0.15±0.01	0.65±0.02	0.72±0.05	0.09±0.02
18:1	16.25±0.06	16.26±0.05	18.84±0.08	1.62±0.04	1.65±0.08	17.62±0.05
20:1	0.52±0.01	0.57±0.02	0.78±0.01	0.15±0.01	0.12±0.02	0.27±0.01
22:1	1.97±0.02	1.77±0.03	1.76±0.02	1.07±0.06	1.14±0.04	0.00±0.00
24:1	0.58±0.02	0.46±0.02	0.48±0.01	0.34±0.03	0.22±0.03	0.15±0.03
ΣMUFA	**28.98±0.11**	**30.44±0.16**	**30.71±0.13**	**7.98±0.15**	**8.32±0.19**	**18.76±0.06**
18:2*n*-6	2.51±0.02	2.48±0.02	2.23±0.05	3.94±0.04	4.45±0.04	19.61±0.07
18:3*n*-6	2.85±0.10	2.19±0.02	3.13±0.03	3.17±0.02	4.57±0.07	0.18±0.01
18:3*n*-3	1.27±0.02	1.15±0.01	1.25±0.03	0.26±0.03	0.34±0.03	22.06±0.01
18:4*n*-3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	6.18±0.04	0.00±0.00
20:2*n*-6	1.13±0.04	1.21±0.01	1.05±0.01	5.20±0.01	7.70±0.02	0.32±0.04
20:3*n*-6	0.20±0.03	0.15±0.01	0.28±0.02	6.83±0.02	7.72±0.03	0.14±0.01
20:4*n*-6	0.42±0.01	0.44±0.06	1.39±0.01	7.17±0.02	8.81±0.04	2.60±0.02
20:5*n*-3	11.40±0.01	9.14±0.01	9.23±0.02	8.76±0.02	5.42±0.05	0.17±0.01
22:5*n*-3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.11±0.02	0.00±0.00
22:6*n*-3	3.76±0.01	2.96±0.02	3.47±0.02	4.29±0.01	4.33±0.05	0.13±0.03
ΣPUFA	**23.55±0.13**	**19.72±0.05**	**22.03±0.10**	**39.63±0.04**	**49.63±0.08**	**45.21±0.07**
n-3	16.44±0.17	13.25±0.13	13.95±0.22	13.31±0.12	16.38±0.34	22.36±0.10
n-6	7.11±0.23	6.47±0.13	8.08±0.30	26.32±0.36	33.25±0.35	22.85±0.44
n-3/*n*-6	2.31±0.04	2.05±0.18	1.73±0.06	0.51±0.02	0.49±0.14	0.98±0.07

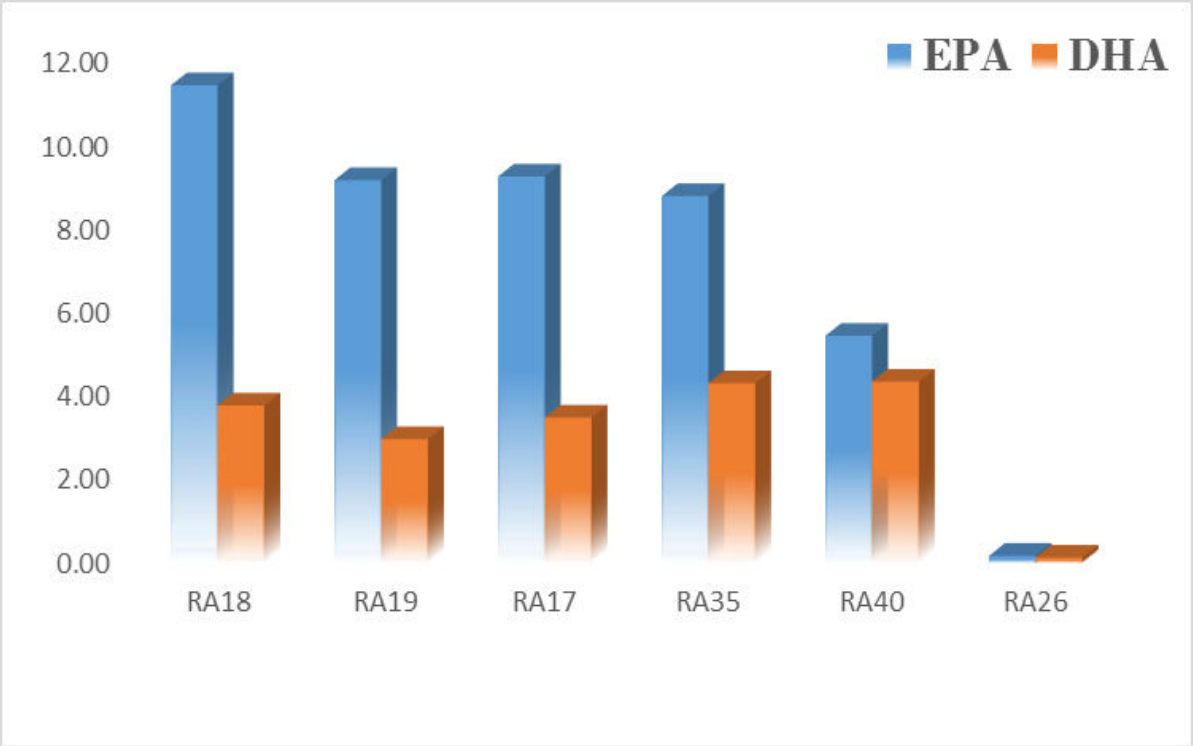


Figure 4.12. Differential content of aggregate EPA & DHA in the experimental microalgae (RA18, 19, 17, 35, 40, & 26)

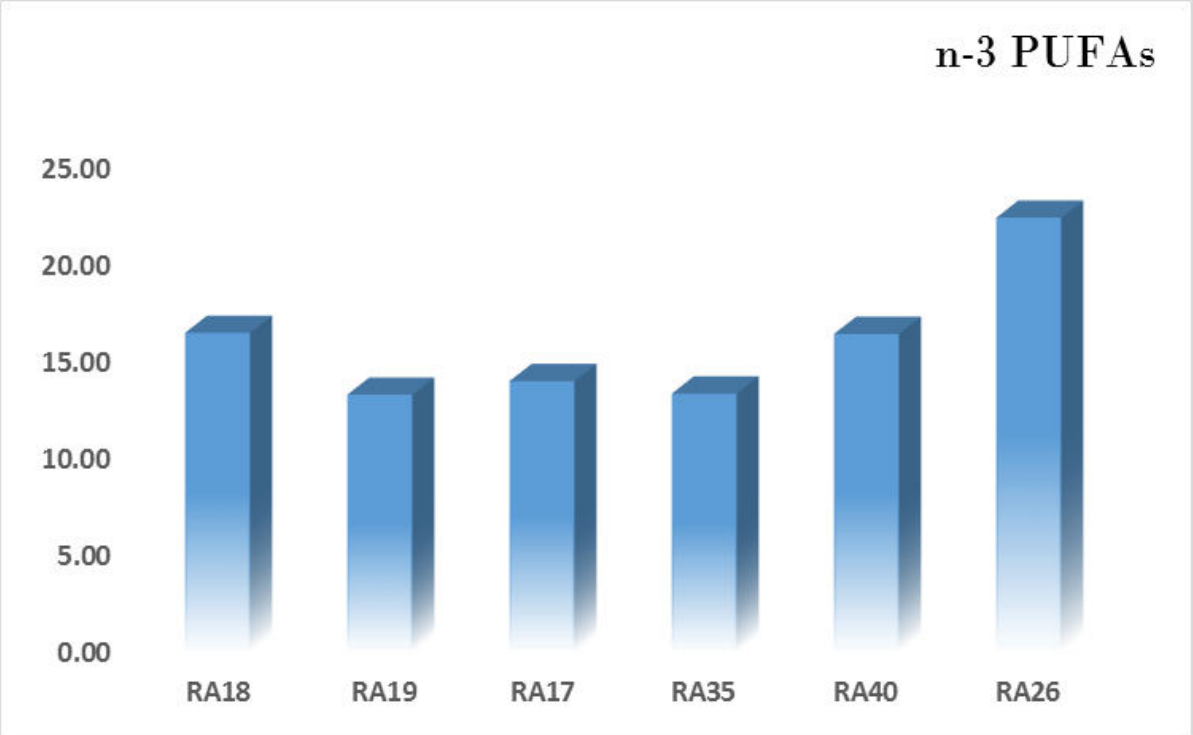


Figure 4.13. Differential content of aggregate *n*-3 PUFAs in the experimental microalgae (RA18, 19, 17, 35, 40, & 26)

Table 4.2. (Continue….)

Fatty acids (%)	RA_{24}	RA_{45}	RA_{46}	RA_{20}	RA_{47}	RA_{14}
14:0	11.54±0.02	13.37±0.34	11.50±0.34	0.76±0.01	8.12±0.01	1.44±0.06
15:0	2.82±0.01	2.10±0.11	0.84±0.08	0.22±0.01	1.17±0.01	0.63±0.09
16:0	14.08±0.04	10.21±0.03	19.23±0.05	21.78±0.14	30.36±0.10	18.81±0.06
17:0	1.94±0.03	0.46±0.04	0.00±0.00	0.00±0.01	1.05±0.02	0.00±0.00
18:0	0.45±0.03	0.57±0.05	1.89±0.04	20.83±0.04	4.57±0.02	0.08±0.02
20:0	1.23±0.02	0.48±0.03	0.00±0.00	4.25±0.03	0.53±0.01	0.00±0.00
22:0	0.49±0.01	0.99±0.06	0.00±0.00	1.55±0.02	0.37±0.02	0.00±0.00
24:0	0.25±0.02	0.47±0.04	1.18±0.03	2.08±0.02	0.53±0.03	0.72±0.04
ΣSFA	**32.79±0.14**	**28.66±0.18**	**34.65±0.26**	**51.48±0.15**	**46.69±0.13**	**21.67±0.11**
14:1	0.36±0.02	0.15±0.02	0.00±0.00	2.57±0.01	0.85±0.03	0.00±0.00
15:1	2.86±0.01	1.90±0.04	0.00±0.00	0.26±0.02	0.20±0.01	0.00±0.00
16:1	6.83±0.09	5.44±0.26	27.56±0.31	4.59±0.04	10.75±0.16	0.63±0.19
18:1*n*-7	0.23±0.02	0.05±0.01	0.00±0.00	0.34±0.02	0.27±0.02	0.00±0.00
18:1	15.36±0.09	15.11±0.30	7.65±0.29	11.21±0.06	17.37±0.04	25.42±0.32
20:1	0.37±0.02	0.62±0.05	0.00±0.00	1.48±0.01	0.47±0.01	0.00±0.00
22:1	0.32±0.01	0.86±0.05	0.00±0.00	0.00±0.00	1.45±0.02	0.00±0.00
24:1	0.59±0.02	0.43±0.04	0.84±0.07	0.27±0.07	0.83±0.02	0.38±0.03
ΣMUFA	**26.91±0.07**	**24.56±0.14**	**36.05±0.10**	**20.70±0.09**	**32.18±0.21**	**26.43±0.52**
18:2*n*-6	8.50±0.07	10.73±0.04	3.43±0.04	7.92±0.08	1.61±0.04	6.38±0.05
18:3*n*-6	5.30±0.03	5.08±0.04	1.05±0.04	2.27±0.03	1.94±0.02	0.39±0.05
18:3*n*-3	0.34±0.03	0.52±0.02	1.40±0.06	1.64±0.02	1.26±0.02	12.84±0.08
18:4*n*-3	0.00±0.00	14.76±0.05	2.89±0.03	0.00±0.00	0.00±0.00	8.60±0.02
20:2*n*-6	11.59±0.02	0.17±0.05	0.26±0.03	2.45±0.13	2.36±0.02	0.22±0.05
20:3*n*-6	0.37±0.02	0.72±0.06	0.13±0.03	1.86±0.02	0.21±0.04	1.07±0.04
20:4*n*-6	0.74±0.01	0.95±0.04	1.35±0.04	3.58±0.02	0.43±0.01	0.93±0.05
20:5*n*-3	3.16±0.02	0.37±0.04	9.54±0.04	2.20±0.01	7.66±0.02	6.87±0.05
22:5*n*-3	0.00±0.00	1.52±0.05	0.19±0.03	0.00±0.00	0.00±0.00	0.33±0.04
22:6*n*-3	6.53±0.01	6.38±0.07	1.84±0.08	3.20±0.02	2.44±0.03	1.31±0.04
ΣPUFA	**36.54±0.11**	**41.19±0.17**	**22.09±0.18**	**25.12±0.07**	**17.92±0.10**	**38.94±0.07**
n-3	10.04±0.23	23.54±0.71	15.87±0.70	7.04±0.13	11.36±0.26	29.95±0.62
n-6	26.50±0.25	17.65±0.37	6.22±0.36	18.08±0.33	6.56±0.21	8.99±0.19
n-3/*n*-6	0.38±0.05	1.33±0.20	2.55±0.21	0.39±0.05	1.73±0.12	3.33±0.22

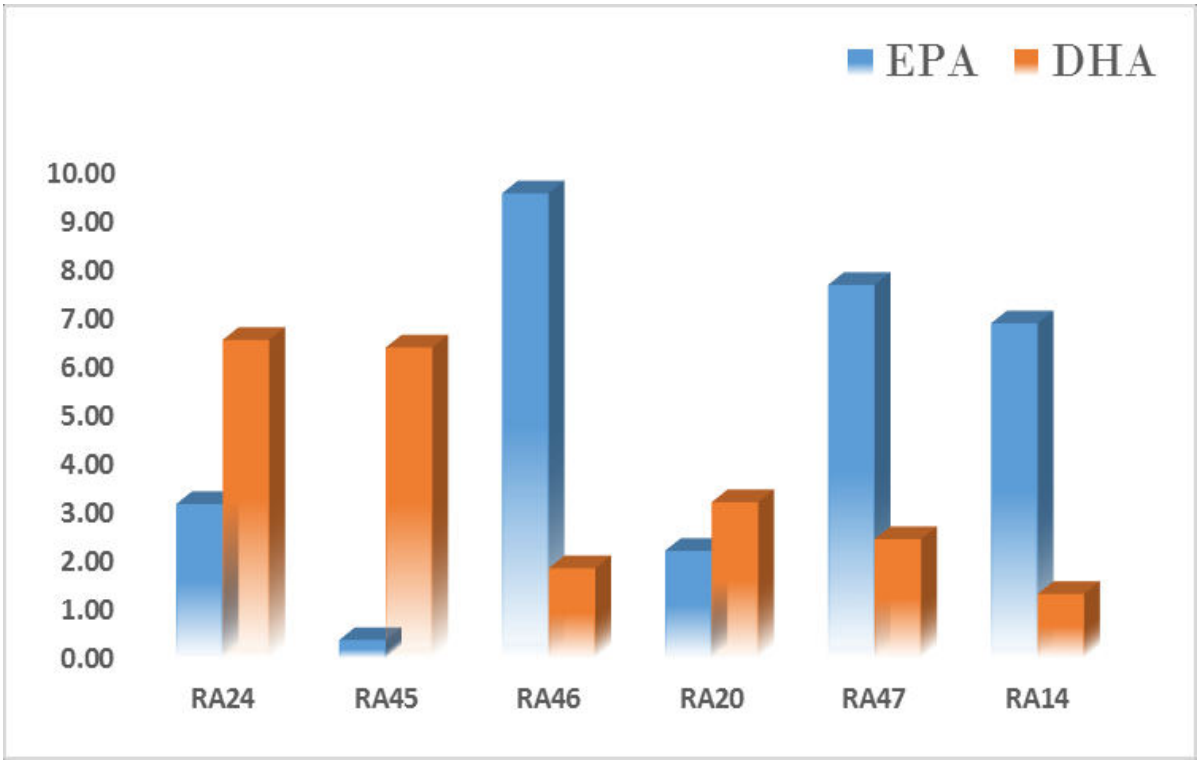


Figure 4.14. Differential content of aggregate EPA & DHA in the experimental microalgae (RA24, 45, 46, 20, 47, & 14)

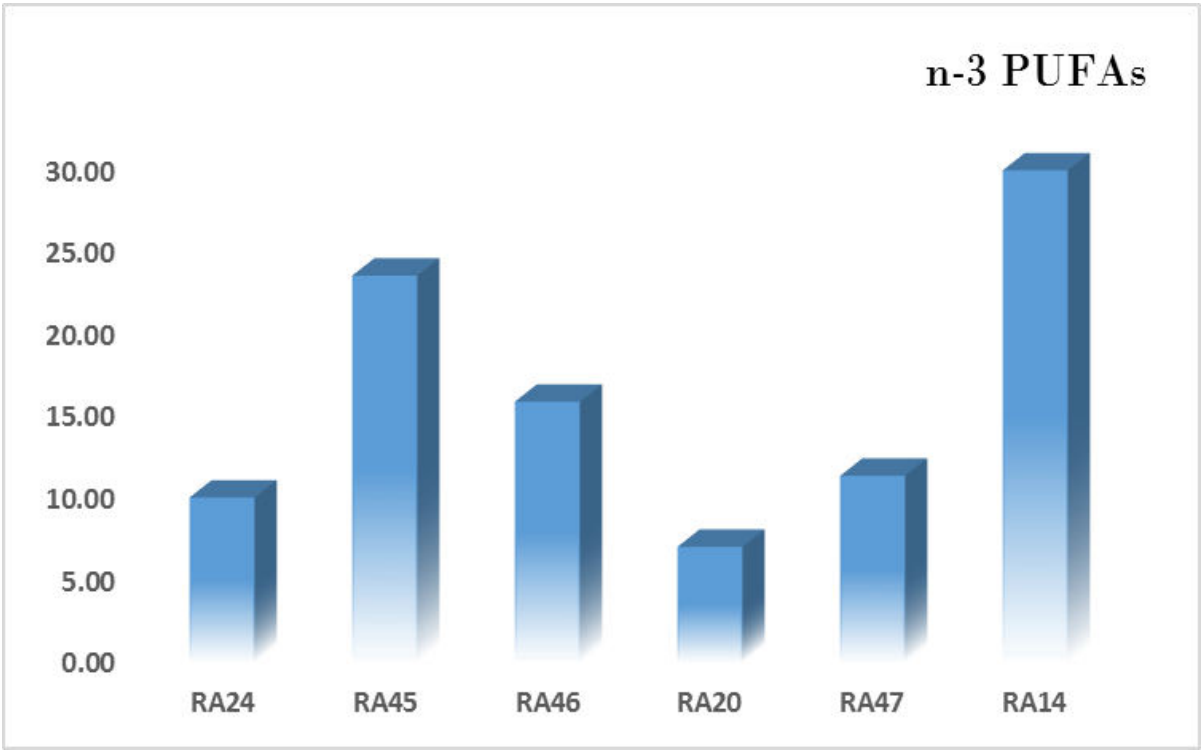


Figure 4.15. Differential content of aggregate *n*-3 PUFAs in the experimental (RA24, 45, 46, 20, 47, & 14)

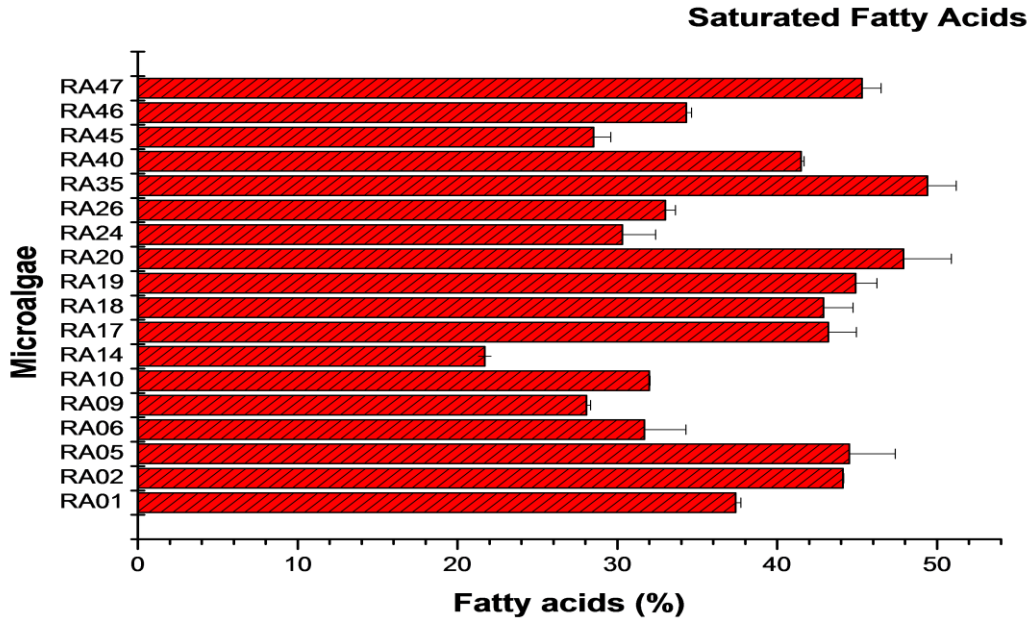


Figure 4.16. Differential content of aggregate SFAs in the experimental microalgae

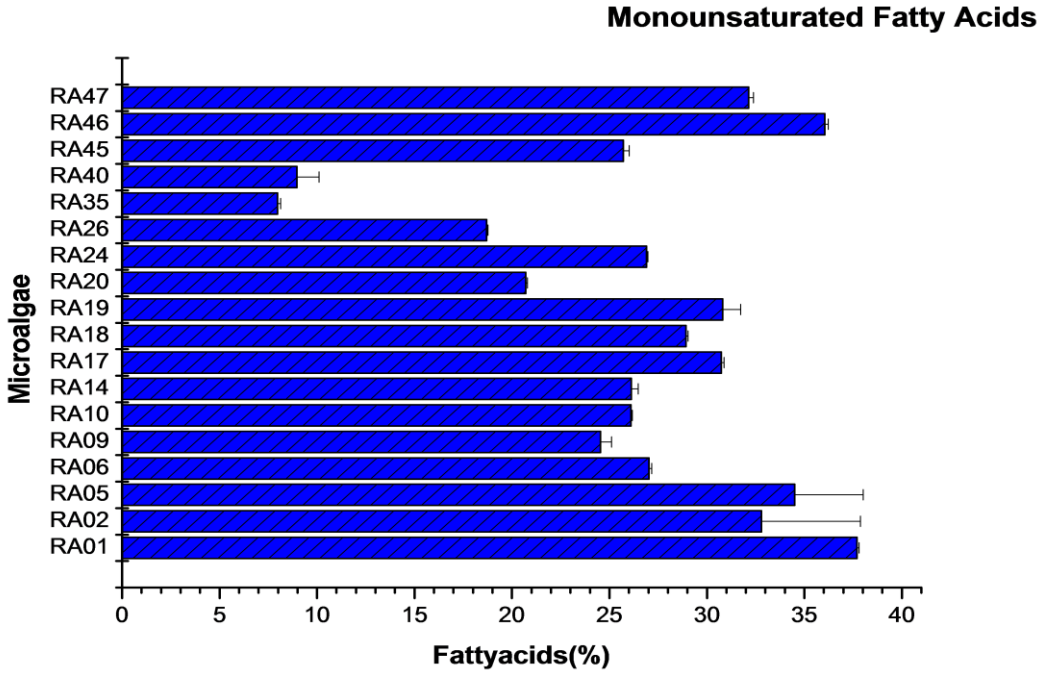


Figure 4.17. Differential content of aggregate MUFAs in the experimental microalgae

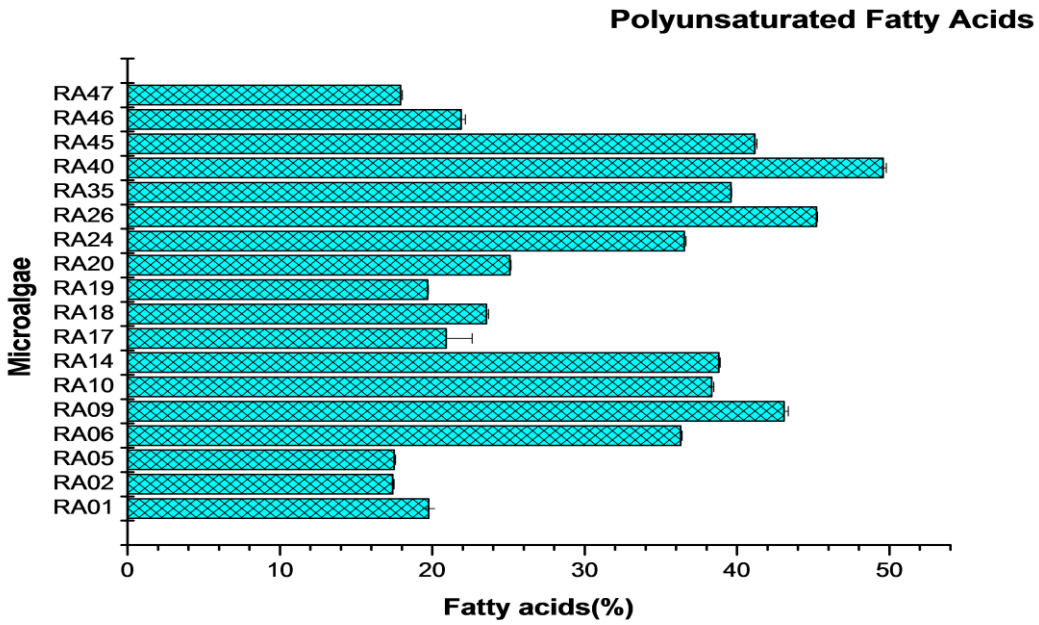


Figure 4.18. Differential content of aggregate PUFAs in the experimental microalgae

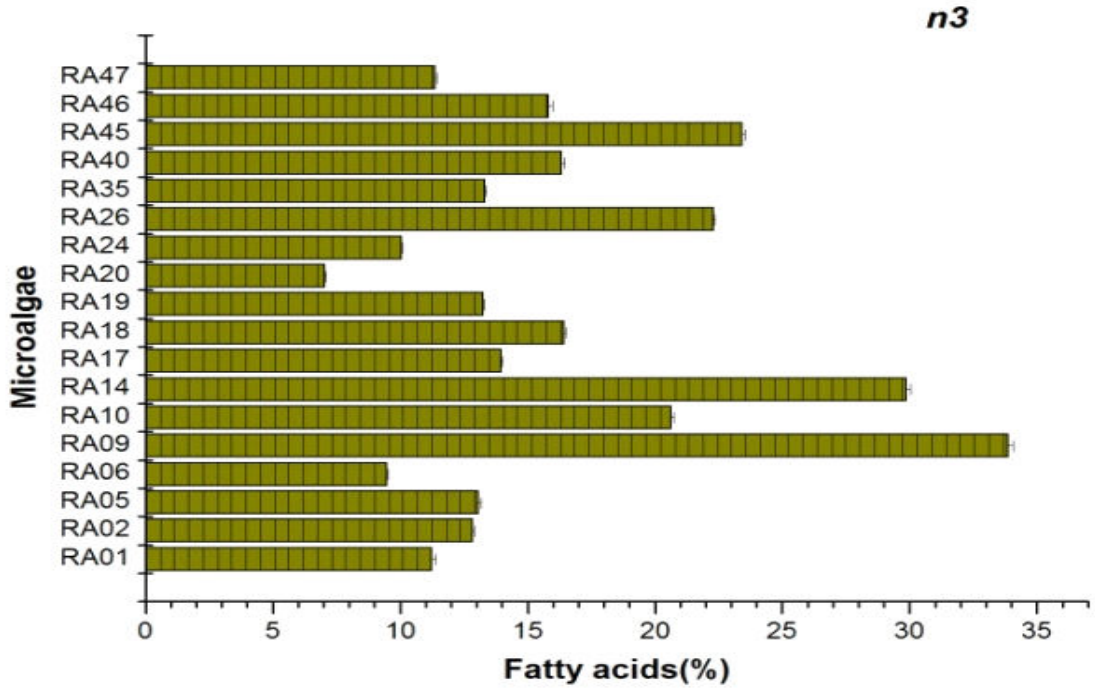


Figure 4.19. Differential content of aggregate *n*-3 PUFAs in the experimental microalgae

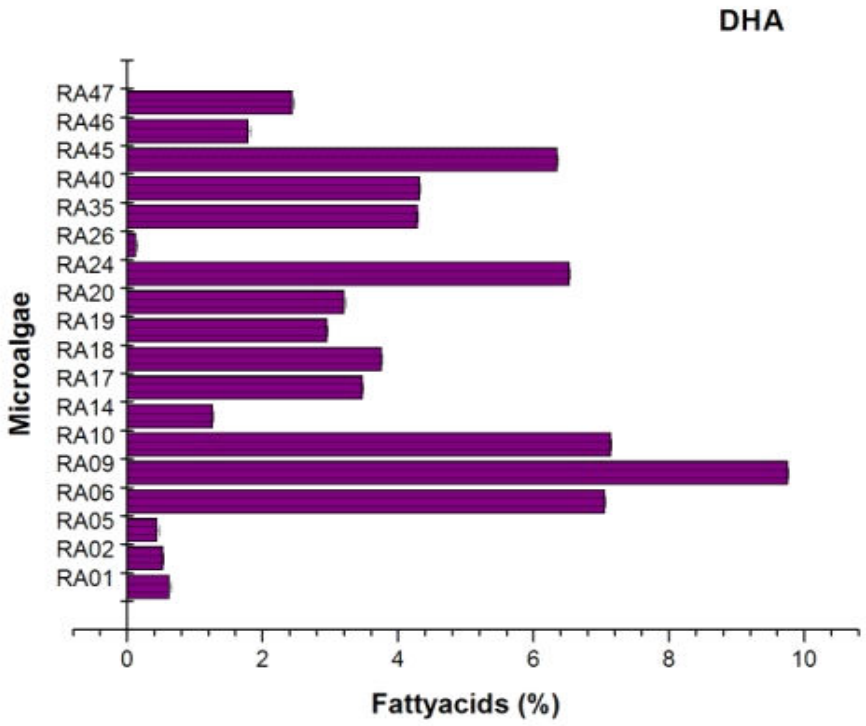


Figure 4.20. Differential content of aggregate DHA in the experimental microalgae

4.3.2. Fatty acid composition of marine microalgal powder

It is well - known that the success of a hatchery set-up always be influenced by the readiness of microalgae as basic food (Silas, 1982). This present study has been carried out to make a readily available microalgal powder for use in aquaculture, and a suitable replacement of commercially available analogues products, which are expensive. Among the different experimental microalgae, *Chaetoceros calcitrans* was found to contain the greater content of aggregate PUFAs as compared to other microalgae species. This was followed by the microalgae, namely, *Isochrysis galbana, Pavlova lutheri,* and *Chlorella* sp recorded an aggregate content of greater than 40% TFA.

In this study *Chaetoceros calcitrans* was shortlisted based on its characteristic physical and chemical features. It is the fast growing, chain type, easy to filter among the other marine microalgae species studied in the present study (4.2.3.). *Chaetoceros calcitrans*

can be considered as the suitable marine microalgae to develop live feed enrichment product, and for use in aquaculture because of its greater nutritional value of essential fatty acids, particularly arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid (Table 4.5.).It is interesting to note for the first time that the balanced proportion of arachidonic acid, eicosapentaenoic acid and docosahexaenoic acids (Table 4.2), is present only in *Chaetoceros calcitrans.* It is of note that *Chaetoceros* sp, *Isochrysis*sp (T.ISO), *Tetraselmis suecica, Pavlova lutheri,* and *Nannochloropsis* spp usually fed together as a mixed diet, are the most common species used to feed the bivalve molluscs, crustacean larvae, juvenile abalone and etc., (Brown, 2002; O'Connor and Heasman, 1997). The mass culture of the shortlisted species was done in outdoor condition. The maximum concentrated culture was filtered using the methodology mentioned above (4.2.3).

Successively, the filtered sample was initially lyophilized to remove the moisture, which was thereafter used as rotifer enrichment media. This species was shortlisted to develop as a live feed (rotifer) enrichment product due to its balanced proportion of ARA (7.55% TFA), EPA (9.20% TFA), and DHA (5.13% TFA). The detailed results are shown in the Table 4.3. The present study attempted to develop an indigenous microalgal powder, which can be used as a readily available product to culture rotifer or to feed finfish and shellfish larvae. In future, the same product can be used by adding other essential nutrients as a cost effective alternative to the imported products, for example, Algamac.

Table 4.3. Fatty acid composition of microalgal powder

Fatty acids (%)	**RA_{40}powder**
C14:0	13.01±0.16
C15:0	0.84±0.11
C16:0	16.13±0.21
C17:0	0.81±0.07
C18:0	6.31±0.21
C20:0	0.61±0.07
C22:0	1.64±0.11
C24:0	0.18±0.07
ΣSFA	**39.53±0.54**
C14:1	0.22±0.04
C15:1	0.12±0.02
C16:1n7	4.47±0.21
C18:1n9	6.67±0.23
C20:1	0.15±0.04
C22:1	0.74±0.09
C24:1	0.15±0.03
ΣMUFA	**12.53±0.43**
C18:2n6	4.12±0.25
C18:3n6	2.85±0.18
C18:3n3	0.31±0.04
C20:2n6	4.61±0.32
C20:3n6	6.62±0.33
C20:4n6	7.55±0.25
C20:5n3	9.20±0.39
C22:6n3	5.13±0.16
ΣPUFA	**40.40±0.45**
Others (unidentified)	**7.86±0.16**

4.4. Conclusions

Among different *n*-3 long chain-PUFAs, the C_{22}*n*-3 fatty acid DHA has been considered to be indispensable for the growth and survival of the marine fish larvae at the initial stage of their feeding. The fatty acid composition of DHA in the microalgae was found to be present in considerable amount (7-9% TFA) in *Isochrysis galbana* and *Isochrysis* sp strain RA_{10} and RA_{10}, followed by *Chlorella salina* and *Pavlova lutheri*, which shared about 6% TFA of the aggregate content of fatty acids. Among the different experimental microalgae, *Chaetoceros calcitrans* was found to contain the greater content of aggregate PUFA as compared to other microalgae species. The present study also attempted to develop

an indigenous microalgal powder which is readily available product to culture rotifer or to feed finfish and shellfish larvae. *Chaetoceros calcitrans* was shortlisted to develop a microalgal formulation based on its characteristic physical and chemical features. In future the same product can be used by adding other essential nutrients as a cost effective alternative to the imported products, for example, Algamac.

CHAPTER 5

Fatty acid composition of rotifer enriched with experimental media

Chapter 5

Fatty acid composition of rotifers enriched with experimental media

5.1. Background of the study

Rotifers popularly called as wheel animalcules. They are an important group of live food organisms for use in aquaculture. *Brachionus* sp, which is the most common form of all known rotifers, serve as an ideal starter feed for early larval stages of many fish and crustacean species in marine as well as freshwater (Das *et al.*, 2012). Species of the genus *Brachionus* (Brachionidae: Rotifera) are well characterized in different water bodies worldwide (Pejler, 1977). Depending on the mouth size of the cultured organisms, small (50 to 110 µ) and large (100 to 200µ) rotifers are used. There are about 2,500 species of rotifers that have been known from global freshwater, brackishwater, and seawater (Lubzens and Zmora, 2003). Globally *B. plicatilis* is the species used most commonly to feed fish larvae in hatcheries. It is a euryhaline species, slow swimming, with good nutritional value.

They are commonly used as live feed, with their size ranging from 50 to 350 µm (Howey, 1999), and were initially described by Rev. John Harris (1696). Most rotifers are of freshwater origin, whilst few are from the marine origin. The intensive larval culture of most marine fish depends on the supply of zooplanktons, especially rotifers. In mariculture, rotifers especially *Brachionus plicatilis* are popularly used as live feed for larval growth and development (Philippe*et al*., 2001). These species were reported to have a greater reproduction rate (0.7 - 1.4 offspring female^{-1} day^{-1}) (Hada *et al*., 1997; Maeda and Hino, 1991). It is a euryhaline species, and one of the suitable preys for the fish larvae. Rotifers are slow swimming planktonic filter feeders ingesting many types of feeds, including bacteria (Philippe*et al.,* 2001). This is essential by keeping in mind that the rotifers carry the vital

nutrients to the fish larvae.

Since 1960s, the rotifers had been used as a live larval food, and thereafter, the mass culture of rotifers become popular as reported in earlier literature (Kitajima *et al*., 1979, Yoshimura *et al*., 1997). There are failed reports of culturing fish larvae under captivity using these live feed. This was attributed due to the absence of vital fatty acids, for example, C_{20-22} long chain *n*-3 polyunsaturated fatty acids in the desired quantities (Lubzens *et al*., 2001). This often resulted in the mass mortality of the fish larvae (Watanabe *et al*., 1978). This drawback could easily overcome by enriching the rotifers with external source with greater content of these polyunsaturated fatty acids (Watanabe *et al*., 1983; Sargent *et al*., 1997; Olivotto *et al*., 2006 and Avella *et al*., 2007). These polyunsaturated fatty acids incorporated through different avenues, for example, microalgae and PUFA enriched fish oil emulsion. The microalgae enriching method of rotifers were reported to increase the long chain *n*-3 PUFA in a meagre quantity, which is not sufficient to supplement the required quantities of these essential fatty acids to the fish larvae (Whyte *et al*., 1994; Oie *et al*., 1994; Fabregas *et al*., 2001). Nutritional enrichment of rotifers developed through various methods (Watanabe *et al*., 1983). Usually commercial enrichment diets have been preferred to enrich rotifers (Reiriz *et al*., 1993). Each enrichment diet manufacturer recommends certain directions for their products, including quantities and durations. The recommended direction is possibly effective for providing optimum nutritional value for larvae, and many previous studies have followed the directions (Kotani *et al*., 2010).

The type of feed given to rotifers was important and it influences the nutritive value. Microalgae could be fed to zooplankton (rotifers) to enrich them, which contain greater quantities of long chain C_{20-22} fatty acids EPA and DHA. However, often these do not provide the level of enrichment often sought for zooplankton. Therefore, in the commercial

hatcheries it is the common practice to enhance nutritional values of rotifers by enriching them with enrichment products (eg. DHA Selco from INVE), which are often used to enrich rotifers (Brown, 2002). Live prey feeding remains essential in commercial marine hatchery operations and rotifers are relatively poor in essential fatty acids like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Hence, it is a common practice to enrich this live prey with emulsions of marine oils (Philippe *et al*., 2001).

In the present study, the feasibility of marine microalgae, for example, *Isochrysis galbana* to enrich the live feed rotifer, *Brachionus plicatilis* was examined due to the presence of greater content of DHA (~10 %) than other experimental microalgae. This particular fatty acid has a multiple roles in the structural and functional development of live feed. Rotifers are often used as the carriers of the long chain fatty acids by means of enrichment with the microalgae for different time interval. In this way, the optimum enrichment period recorded to harvest greater quantities of long chain *n*-3 fatty acids from microalgae to the live feed. Further, different combinations of experimental media were used to enrich the rotifers, and to understand their effect on the $C_{20\text{-}22}$ PUFA composition of rotifers. The PUFA enriched oil emulsion prepared from marine finfish and microalgal powder derived from the *Chaetoceros* sp were taken into account to enrich the live feed (rotifer). Further, the enrichment protocol of the rotifers with the PUFA enriched formulations with respect to time duration and concentration were optimiszd in this study.

5.2 Materials and Methods

5.2.1 Culture of rotifer

The marine rotifers, *Brachionus plicatilis* stock culture was obtained from the Vizhinjam research centre of CMFRI, and upscaled as mass culture. The size of rotifers,

Brachionus plicatilis was ranged 40-300 µm. The microalgal culture of *Isochrysis galbana* was pumped into the FRP tanks of 1 ton capacity. Moderate aeration was provided, and the rotifers were introduced at the rate of 50 numbers/ml. The salinity of the culture medium was 24-28‰, and the temperature ranged between 28-32°C. The rotifers were multiplied parthenogenetically, and reached the maximum concentration of 100-150 numbers/mL within a period of 3-4 days. Harvesting was carried out on alternative days by siphoning out through the 50 µm filter cloth, and the culture was maintained in good condition for a period of about 10 days. After the final harvest, the tanks were drained, cleaned, and dried for one day to start the new culture. The collected rotifers were fed to the larvae after proper wash.

Figure 5.1. Rotifer *Brachionus plicatilis* culture in mass

Figure 5.2. Rotifer culture tank for experimental usage

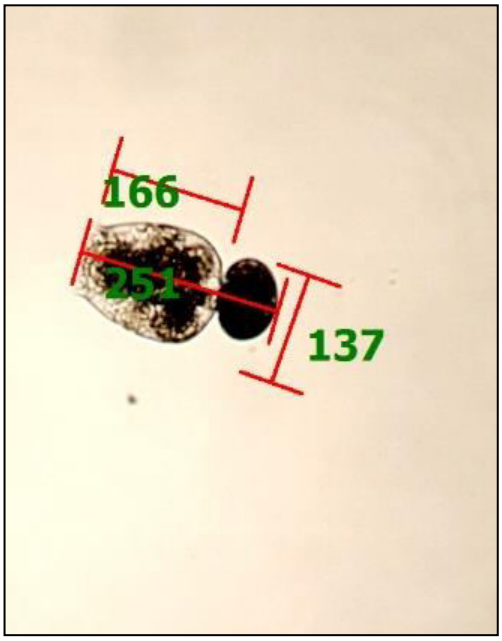


Figure 5.3. Rotifer (*B. plicatilis*) at 10x

5.2.2 Rotifers enriched with microalgae

Initially, the levels of fatty acids were assessed in the microalga *Isochrysis galbana* that were utilized to enrich the rotifers (*B. plicatilis*) at different time intervals (3, 6, 12, 30, and 48 h). The rotifers were thereafter analyzed for their fatty acid composition to determine the optimum time duration, and to ascertain the time taken to attain the maximum level of enrichment. The nutritional enrichment experiments with rotifers were conducted in triplicate at a final volume of 10 L.

5.2.3 Rotifers enriched with oil emulsion

Sardine oil enrichment carried out by biochemical and microbiological procedures as explained under the chapter 3,was used to formulate enrichment emulsion, which contained grossly 90% PUFA enriched fish oil and 10% lecithin extracted from roe of seer fish *Scomberomorus guttatus*. In brief, the preparation of oil emulsion done by following method is used to standardize the concentration of oil required to be used for the further experiments. Two concentrations of oil, 1 ml^{-1} and 0.1 ml^{-1} were chosen to correlate and standardise the required amount of oil to betaken for enrichment. The prepared stock concentrate of long chain polyunsaturated oil was pipetted into the homogenizer (mixer and grinder) containing 500 ml of filtered seawater, and the contents were homogenized for 1 minute. An amount of 0.1 % emulsifier (Tween 20) was added to it before being homogenized for 3 minutes until the solution became milky in appearance. The prepared oil emulsion was thereafter transferred to a container of 3L capacity bearing a total volume of 1.5 L of filtered seawater. The mix was strongly aerated before being introduced to the rotifer culture. The rotifers were subjected to an enrichment period of 24 h. The samples were harvested and washed with fresh, filtered seawater before being stored at -20 °C for further analyses for every 3 h.

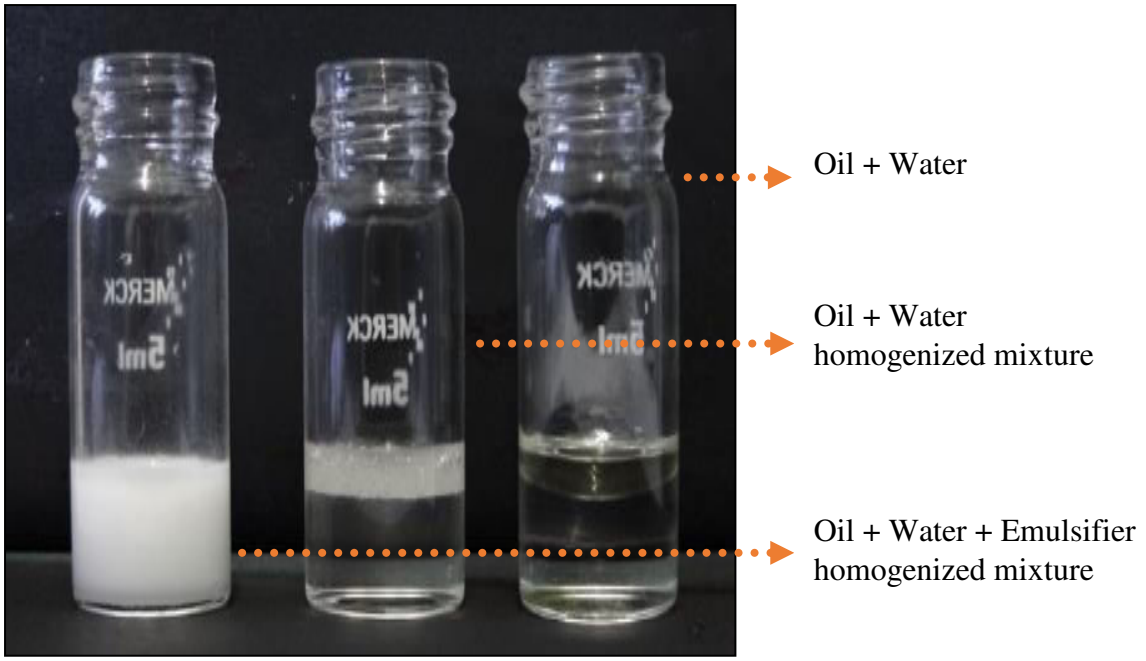


Figure 5.4. Oil emulsion

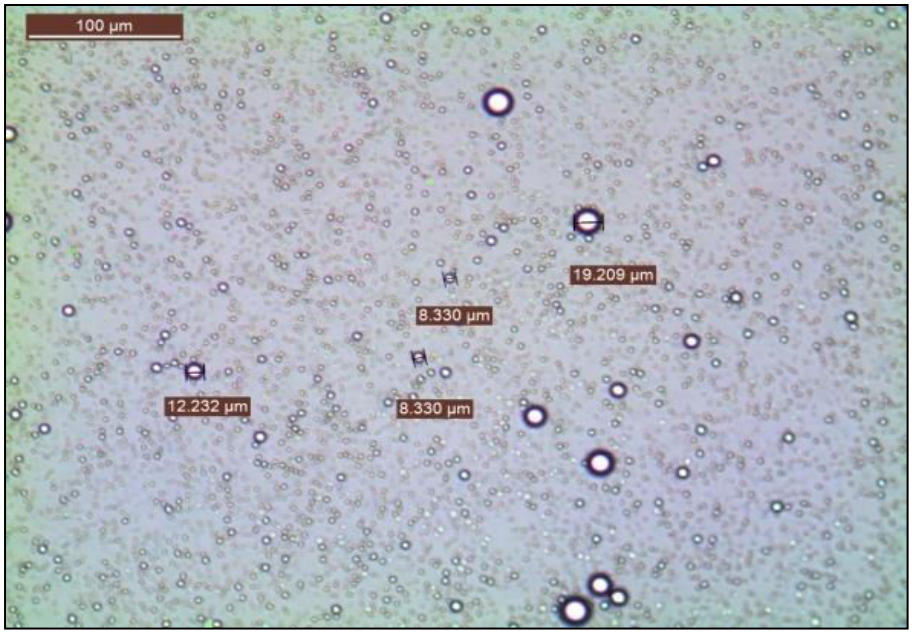


Figure 5.5. Microscopic view of oil emulsion (globules)

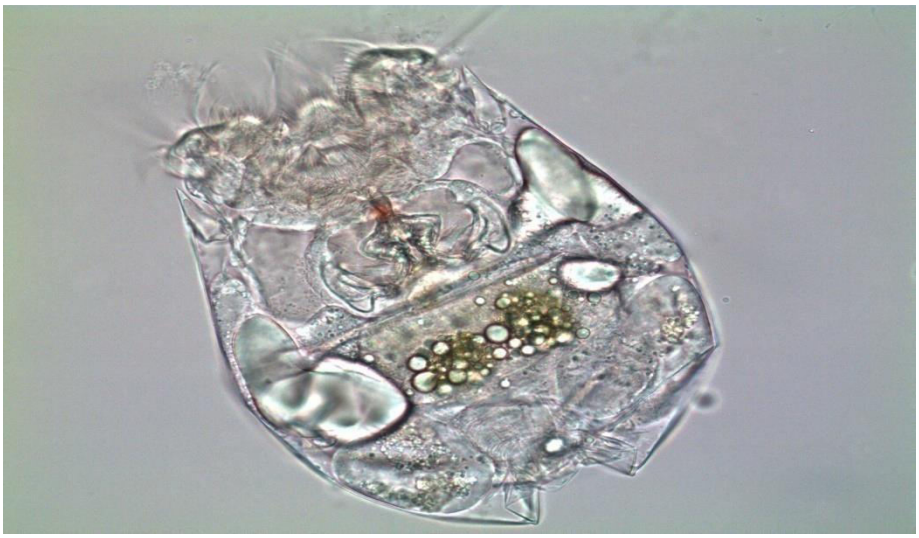


Figure 5.6. Oil emulsified rotifer (bio-encapsulation) at 100x

5.2.4 Rotifers enriched with different media

The rotifers were enriched with five different media with an aim to enrich the C_{20-22} long chain *n*-3 PUFA in the live feed rotifers. The PUFA – rich experimental media used in the present study were as follows: (1) Algamac – 2000 (Biomarine Inc., Aquafauna, USA); (2) S. presso (Selco S. presso®, INVE Aquaculture); (3) marine fish derived oil emulsion; (4) *Cheatoceros calcitrans* derived microalgal powder, and (5) marine microalgae (*Isochrysis galbana*).

Accordingly, the optimum fatty acid enrichment period of the rotifers by using the oil emulsion was taken into an account. To understand the comparative effects on other experimental enrichment media, such as, (1) Algamac – 2000 (Biomarine Inc., Aquafauna, USA); (2) S. presso (Selco S. presso ®, INVE Aquaculture); including the indigenously produced (3) marine fish derived oil emulsion; (4) *Cheatoceros calcitrans* derived microalgal powder and the shortlisted (5) marine microalgae (*Isochrysis galbana*) with considerably greater content of C_{22} long chain *n*-3 fatty acid (chapter 4). The rotifer samples were

harvested then washed with filtered seawater and stored at -20 °C for further analysis.

5.2.5 Statistical analysis

A suite of statistical analyses were carried out by using statistical packages for triplicate values, and the results were expressed as mean $\pm$ standard deviation. One way analysis of variance (ANOVA) was used to detect difference ($P< 0.05$) in the fatty acid composition of rotifers enriched with different diets, the source of variation includes saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, and *n*-3 long chain polyunsaturated fatty acids. The mean of enrichment diets were compared using Duncan's multiple range test (DMRT), whereas all the elucidated graph for the fatty acids were analysed through the Microcal Origin (Version 8).

5.3 Results and discussion

5.3.1 Fatty acid composition of enriched rotifers

Rotifers were grown or enriched with the microalgae *Isochrysis galbana* before being profiled (Figure 5.2.) to ascertain the optimum time required to obtain maximum enrichment of long chain *n*-3 fatty acids. The microalga *Isochrysis galbana* was selected to enrich the live feed rotifer based on the greater content of $C_{20\text{-}22}$ long chain *n*-3 PUFAs, particularly docosahexaenoic acid (DHA) among other microalgae considered in the present study. DHA has been considered as most predominant fatty acid for the fish larval growth and development (Glencross, 2009). In the present study, the rotifer enrichment process has been carried out with the shortlisted microalgae (*Isochrysis galbana*) for a period of 48 h, and the sampling intervals were arbitrarily chosen as 3 h, 6 h, 12 h, 30 h, and 48 h. The washed and filtered rotifers were thereafter analyzed for their fatty acid composition. The results revealed

that the aggregate content of PUFAs of rotifers enriched with *Isochrysis galbana* attained its maximum peak during 12 h (20 %TFA) of enrichment period, and thereafter a diminishment in their content was apparent (Table 5.2.). It is interesting to note that the content of PUFA and SFA exhibited an inverse proportion in the rotifers enriched with *I. galbana* for different time interval. This could be explained by the fact that the PUFAs might undergo the desaturation process during the course of longer period. It is therefore apparent that an enrichment period of about 12 h was found to apparently upkeep the long chain fatty acids at their nativity. The content of MUFAs apparently did not exhibit any correlation with the time interval during the enrichment period. The *n*-3 PUFAs exhibited a significant reduction in its content after 12 – 30 h of enrichment period, and its content was found to be about 8% TFA after 12 h, which reduced as 5% TFA after 48 h. The rotifers enriched with the microalgae *I. galbana* after 12 h showed an aggregate PUFA content of about 20% TFA, which was found to diminish at about 14 % TFA after the end of the enrichment period.

Maximum polyunsaturated fatty acids (PUFAs) enrichment was observed within six to twelve hours of enrichment period. Even though increase in the individual fatty acids occurred up to 30 h, the overall trend was an increase in saturated fatty acids (SFAs) and decline in monounsaturated fatty acids (MUFAs). The same trend was noticed by Lie *et al*. (1997), whereas thepolyunsaturated fatty acids (PUFAs) were found to decline beyond 30 h. In the present study, it was found that the content of the long chain *n*-3 PUFAs was greater during 12 h of enrichment period. DHA enrichment in rotifers with *I. galbana* increased to a total of 1.13% at 30 h, and a gradual decline in the content of this fatty acid was discernible afterwards. However, it is of note that this was lower than the enrichment level obtained by Faulk and Holt (2005), which was recorded at 3.4% for DHA after 24 h of enrichment duration.

It is of note that *I. galbana* has been used as the initial culture media with rotifers instead of yeast to enable the live feed to grow on a nutritionally enriched healthy diet. However, it is not suspected to derive significant quantities of polyunsaturated fatty acids, for example, DHA from the microalgae by the rotifers due to their noteworthy distinct genetic traits. It is apparent that the inert subjects need to use to enrich these polyunsaturated C_{20-22} fatty acids in greater quantities. It is therefore, that the microalga *I. galbana* used not only as an enrichment media *per say*, but to impart better growth and development traits of the live feeds.

For the optimum growth and development of rotifers, and to harness greater content of the C_{20-22} long chain *n*-3 PUFAs in the live feed, it is imperative to standardize the time to enrich the rotifer under the culture condition with microalga *I. galbana* used in the present study. It was demonstrated that the *n*-3 PUFA content of the rotifers gradually increased from 0 to 12 h, and thereafter a decline in the content of these group of fatty acids were discernible. Therefore, it was recommended to enrich these live feed for a period of not more than 12 h for growth, development, and greater enrichment of *n*-3 long chain (LC)-PUFAs, under the culture with the microalga *I. galbana*. Moreover, the essential fatty acid (EFAs) composition of rotifers enriched with *I. galbana* might use to develop rotifer as the healthier starter live feed. To achieve the required amount of EFAs for fish larvae, it is necessary to enrich live feed rotifer with enrichment diets having greater content of HUFAs. The ANOVA (Table 5. 3.) showed the fatty acid composition of rotifer enriched with *I. galbana* in different time interval, whereas the enrichment effects were found to be highly significant ($P < 0.001$).

Table 5.1. Fatty acid composition of unenriched rotifer

Fatty acids	Unenriched rotifer
14:0	5.03±025
16:0	29.76±0.08
18:0	3.58±0.06
ΣSFA	**38.44±0.05**
16:1*n*-7	13.16±0.11
18:1*n*-9	21.45±0.12
24:1	1.52±0.04
ΣMUFA	**36.07±0.13**
18:2*n*-6	19.57±0.06
18:3*n*-3	1.39±0.07
20:2*n*-6	0.91±0.04
20:3*n*-6	0.37±0.05
20:4*n*-6	0.58±0.06
20:5*n*-3	0.22±0.05
22:6*n*-3	0.03±0.02
ΣPUFA	**22.89±0.05**

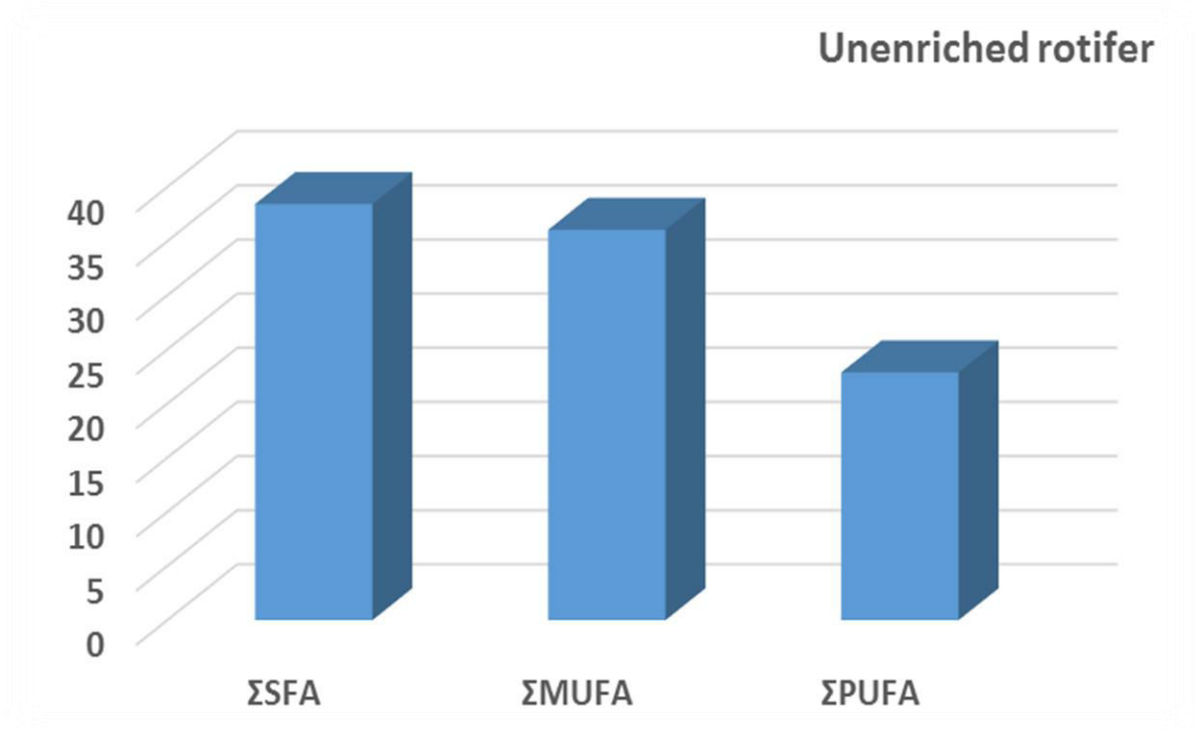


Figure 5.7. Differential content of aggregate SFA, MUFA, & PUFA in the unenriched rotifer

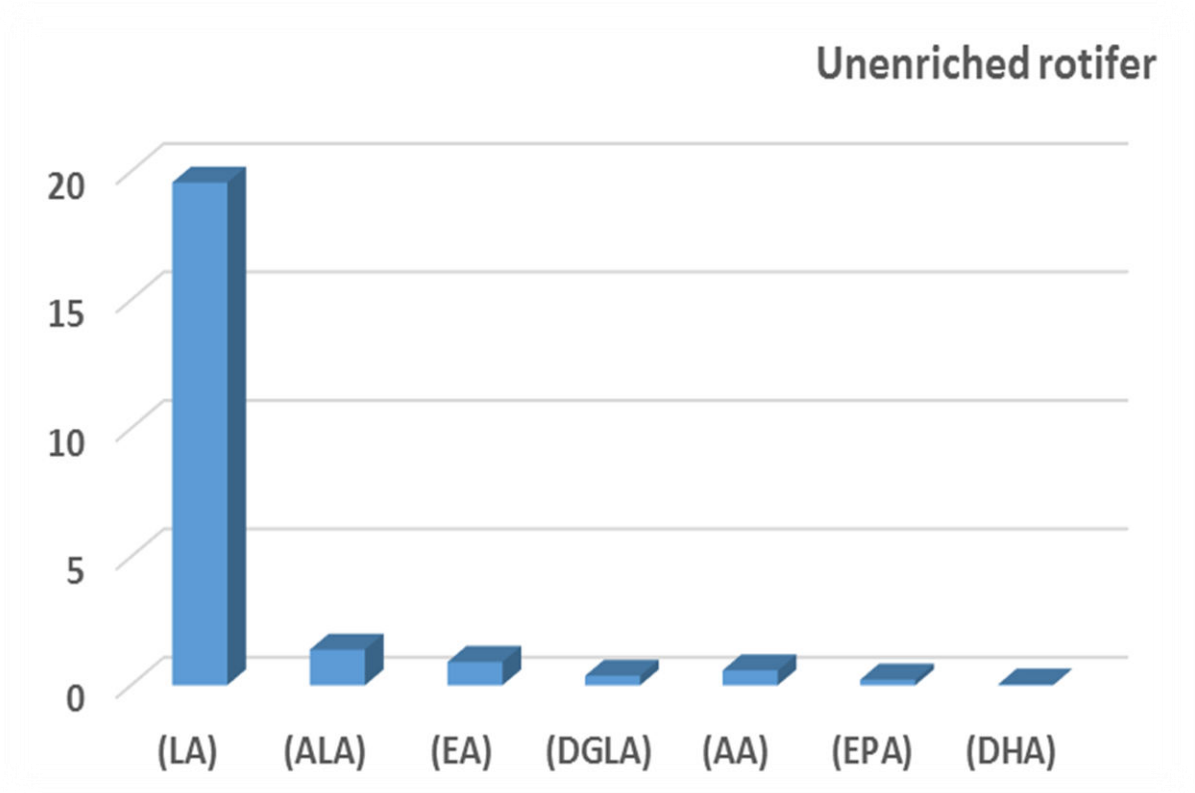


Figure 5.8. Differential content of aggregate PUFAs in the unenriched rotifer

Table 5.2. Fatty acid composition of rotifer enriched with *Isochrysis galbana* in different time interval

Fatty acids (%)	3 h	6 h	12 h	30 h	48 h
14:0	6.55±0.25	6.63±0.05	6.83±0.11	11.10±0.06	15.35±0.04
15:0	0.68±0.25	0.83±0.15	1.22±0.14	2.40±0.02	2.74±0.15
16:0	23.17±0.15	22.20±0.05	23.57±0.24	25.74±0.07	27.82±0.04
18:0	3.19±0.06	3.55±0.28	3.71±0.21	5.40±0.06	7.48±0.08
24:0	0.19±0.03	0.27±0.07	0.52±0.20	0.55±0.06	1.26±0.02
ΣSFA	**33.78±0.11**	**33.48±0.36**	**35.86±0.40**	**45.20±0.19**	**54.65±0.29**
16:1	14.43±0.36	14.17±0.11	13.43±0.14	11.47±0.10	9.20±0.04
18:1	24.46±0.49	23.34±0.20	22.32±0.19	17.14±0.10	16.92±0.04
24:1	0.82±0.10	1.09±0.04	1.78±0.18	2.21±0.13	3.24±0.03
ΣMUFA	**39.71±0.38**	**38.61±0.20**	**37.53±0.46**	**30.83±0.17**	**29.37±0.02**
18:2n-6	12.04±0.15	11.52±0.29	9.09±0.16	8.19±0.07	7.17±0.04
18:3n-6	0.71±0.10	0.56±0.25	0.58±0.19	0.59±0.06	0.33±0.01
18:3n-3	3.65±0.11	3.76±0.23	4.23±0.05	3.05±0.03	1.84±0.05
18:4n-3	0.49±0.03	0.51±0.12	0.73±0.11	0.89±0.06	0.65±0.04
20:2n-6	1.36±0.07	1.41±0.11	1.61±0.04	1.71±0.03	0.77±0.06
20:3n-6	0.34±0.08	0.38±0.06	0.55±0.08	0.66±0.05	0.24±0.04
20:4n-6	0.36±0.07	0.43±0.06	0.47±0.04	0.28±0.06	0.18±0.02
20:5n-3	0.38±0.10	0.60±0.13	1.62±0.08	1.74±0.05	1.92±0.03
22:5n-3	0.02±0.03	0.06±0.03	0.19±0.06	0.15±0.02	0.08±0.04
22:6n-3	0.23±0.06	0.36±0.07	0.92±0.07	1.19±0.06	0.57±0.06
ΣPUFA	**19.59±0.27**	**19.58±0.35**	**20.00±0.40**	**18.45±0.15**	**13.75±0.14**
***n*-3 LC-PUFAs**	**4.77±0.32**	**5.28±0.58**	**7.69±0.35**	**7.02±0.21**	**5.06±0.21**

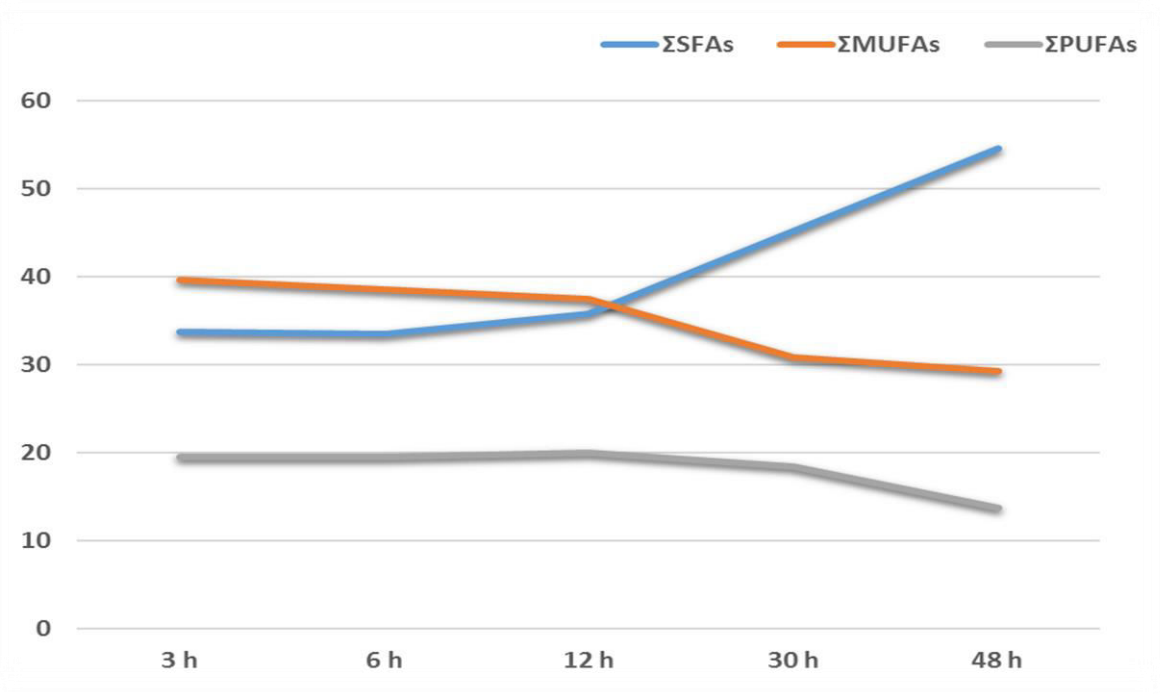


Figure 5.9. Differential content of aggregate SFA, MUFA, & PUFA in the rotifer enriched with *Isochrysis galbana* in different time interval

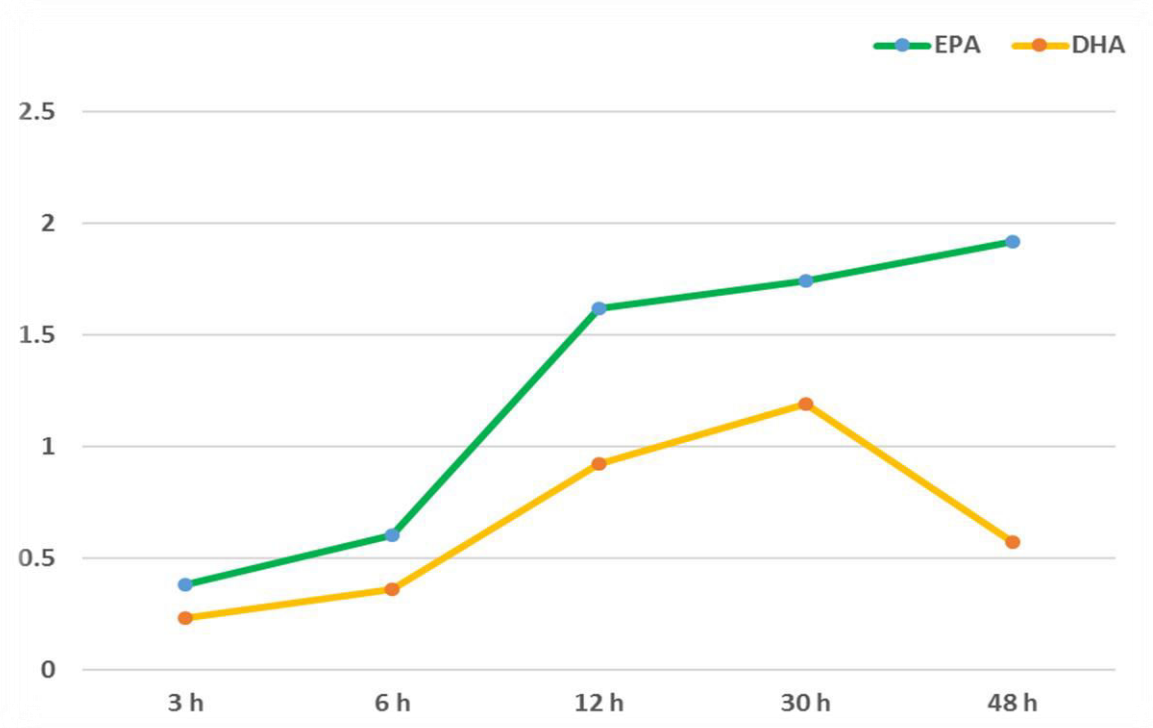


Figure 5.10. Differential content of aggregate EPA & DHA in the rotifer enriched with *Isochrysis galbana* in different time interval

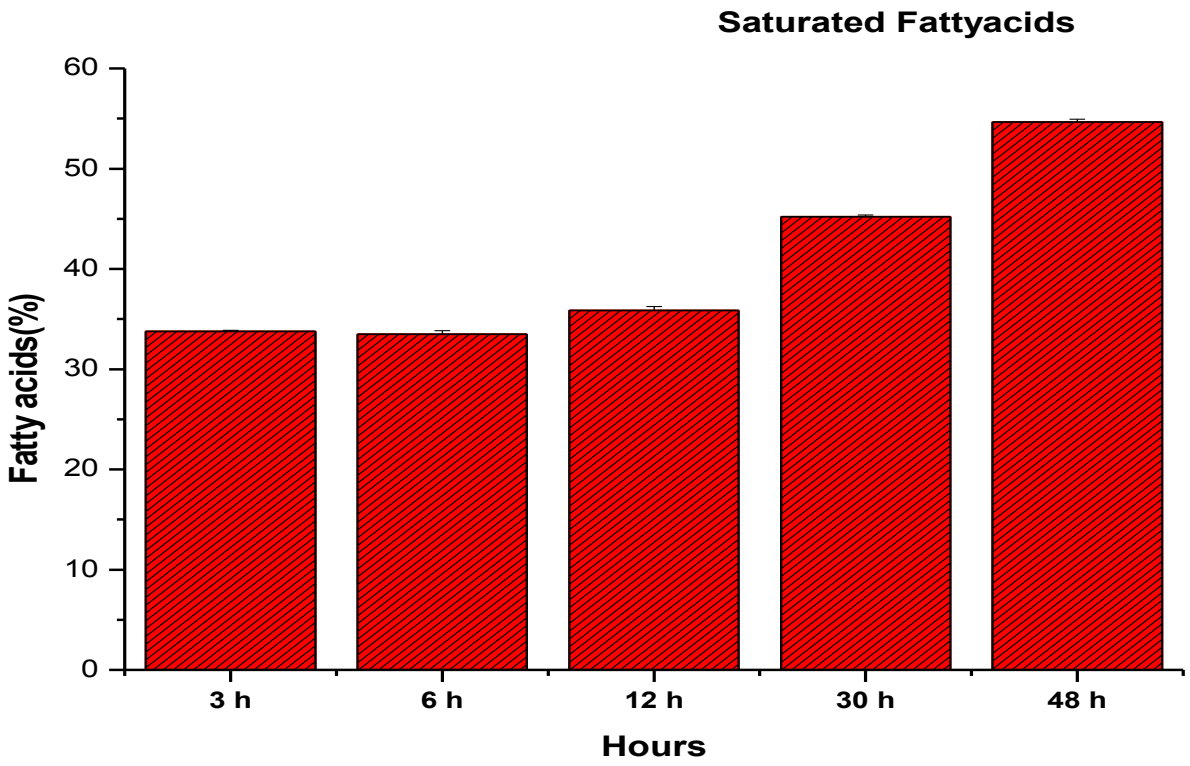


Figure 5.11. Total content of saturated fatty acids in microalgae *Isochrysis galbana* during different time intervals

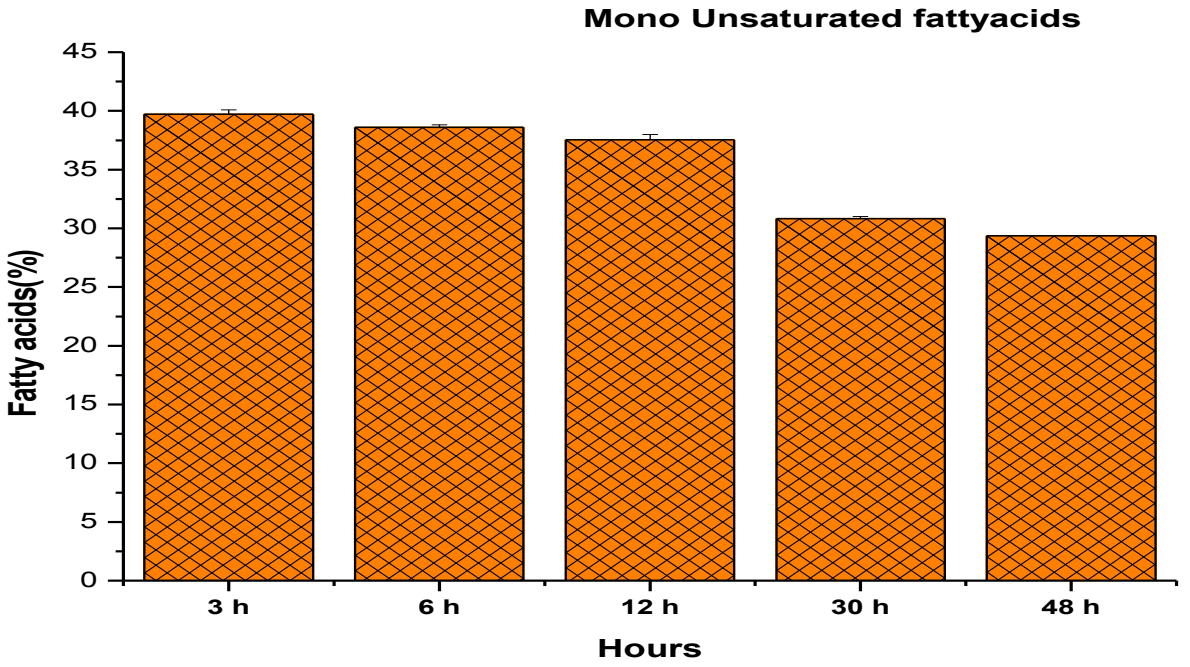


Figure 5.12. Total content of monounsaturated fatty acids in microalgae *Isochrysis galbana* during different time intervals

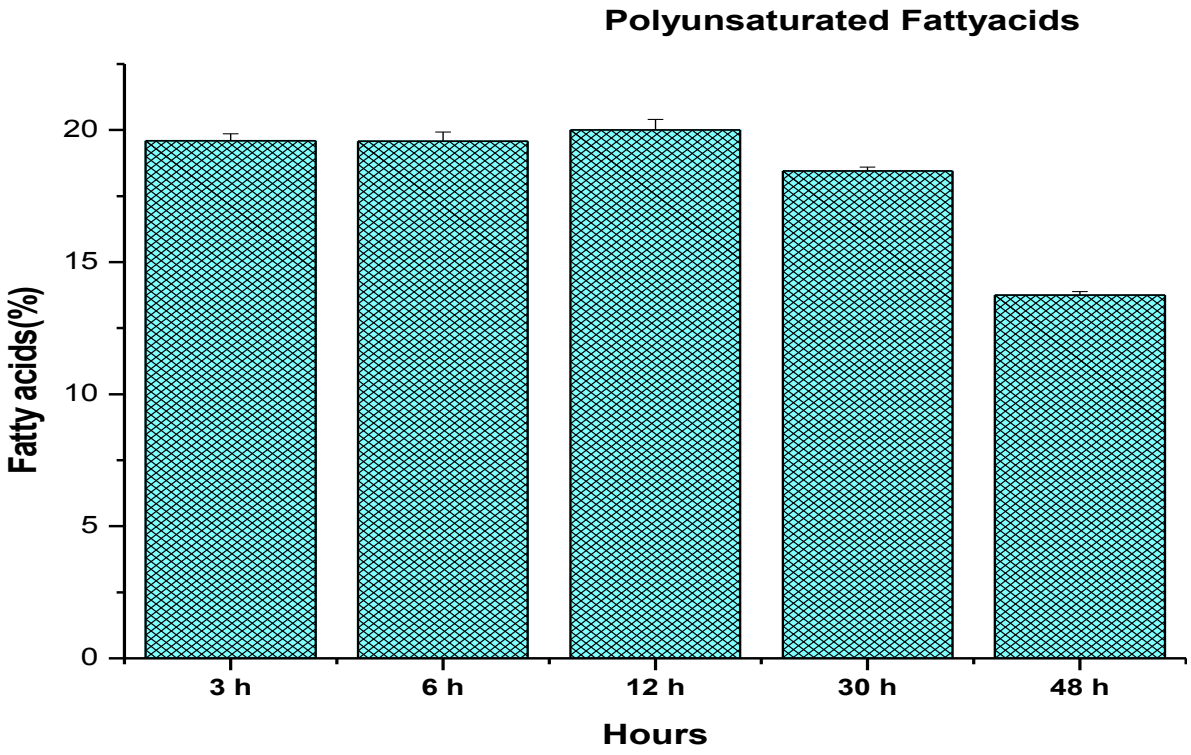


Figure 5.13. Total content of polyunsaturated fatty acids in microalgae *Isochrysis galbana* during different time intervals

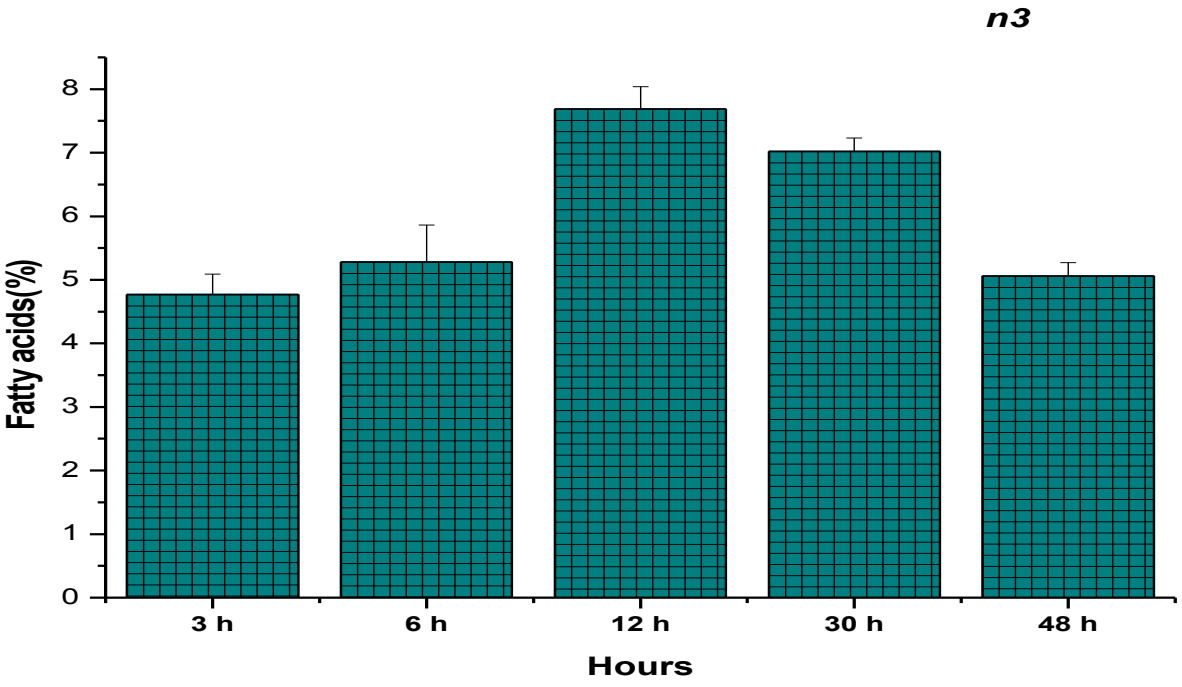


Figure 5.14. Total content of *n*-3polyunsaturated fatty acids in microalgae *Isochrysis galbana* during different enrichment time intervals

Table 5.3. ANOVA for fatty acid composition of rotifer enriched with *Isochrysis galbana* in different time interval of enrichment

Fatty acids (%)	**3 h**	**6 h**	**12 h**	**30 h**	**48 h**
ΣSFAs	33.78±0.11^{a}	33.48±0.36^{a}	35.86±0.40^{b}	45.20±0.19^{c}	54.65±0.29^{d}
ΣMUFAs	39.71±0.38^{e}	38.61±0.20^{d}	37.53±0.46^{c}	30.83±0.17^{b}	29.37±0.02^{a}
20:5n-3	0.38±0.10^{a}	0.60±0.13^{b}	1.62±0.08^{c}	1.74±0.05^{c}	1.92±0.03^{d}
22:6n-3	0.23±0.06^{a}	0.36±0.07^{b}	0.92±0.07^{d}	1.19±0.06^{e}	0.57±0.06^{c}
ΣPUFAs	19.59±0.27^{c}	19.58±0.35^{c}	20.00±0.40^{c}	18.45±0.15^{b}	13.75±0.14^{a}
n-3 LC-PUFAs	4.77±0.32^{a}	5.28±0.58^{c}	7.69±0.35^{e}	7.02±0.21^{d}	5.06±0.21^{b}

Mean sample size, 3.0

The $C_{20\text{-}22}$ long chain *n*-3 PUFA enriched sardine oil are good start material to develop cost effective live feed enrichment products for domestic markets, and could aid as an cost-effective import substitute. Refining the product to the extent of achieving the optimum fatty acid levels for translating into improved larval survival cycles and healthy fry for farming and grow out is the challenging objective (Vijayagopal *et al*., 2012). The development of an indigenous fish oil based enrichment emulsion for live feeds was therefore attempted. The crude sardine oil was available locally at a cost of ₹ 45 kg^{-1} ($1 kg^{-1}). Fish roe of quality fishes collected and sold by the fish vendors was available for ₹ 80 kg^{-1} (<$2 kg^{-1}). The fish oil was enriched as described earlier (Chakraborty and Paul Raj, 2009; Chakraborty *et al.,* 2010). The blended fish roe phospholipids (Chapter 3) was thereafter emulsified and stabilized with enriched sardine oil. The DHA rich oil emulsion, with DHA content of 39% TFA and EPA content of 19% TFA, was used to enrich rotifers, which resulted in an enrichment level of 8.76% DHA and 3.17% EPA in six hours of enrichment period, and after which the decline in their contents were discernable (Table 5.3.). The enrichment levels of 27% DHA and 15% EPA were reported with a commercial product, DHA Super Selco (INVE, SA, Belgium) (Lie *et al.,* 1997). However, the contents of DHA and EPA levels in the present study were found to be comparatively greater (DHA, 38% and EPA, 19%) than those reported by Lie *et al.* (1997). The ratio of DHA: EPA was reported to be most

appropriate (2:1) that could obtain by enriching the live feed rotifers in the DHA rich emulsion (Parrish *et al*., 1994; Sargent *et al*., 1999). The ratio of DHA: EPA in rotifers after enrichment duration of 6 h was found to be 2.76: 1. Another set of enrichment products, such as, Algamac 2000 and Algamac 3050, which are the spray dried forms of the marine microalgae *Schizochytrium* were reported in the earlier literature (Faulk and Holt, 2005). It was demonstrated that the microalgl powder was rich in DHA and EPA (20 and 30%, respectively) (Faulk and Holt, 2005). These products are utilized as spray dried powder (Algamac 2000) and flakes (Algamac 3050) with a content of 27% and 47% DHA, respectively (www.aquafauna.com).

To standardize the duration of enrichment, the rotifers were enriched with the experimental oil emulsion (1 ml/l and 0.1 ml/l) for a period of 24 h. The live feed samples (rotifers) were harvested from the culture tank for every 3 h interval before being analyzed for their fatty acid composition. The results revealed that the a maximum content of polyunsaturated fatty acids (~ 40%) were apparent after a period of 6 h, whilst the oil emulsion was used at the dose of 1 ml/l. It is of note that the C_{22} *n*-3 fatty acid (DHA, 22:6*n*-3) in the rotifers exhibited a significant increase (~ 9 %) after the enrichment period of 6 h, while the oil emulsion was used at the identical dose (1 ml/l). It was therefore conceivably demonstrated through the present study that the rotifers enriched with the PUFA enriched oil emulsion for a period of six hours could yield greater quantities of the desired fatty acids ($C_{20\text{-}22}$*n*-3 fatty acid, EPA, 20:5*n*-3 and DHA, 22:6*n*-3). The desired fatty acids had maximum content of PUFA and lesser SFA and MUFA during 6 h of enrichment, whereas, a reduction in the content of PUFA was recorded afterwards. Similar results were reported by Hache and Plante (2011) who observed a reduction of total PUFA and an increase of total SFA and MUFA in rotifers enriched with twice the amount of enrichment product. The ANOVA (Table 5. 5. & 5. 6.) showed the fatty acid composition of rotifer enriched with oil emulsion

(0.1 & 1ml/l)in different time intervals. The enrichment effects were found to be highly significant ($P< 0.001$).

Table 5.4. Fatty acid composition of rotifer enriched with oil emulsion (1 ml^{-1})

Fatty acids	**3 h**	**6 h**	**9 h**	**12 h**
C14:0	3.86±0.10	3.21±0.02	4.48±0.04	7.62±0.08
C15:0	1.37±0.04	0.44±0.02	3.27±0.07	0.58±0.06
C16:0	18.84±0.12	16.46±0.08	22.09±0.06	20.27±0.05
C18:0	9.65±0.02	6.20±0.02	7.18±0.06	7.57±0.06
C24:0	0.92±0.03	0.68±0.04	2.18±0.08	2.25±0.04
ΣSFA	**34.64±0.23**	**26.99±0.06**	**39.19±0.22**	**38.29±0.08**
C16:1	2.62±0.53	2.51±0.08	5.22±0.05	5.91±0.08
C18:1	14.87±0.06	21.14±0.03	24.41±0.03	28.16±0.08
C24:1	4.73±0.11	2.83±0.04	1.14±0.02	2.44±0.06
ΣMUFA	**22.22±0.68**	**26.47±0.11**	**30.77±0.04**	**36.51±0.13**
C18:2*n*-6	9.47±0.07	6.91±0.06	4.33±0.01	2.48±0.05
C18:3*n*-6	2.47±0.04	0.55±0.04	2.24±0.03	1.46±0.06
C18:3*n*-3	6.18±0.06	4.99±0.03	2.53±0.06	1.43±0.08
C18:4*n*-3	1.38±0.05	1.66±0.03	0.78±0.11	0.41±0.02
C20:2*n*-6	0.46±0.05	0.20±0.03	1.38±0.09	1.55±0.05
C20:3*n*-6	1.53±0.04	1.64±0.06	1.78±0.07	1.90±0.03
C20:4*n*-6	6.16±0.05	9.82±0.06	8.89±0.05	7.58±0.04
C20:5*n*-3	2.57±0.05	2.34±0.05	2.10±0.06	1.53±0.09
C22:5*n*-3	1.58±0.05	3.19±0.02	0.84±0.03	0.28±0.03
C22:6*n*-3	4.30±0.03	8.74±0.02	1.43±0.04	0.91±0.07
ΣPUFA	**36.11±0.23**	**40.04±0.18**	**24.87±2.66**	**19.51±0.22**

Table 5.4. (Continued…)

Fatty acids	15 h	18 h	21 h	24 h
C14:0	7.88±0.04	7.96±0.05	7.86±0.06	7.57±0.05
C15:0	0.67±0.04	0.62±0.04	0.73±0.04	0.89±0.02
C16:0	33.36±0.10	34.61±0.37	32.64±0.40	34.26±0.07
C18:0	8.10±0.04	8.20±0.07	7.19±0.04	7.22±0.04
C24:0	0.56±0.04	0.52±0.05	0.67±0.05	0.78±0.05
ΣSFA	**50.57±0.09**	**51.91±0.36**	**49.09±0.35**	**50.72±0.17**
C16:1	3.57±0.07	4.16±0.08	4.17±0.07	5.67±0.06
C18:1	26.32±0.50	27.09±0.06	25.67±0.05	28.37±0.06
C24:1	3.62±0.04	1.56±0.06	2.18±0.06	0.96±0.03
ΣMUFA	**33.51±0.60**	**32.81±0.09**	**32.01±0.11**	**35.00±0.11**
C18:2*n*-6	1.17±0.04	2.43±0.10	2.57±0.06	1.19±0.03
C18:3*n*-6	0.33±0.03	0.19±0.04	1.94±0.03	0.86±0.07
C18:3*n*-3	2.35±0.15	2.09±0.08	1.71±0.04	2.40±0.05
C18:4*n*-3	0.56±0.04	0.26±0.09	0.46±0.07	0.57±0.05
C20:2*n*-6	0.38±0.04	0.58±0.08	0.82±0.03	1.08±0.04
C20:3*n*-6	1.18±0.05	1.66±0.21	1.49±0.08	1.47±0.08
C20:4*n*-6	5.93±0.05	3.75±0.08	2.61±0.06	3.19±0.05
C20:5*n*-3	1.45±0.06	0.91±0.05	0.88±0.07	0.67±0.05
C22:5*n*-3	0.49±0.03	0.61±0.03	0.57±0.06	0.23±0.05
C22:6*n*-3	0.18±0.05	0.55±0.27	0.25±0.04	0.20±0.04
ΣPUFA	**14.05±0.20**	**13.04±0.58**	**13.31±0.14**	**11.87±0.17**

Table 5.5. ANOVA for fatty acid composition of rotifer enriched with oil emulsion (1 ml^{-1}) in different time intervals.

Fatty acids	ΣSFA	ΣMUFA	EPA	DHA	ΣPUFA	∑*n*-3 LC – PUFAs
3 h	34.64 ± 0.23^{b}	22.22 ± 0.68^{a}	2.57 ± 0.05^{f}	4.30 ± 0.03^{e}	36.11 ± 0.23^{e}	16.01 ± 0.22^{e}
6 h	26.99 ± 0.06^{a}	26.47 ± 0.11^{b}	2.34 ± 0.05^{e}	8.74 ± 0.02^{f}	40.04 ± 0.18^{f}	20.93 ± 0.15^{f}
9 h	39.19 ± 0.22^{d}	30.77 ± 0.04^{c}	2.10 ± 0.06^{d}	1.43 ± 0.04^{d}	24.87 ± 2.66^{d}	7.69 ± 0.30^{d}
12 h	38.29 ± 0.08^{c}	36.51 ± 0.13^{h}	1.53 ± 0.09^{c}	0.91 ± 0.07^{c}	19.51 ± 0.22^{c}	4.55 ± 0.29^{b}
15 h	50.57 ± 0.09^{f}	33.51 ± 0.60^{f}	1.45 ± 0.06^{c}	0.18 ± 0.05^{a}	14.05 ± 0.20^{b}	5.05 ± 0.32^{c}
18 h	51.91 ± 0.36^{g}	32.81 ± 0.09^{e}	0.91 ± 0.05^{b}	0.55 ± 0.27^{b}	13.04 ± 0.58^{b}	$4.42\pm0.51a^{b}$
21 h	49.09 ± 0.35^{e}	32.01 ± 0.11^{d}	0.88 ± 0.07^{b}	0.25 ± 0.04^{a}	13.31 ± 0.14^{b}	3.87 ± 0.26^{a}
24 h	50.72 ± 0.17^{f}	35.00 ± 0.11^{g}	0.67 ± 0.05^{a}	0.20 ± 0.04^{a}	11.87 ± 0.17^{a}	4.07 ± 0.23^{a}

Mean Sample Size = 3.000.

Table 5.6. Fatty acid composition of rotifer enriched with oil emulsion (0.1 ml^{-1})

Fatty acids	3 h	6 h	9 h	12 h
14:0	3.30±0.06	3.28±0.06	4.14±0.04	7.63±0.04
15:0	0.47±0.04	1.43±0.05	1.43±0.06	0.54±0.06
16:0	28.15±0.03	26.33±0.04	30.75±0.10	30.27±0.05
18:0	5.24±0.07	8.71±0.06	7.29±0.04	7.58±0.09
24:0	0.29±0.04	0.76±0.06	0.38±0.04	0.38±0.04
∑SFA	**37.45±0.13**	**40.51±0.07**	**43.99±0.07**	**46.41±0.15**
16:1	13.23±0.03	9.50±0.07	9.46±0.05	5.88±0.04
18:1*n*-9	28.36±0.09	29.07±0.06	29.76±0.11	28.23±0.18
24:1	0.91±0.06	1.44±0.06	0.67±0.07	2.64±0.19
∑MUFA	**42.51±0.17**	**40.01±0.05**	**39.90±0.07**	**36.75±0.35**
18:2*n*-6	7.73±0.11	6.19±0.04	2.70±0.12	2.65±0.22
18:3*n*-6	1.29±0.07	1.47±0.05	1.73±0.05	0.48±0.07
18:3*n*-3	2.90±0.05	1.62±0.08	1.42±0.04	1.38±0.07
18:4*n*-3	0.24±0.03	0.35±0.02	0.48±0.06	1.57±0.05
20:2*n*-6	0.92±0.06	0.79±0.07	0.68±0.06	0.47±0.04
20:3*n*-6	0.46±0.03	0.91±0.04	1.08±0.05	0.73±0.04
20:4*n*-6	1.87±0.06	2.30±0.19	2.45±0.25	3.58±0.06
20:5*n*-3	1.26±0.04	1.45±0.07	1.57±0.05	2.11±0.06
22:6*n*-3	0.36±0.05	0.58±0.04	0.80±0.04	0.78±0.05
∑PUFA	**16.06±1.58**	**15.66±0.20**	**12.43±0.46**	**13.75±0.21**

Table 5.6. (Continued…)

Fatty acids	15 h	18 h	21 h	24 h
14:0	7.85±0.10	7.81±0.13	7.82±0.06	7.50±0.04
15:0	0.61±0.08	0.65±0.06	0.71±0.04	0.88±0.06
16:0	33.52±0.06	34.65±0.06	32.82±0.40	33.83±0.75
18:0	8.07±0.05	8.17±0.07	7.21±0.04	7.27±0.09
24:0	0.56±0.06	0.49±0.05	0.65±0.05	0.77±0.06
SFA	**50.62±0.04**	**51.76±0.13**	**49.20±0.35**	**50.24±0.78**
16:1	3.58±0.08	4.20±0.07	4.17±0.07	5.66±0.07
18:1n-9	26.07±0.04	27.08±0.04	25.62±0.05	28.35±0.10
24:1	3.64±0.06	1.38±0.20	2.24±0.06	0.91±0.06
MUFA	**33.29±0.09**	**32.65±0.12**	**32.03±0.11**	**34.91±0.08**
18:2n-6	1.20±0.06	2.52±0.11	2.60±0.06	1.18±0.06
18:3n-6	0.37±0.05	0.19±0.04	1.91±0.03	0.83±0.06
18:3n-3	2.24±0.06	2.09±0.08	1.70±0.04	2.46±0.09
18:4n-3	0.57±0.06	0.26±0.06	0.48±0.07	0.58±0.03
20:2n-6	0.39±0.07	0.57±0.06	0.89±0.03	1.08±0.04
20:3n-6	1.18±0.06	1.50±0.03	1.46±0.08	1.45±0.06
20:4n-6	3.82±0.11	3.76±0.06	2.58±0.06	3.19±0.06
20:5n-3	1.54±0.09	0.93±0.08	0.86±0.07	0.62±0.04
22:6n-3	1.15±0.10	0.38±0.05	0.27±0.06	0.20±0.04
PUFA	**12.47±0.22**	**12.19±0.13**	**12.75±0.04**	**11.60±0.14**

Table 5.7. ANOVA for fatty acid composition of rotifer enriched with oil emulsion (0.1 ml^{-1}) in different time interval.

Fatty acids	∑SFA	∑MUFA	EPA	DHA	∑PUFA	∑*n*-3 LC -PUFAs
3 h	37.45±0.13^{a}	42.51±0.17^{g}	1.26±0.04^{c}	0.36±0.05b^{c}	16.06±1.58^{d}	4.75±0.16^{e}
6 h	40.51±0.07^{b}	40.01±0.05^{f}	1.45±0.07^{d}	0.58±0.04^{d}	15.66±0.20^{d}	4.00±0.20^{c}
9 h	43.99±0.07^{c}	39.90±0.07^{f}	1.57±0.05^{e}	0.80±0.04^{e}	12.43±0.46^{ab}	4.27±0.18^{d}
12 h	46.41±0.15^{d}	36.75±0.35^{e}	2.11±0.06^{f}	0.78±0.05^{e}	13.75±0.21^{c}	5.84±0.22^{f}
15 h	50.62±0.04^{f}	33.29±0.09^{c}	1.54±0.09^{e}	1.15±0.10^{f}	12.47±0.22^{ab}	5.50±0.30^{e}
18 h	51.76±0.13^{g}	32.65±0.12^{b}	0.93±0.08^{b}	0.38±0.05^{c}	12.19±0.13^{ab}	3.66±0.26^{b}
21 h	49.20±0.35^{e}	32.03±0.11^{a}	0.86±0.07^{b}	0.27±0.06^{ab}	12.75±0.04^{bc}	3.31±0.22^{a}
24 h	50.24±0.78^{f}	34.91±0.08^{d}	0.62±0.04^{a}	0.20±0.04^{a}	11.60±0.14^{a}	3.86±0.19^{c}

Mean sample size = 3.0.

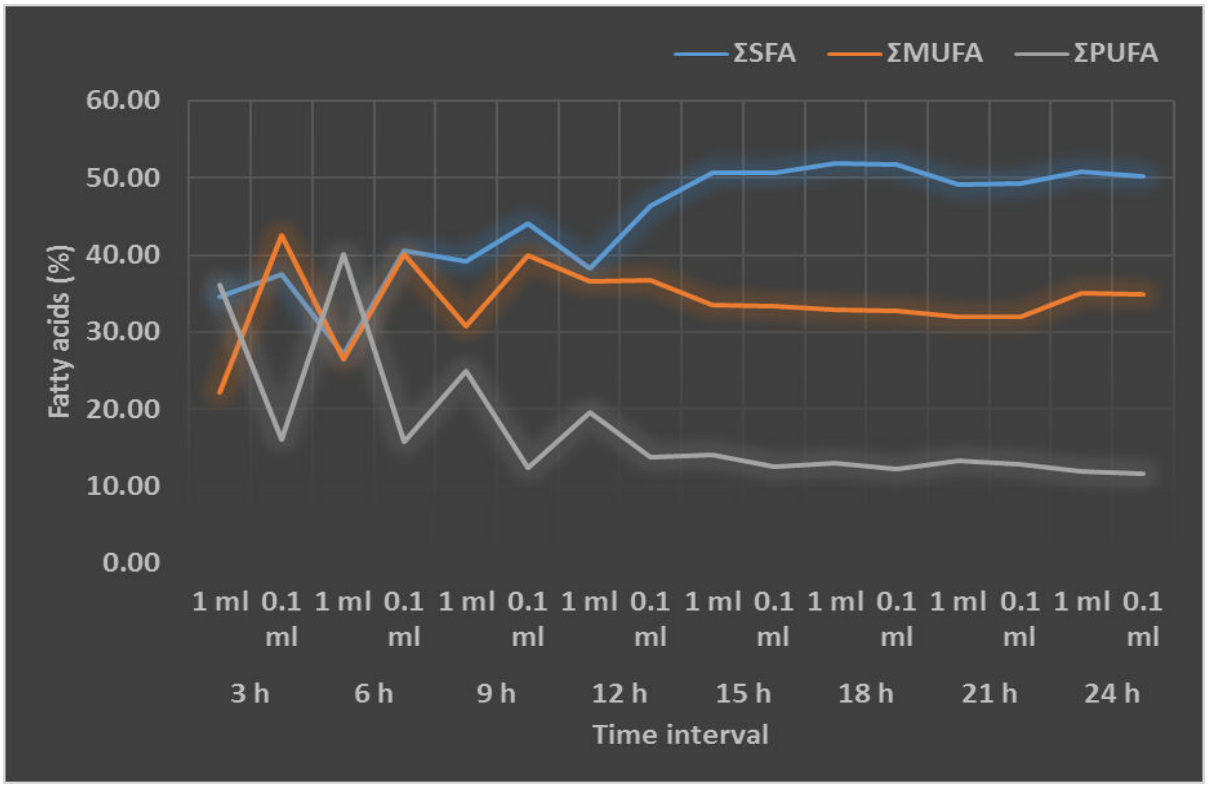


Figure 5.15. Differential content of aggregate SFA, MUFA, & PUFA in the rotifer enriched with oil emulsion

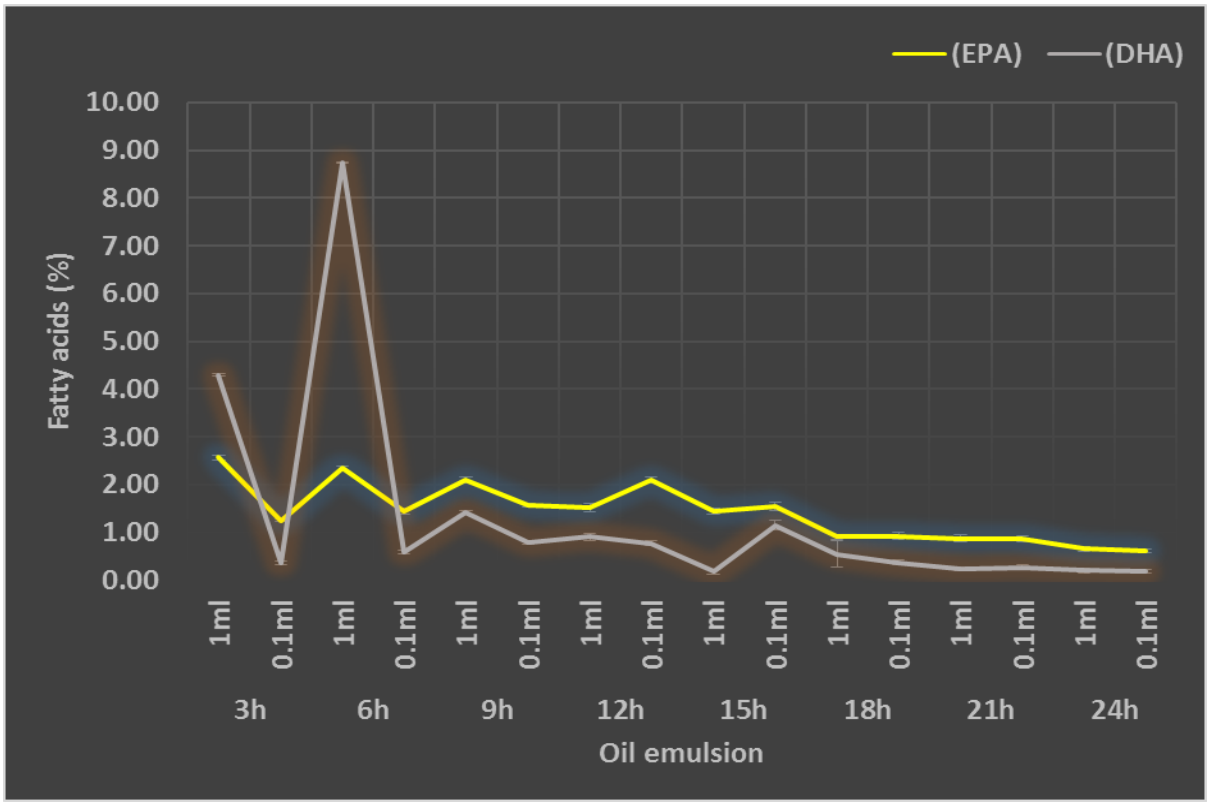


Figure 5.16. Differential content of aggregate EPA & DHA in the rotifer enriched with oil emulsion

In the present work, the indigenously produced $C_{20\text{-}22}$ PUFA rich oil emulsion (chapter 3) and its effect on live feed rotifers analyzed. Concurrently, different enrichment media, for example, (A) Algamac 2000 (Biomarine Inc., Aquafauna, USA), (B) S. presso (Selco S. presso ®, INVE Aquaculture), and (C) an indigenously powdered microalgal derived from *Chaetoceros calcitrans* and marine microalgae (*Isochrysis galbana*) were utilized to compare their efficacy with the $C_{20\text{-}22}$ PUFA rich oil emulsion.

This present protocol was followed with different experimental enrichment media as mentioned earlier to apparently understand the comparative efficacy of these formulations to yield $C_{20\text{-}22}$ *n*-3 fatty acid enriched rotifers. Consequently, the rotifers enriched with Algamac-2000 yielded the aggregate PUFA content of about 30% of the total fatty acids. It is interesting to note that this microalgal formulation is marketed by Biomarine Inc., Aquafauna, USA, and is being popularly used in enriching the live feed, for example,

Artemia and rotifer. However, the PUFA enriched oil emulsion developed in the present study could yield the rotifer with greater content of these fatty acids (~40 %) than those recorded with Algamac-2000. Likewise, S. presso (Selco S. presso®, INVE Aquaculture) is an imported rotifer enrichment medium used in aquaculture industries. However, the rotifers enriched with S. presso (Selco S. presso ®, INVE Aquaculture) were found to possess an aggregate amount of 38 % PUFA, which was lesser than those obtained by using the indigenously developed $C_{20\text{-}22}$*n*-3 PUFA enriched oil emulsion. No significant difference in the content of C_{20} *n*-3 PUFA (EPA) was recorded in the rotifers enriched with $C_{20\text{-}22}$ *n*-3 PUFA enriched oil emulsion, Algamac 2000 (Biomarine Inc., Aquafauna, USA) and S. presso (Selco S. presso ®, INVE Aquaculture) (2-4 % of total fatty acids). However, the content of C_{22}*n*-3 fatty acid (DHA, 22:6*n*-3) in the rotifers was found to be significantly greater (9 %, used at a concentration of 1 ml/l). The content of C_{22}*n*-3 fatty acid were recorded as 0.4, 0.7, 5.1 and 0.1% of the total content of fatty acids, when different enrichment media, such as, *I. galbana*, Algamac 2000, Biomarine Inc., Aquafauna, USA, S. presso-Selco S.presso ®, INVE Aquaculture, and Algal powder derived from *Chaetoceros calcitrans*, respectively were used in that order (Figure 5.13.). The ANOVA (Table 5.9.) showed the fatty acid composition of rotifer enriched with the experimental diets at 6 h. The enrichment effects were found to be highly significant ($P < 0.001$).

Table 5.8. Fatty acid composition of rotifers enriched with the experimental diets at 6 h

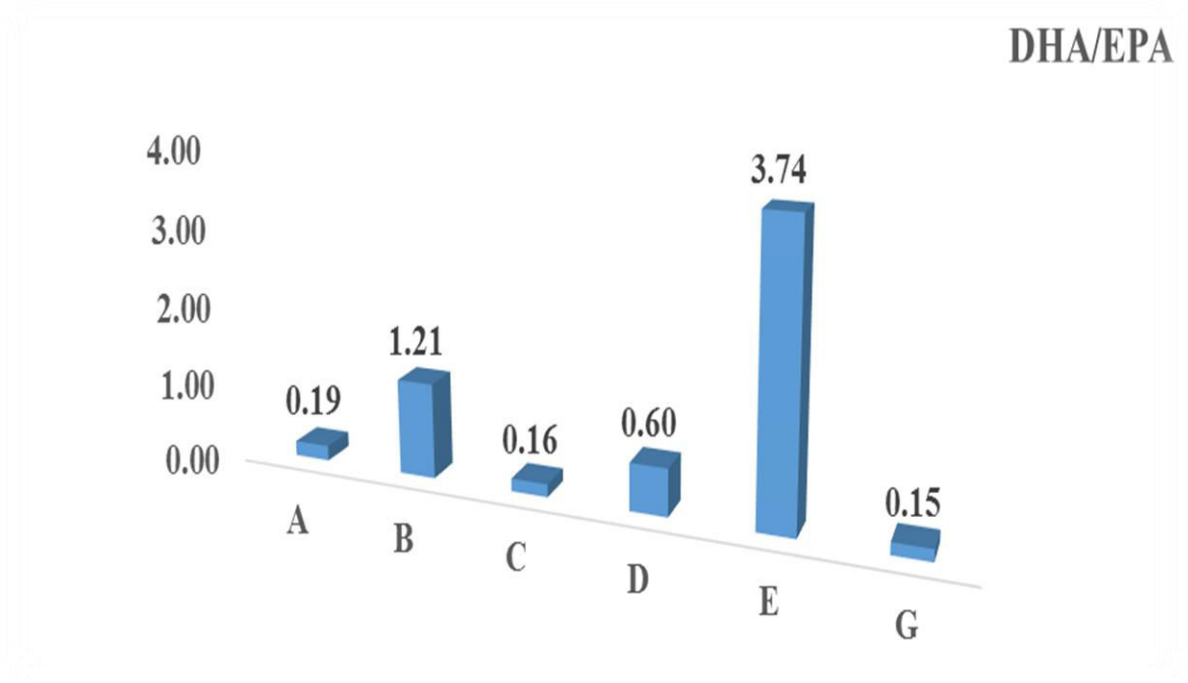
Fatty acids (%)	A	B	C	D	E	F
14:0	6.25±0.09	1.84±0.16	4.79±0.46	6.63±0.05	3.21±0.02	4.03±0.15
15:0	0.04±0.04	0.38±0.07	0.43±0.12	0.83±0.15	0.44±0.02	25.10±0.20
16:0	26.21±0.35	11.37±0.15	20.44±0.41	22.20±0.05	16.46±0.08	3.06±0.05
18:0	2.76±0.18	6.44±0.20	1.67±0.14	3.55±0.28	6.20±0.02	0.00±0.00
24:0	0.02±0.04	0.63±0.08	0.23±0.02	0.27±0.07	0.68±0.04	0.00±0.00
ΣSFA	**35.28±0.38**	**20.66±0.49**	**28.01±0.39**	**33.48±0.36**	**26.99±0.06**	**32.20±0.29**
16:1	14.85±0.16	5.43±0.19	11.38±0.34	14.17±0.11	2.51±0.08	12.56±0.08
18:1	17.57±0.23	28.25±0.11	17.35±0.43	23.34±0.20	21.14±0.03	18.72±0.14
24:1	1.34±0.07	3.57±0.19	0.15±0.03	1.09±0.04	2.83±0.04	1.21±0.16
ΣMUFA	**33.76±0.43**	**37.25±0.21**	**29.69±1.18**	**38.61±0.20**	**26.47±0.11**	**32.48±0.21**
18:2n-6	18.77±0.45	11.89±0.19	10.80±0.39	11.52±0.29	6.91±0.06	16.47±0.13
18:3n-6	0.40±0.37	0.42±0.11	0.24±0.04	0.56±0.25	0.55±0.04	0.00±0.00
18:3n-3	0.00±0.00	0.58±0.05	1.69±0.08	3.76±0.23	4.99±0.03	1.22±0.03
18:4n-3	0.01±0.02	1.79±0.04	0.67±0.06	0.51±0.12	1.66±0.03	0.00±0.00
20:2n-6	1.42±0.06	5.11±0.09	0.67±0.06	1.41±0.11	0.20±0.03	0.62±0.04
20:3n-6	0.36±0.13	5.15±0.08	0.26±0.05	0.38±0.06	1.64±0.06	0.22±0.03
20:4n-6	1.42±0.12	4.39±0.25	0.31±0.06	0.43±0.06	9.82±0.06	0.32±0.05
20:5n-3	3.63±0.10	4.22±0.07	0.74±0.08	0.60±0.13	2.34±0.05	0.13±0.03
22:5n-3	0.00±0.00	0.02±0.00	0.00±0.00	0.06±0.03	3.19±0.02	0.00±0.00
22:6n-3	0.69±0.10	5.10±0.29	0.12±0.01	0.36±0.07	8.74±0.02	0.02±0.01
ΣPUFA	**26.70±6.03**	**38.65±0.80**	**14.78±0.34**	**19.58±0.35**	**40.04±0.18**	**19.00±0.17**

A – Algamac; B – S. presso; C – *Chaetoceros calcitrans* powder; D – *Isochrysis galbana*; E – Oil emulsion (1 ml^{-1}); F – Un-enriched rotifer

Table 5.9. ANOVA for fatty acid composition of rotifer enriched with the experimental diets at 6 h.

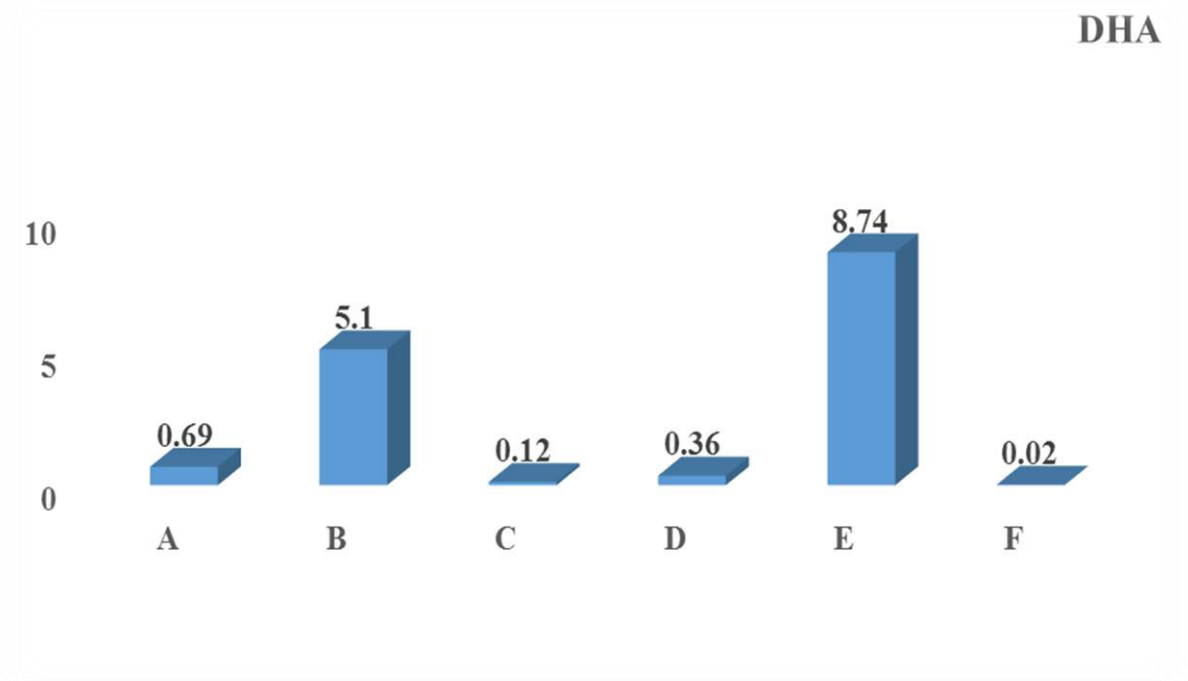
Fatty acids (%)	ΣSFAs	ΣMUFAs	EPA	DHA	ΣPUFAs	∑*n*-3 LC – PUFAs
A	35.28±0.38^{d}	33.76±0.43^{c}	3.63±0.10^{f}	0.69±0.10^{c}	26.70±6.03^{c}	4.33±0.22^{c}
B	20.66±0.49^{a}	37.25±0.21^{e}	4.22±0.07^{g}	5.10±0.29^{d}	38.65±0.80^{d}	11.69±0.45^{e}
C	28.01±0.39^{b}	29.69±1.18^{b}	0.74±0.08^{c}	0.12±0.01^{a}	14.78±0.34^{a}	3.22±0.23^{b}
D	33.48±0.36^{c}	38.61±0.20^{f}	0.60±0.13^{b}	0.36±0.07^{b}	19.58±0.35^{a}	5.29±0.58^{d}
E	26.99±0.06^{b}	26.47±0.11^{a}	2.34±0.05^{e}	8.74±0.02^{e}	40.04±0.18^{d}	20.92±0.15^{f}
F	32.20±0.29^{e}	32.48±0.21^{d}	0.13±0.03^{a}	0.02±0.01^{a}	19.00±0.17b^{c}	1.37±0.07^{a}

Mean sample size = 3.0



A – Algamac; B – S. presso; C – *Chaetoceros calcitrans* powder; D – *Isochrysis galbana*; E – Oil emulsion (1 ml^{-1}); F – Unenriched rotifer

Figure 5.17. Differential content of aggregate DHA/EPA in the rotiferenriched with experimental diets



A – Algamac; B – S. presso; C – *Chaetoceros calcitrans* powder; D – *Isochrysis galbana*; E – Oil emulsion (1 ml^{-1}); F – Unenriched rotifer

Figure 5.18. Differential content of aggregate DHA/EPA in the rotiferenriched with experimental diets

5.4. Conclusions

Isochrysis galbana was selected to enrich the live feed rotifer based on the greater content of DHA and long chain *n*-3 fatty acids among different microalgae considered in the present study. *I. galbana* has been used as the initial culture media with rotifers instead of yeast to enable the live feed to grow in a healthy environment. The aggregate content of PUFAs of rotifers enriched with *Isochrysis galbana* was of its maximum at the 12 h (20 %TFA) of enrichment period, and thereafter a diminishment in their content was noted. However, the microalga *I. galbana* used not only as an enrichment media *per say*, but to impart better growth and development traits of the live feeds. The DHA rich oil emulsion, with DHA content of 39% TFA and EPA content of 19% TFA was used to enrich rotifers. A maximum content of polyunsaturated fatty acids (~ 40%) were apparent after a period of 6 h, while the oil emulsion was used at the dose of 1 mL/L. The C_{22}*n*-3 fatty acid (DHA) in the rotifers exhibited a significant increase (~ 9 %) after the enrichment period of 6 h, while the oil emulsion was used at the identical dose (1 mL/L). The ratio of DHA: EPA in rotifers after enrichment duration of 6 h was 2.76: 1 that has been reported as ideal for marine fish. The rotifers enriched with the PUFA enriched oil emulsion for a period of six hours could yield greater quantities of the desired fatty acids ($C_{20\text{-}22}$*n*-3 fatty acid, EPA, 20:5*n*-3 and DHA, 22:6*n*-3). Concurrently, different enrichment media, for example, Algamac 2000 (Biomarine Inc., Aquafauna, USA), S.presso (Selco S.presso ®, INVE Aquaculture), an indigenously powdered microalgal derived from *Chaetoceros calcitrans* and marine microalgae (*Isochrysis galbana*) were utilized to compare their efficacy with the $C_{20\text{-}22}$ PUFA rich oil emulsion. However, the PUFA enriched oil emulsion developed in the present study could yield the rotifer with greater content of these fatty acids (~40 %) than those recorded with other enrichment formulation.

CHAPTER 6

Effect of enriched rotifers on growth and survival of the clownfish larvae *Amphiprion ocellaris*

Chapter 6

Effect of enriched rotifers on growth and survival of the clownfish larvae *Amphiprion ocellaris*

6.1 Background of the study

Marine ornamental species were traded globally since 1930s. During the last few decades the market demand of ornamental fishery and aquaculture had been consistently increasing, which become a multi-million dollar industry (Hardin and LeGore, 2005; Murray *et al*., 2012). According to the FAO (2001), not less than 99 % of the marine species have been collected directly from the wild environment. Only 1% of the ornamentals collected from their wild habitats were reported to be reared in the hatcheries worldwide (Bunting and Fossa, 2001). This resulted in the impact of destructive fishing practices and overexploitation (Rhyne *et al*., 2012). This is due to lack of hatchery technology and unpredictable early mortality at the larval stages (Maehre *et al*., 2013). It is possible to reduce the early mortality by careful management through intensive culture systems (Rhyne *et al*., 2012). In nature, marine fish larvae mainly feed on the copepod nauplii and copepodites, which are essential and rich in fatty acids especially 20:4*n*-6 (AA), 20:5*n-3* (EPA) and 20:6*n-3* (DHA) (Avella *et al*., 2007). However, the major drawback with copepods is that the species cannot be cultured in captivity, and therefore, may not a suitable choice as an ideal live feed.

In the commercial hatcheries, rotifers and *Artemia* nauplii habe been commonly fed with microalgae. However, the size of the *Artemia* nauplii is bigger than that of rotifers, which substantiate the utility of the latter as an ideal live feed for ornamental fish larvae with comparatively lesser mouth aperture. Particularly during the last four decades, several hundred microalgal species have been tested as feed for rotifers, but probably only few have gained wide acceptance in aquaculture (Shields and Lupatsch, 2012; Vijayagopal *et al.*,

2012). A variety of food sources can be used to rear rotifers and its nutritional value is mainly dependent on the type of the feed given (Jeeja *et al*., 2011). However, the rotifers enriched with microalgae are not a viable option to improve the content of fatty acids due to their distinct genetic characteristics. It is therefore imperative to enrich the rotifers with artificial enrichment media with greater quantities of $C_{20\text{-}22}$*n*-3 PUFAs. The enriched rotifers perform as the live carrier of these essential long chain fatty acids to the ornamental fish larvae. This hypothesis was further supported by earlier reports stating that it is a common practice to enrich the live prey with emulsions of marine oils, which are rich in *n*-3 long chain PUFAs (Philippe*et al*., 2001; Brown, 2002; Wacker and Martin-Creuzburg, 2012).

The fatty acids provided through live prey are essential to satisfy the high-energy demand required to improve the growth and survival of the ornamental fish larvae. Therefore, in recent decades, research work is progressing to improve growth and survival of ornamental fish larvae by supplying polyunsaturated enriched live diets (Olivotto *et al*., 2011). Among these, the requirements of *n*-3 fatty acids, for example, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are vital for successful growth and development of marine fish larvae (Sargent *et al*., 1999; Avella *et al*., 2007; Carla *et al*., 2012; Mies *et al*., 2014). In hatchery operation, the rotifers are the primary diets for the better development of fish larvae, because of their lesser size, greater availability, adaptability for mass cultivation and majority of the marine larvae require prey with in a size range of 50 to 200 µm, which could be satisfied by the live feed rotifers. Current food sources of *n*-3 long chain PUFAs are fish, shellfish, linseed, hemp oil, soya oil, rapeseed oil, pumpkin seeds, sunflower seeds, leafy vegetables, and walnuts. However, the $C_{20\text{-}22}$ long chain *n*-3 PUFAs, for example, EPA and DHA are only present in the marine fish oil. In the present study, the enriched rotifers obtained by using different PUFA enrichment media (Biomarine Inc., Aquafauna, USA); S. presso (Selco S. presso ®, INVE Aquaculture); $C_{20\text{-}22}$ long chain *n*-3 PUFA enriched oil

emulsion derived from marine finfish; microalgal (*Chaetoceros calcitrans*) powder *vis-à-vis* the unenriched rotifers) were used to culture the marine ornamental finfish *Amphipion ocellaris* under the captive condition. The different biological parameters of the ornamental fish, such as, growth and survival were taken into account to understand the effect of differentially enriched rotifers in the biology, growth, and development of the ornamental fish.

One of the most requested species by the aquarium trade market is the false percula clownfish *A. ocellaris* (FAO, 2001). Its maximum total length is about 8 cm, and it inhabits in lagoons and coastal waters up to depths of at least 12 m. It usually feeds on different zooplanktons, small benthic invertebrates, and algae. It distributes in eastern most part of Indian Ocean and western edges of the Pacific, including Andaman and Nicobar Islands, Indo-Malayan Archipelago, north Western Australia, Philippines, and northward to Japan. It is very similar to *A. percula*, but usually has XI dorsal-fin spines and a taller spiny dorsal fin. Current food sources of *n*-3 and *n*-6 PUFAs are fish, shellfish, linseed, hemp oil, soya oil, rapeseed oil, chia seeds, pumpkin seeds, sunflower seeds, leafy vegetables, and walnuts; however, the major sources of EPA and DHA, on a worldwide basis, are still marine fish (Guedes *et al*., 2011). In view of the importance of these facts, this study illustrated the enrichment media used to enrich the live preys and ensure greater survival and faster growth of *A. ocellaris* larvae.

6.2 Materials and methods

6.2.1 Rotifer enrichment

The rotifers enriched with five different media and one unenriched rotifer with an aim to enrich the C_{22} long chain *n*-3 PUFA in the live feed rotifers. The PUFA – rich

experimental media used in the present study were as follows: (1) Algamac – 2000 (Biomarine Inc., Aquafauna, USA); (2) S. presso (Selco S.presso®, INVE Aquaculture); (3) indigenously produced marine fish derived oil emulsion; (4) *Cheatoceros calcitrans* derived microagal powder, (5) marine microalgae (*Isochrysis galbana*), and (6) unenriched rotifer developed with marine microalgae *Isochrysis galbana*.

The rotifer *Brachionus plicatilis* was mass cultured by semi-continuous system and the medium used was microalgae *Isochrysis galbana*. The experiment was conducted in Vizhinjam Research Centre of Central Marine Fisheries Research Institute at Thiruvananthapuram. The optimized period of 6 hours was used to enrich the rotifers with the experimental media during the larval rearing of *Amphiprion ocellaris*. The standardisation and fatty acid composition as explained in chapter 5. The oil emulsion was prepared from the indigenously produced *n*-3 polyunsaturated fatty acid, particularly docosahexaenoic acid rich sardine oil and the standardized quantity (chapter 5) of oil used for rotifer enrichment.

The experimental requirement of rotifers were filtered by using 80 µm mesh bolting silk from the container containing rotifers developed with microalgae *Isochrysis galbana* to separate the rotifer sized above 80 µm. The filtered rotifers were transferred to a fresh seawater container containing enrichment media.

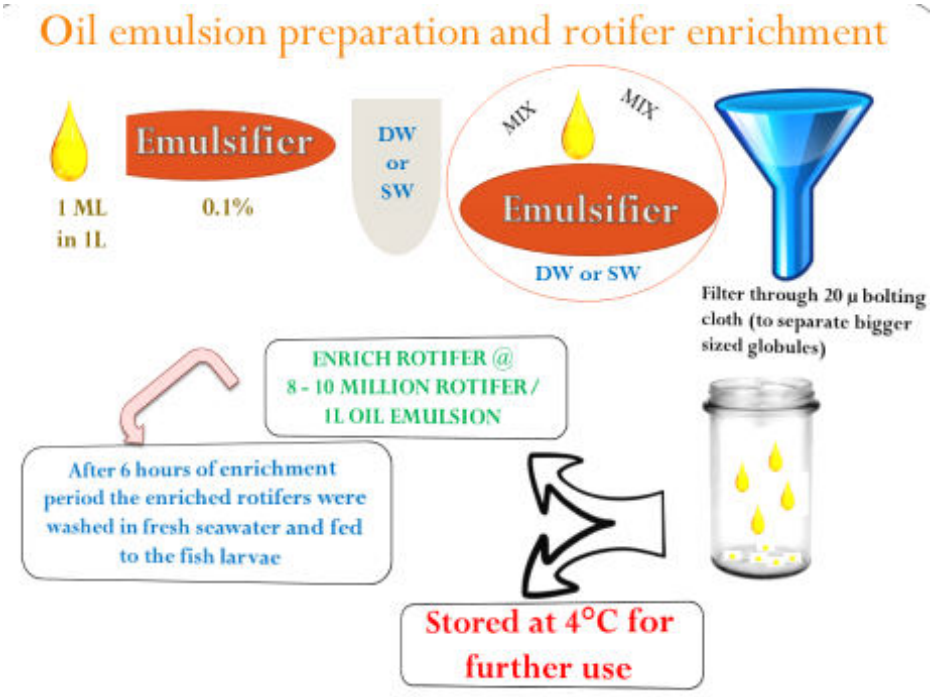


Figure 6.1. Preparation of oil emulsion for experimental usage

Stored (at 4°C) Oil emulsion for daily usage

Oil (ml)	Distilled water (ml)
30	1000
15	500
8	250
4	125
2	63
1	31

Figure 6.2. Oil emulsion stored at 4°C for daily usage

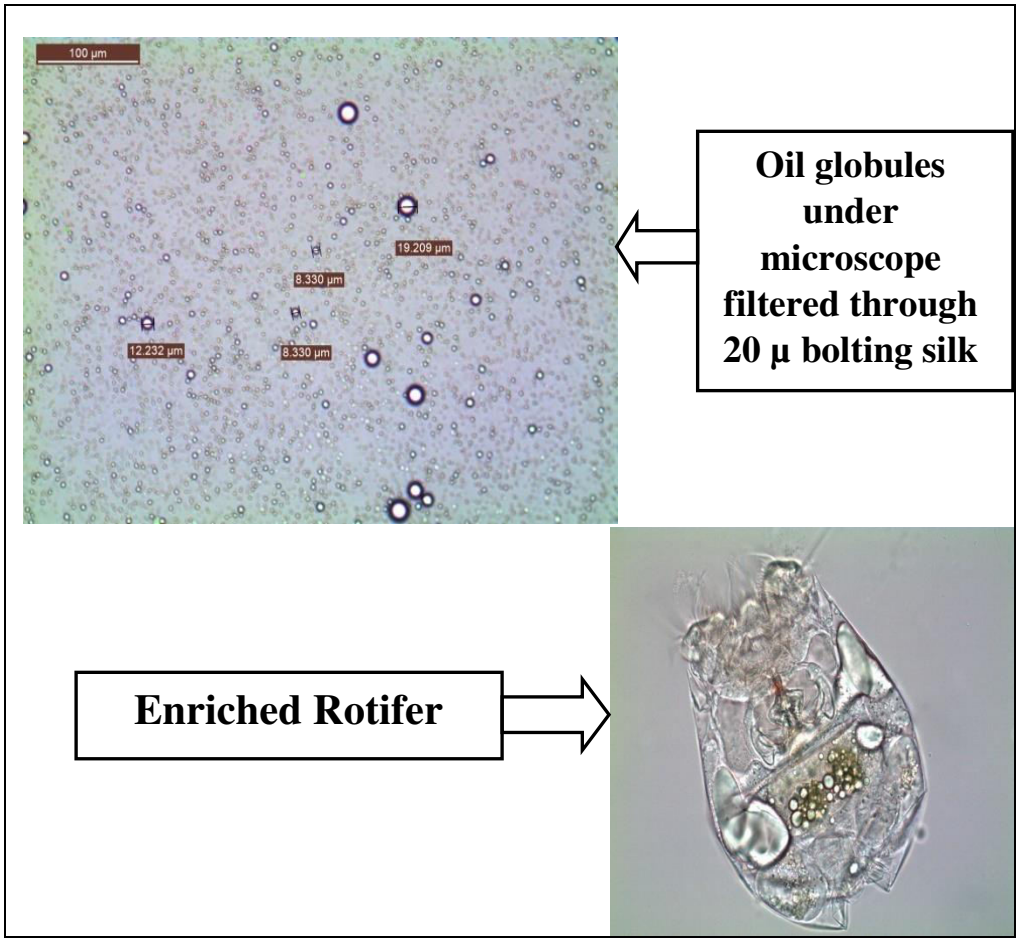


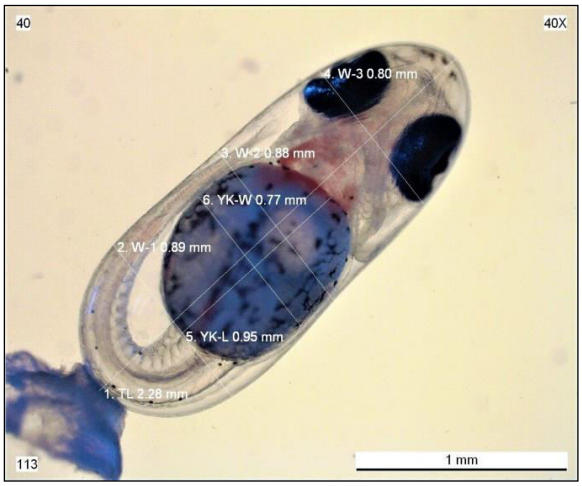
Figure 6.3. Oil emulsion enriched to rotifer *Brachionus*

6.2.3 Larval rearing

The tanks in larval rearing section were installed with thermostat to maintain the constant temperature (26- 28°C). Seawater from the main supply was transferred to the well where the water was naturally filtered by settling the waste particles. The clear water from the well was pumped to the header tank. The stored seawater was thereafter transferred after two days from the well to the strongly aerated fibre tanks with 10 ton capacity for chlorination by using the fine mesh cloth filter to eliminate the zooplankton fauna from entering the system. The chlorinated water was passed through a high intensity ultra-violet (UV) filtering process for a continuous period of two days for removing the unwanted free-floating microscopic water borne bacteria, parasitic, fungal, viral, algae, and other pathogens out of aquarium water for further use in the culturing tanks.

Figure 6.4. Experimental set up of the larval rearing

Fig. 6.5 Experimental fish *Amphiprion ocellaris*



Microscopic view of fish embryo

2 days old fish larva

20 day old fish larva

Fig. 6.6 Brooder rearing and rotifer enrichment setup

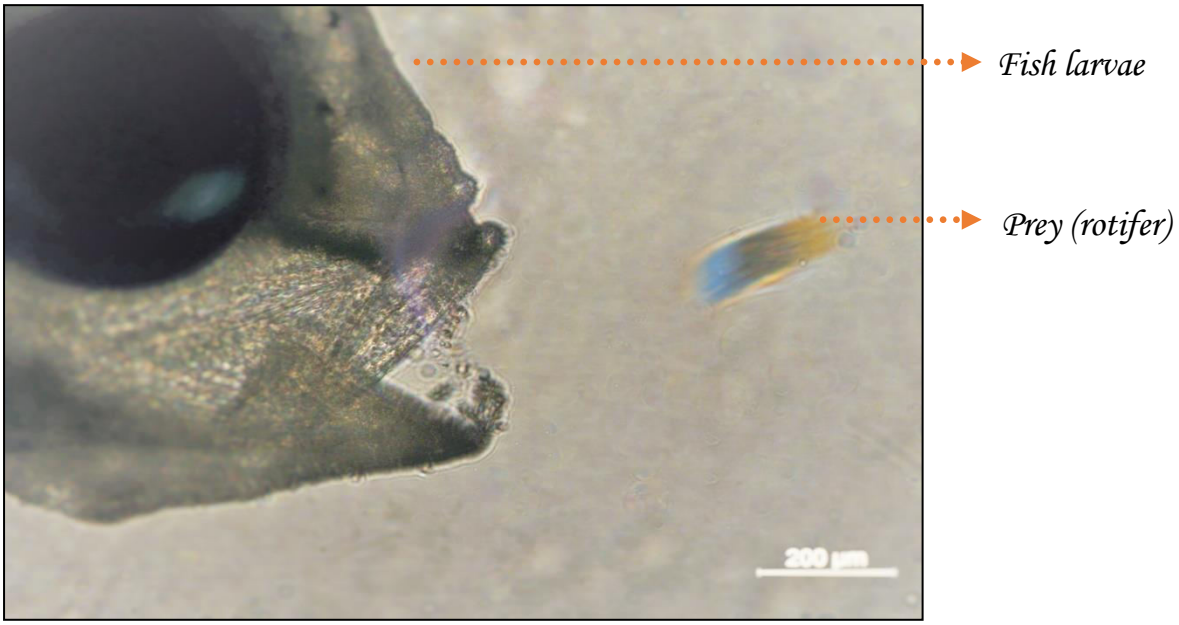


Figure 6.7. Fish larvae and the prey rotifer *Brachionus plicatilis*

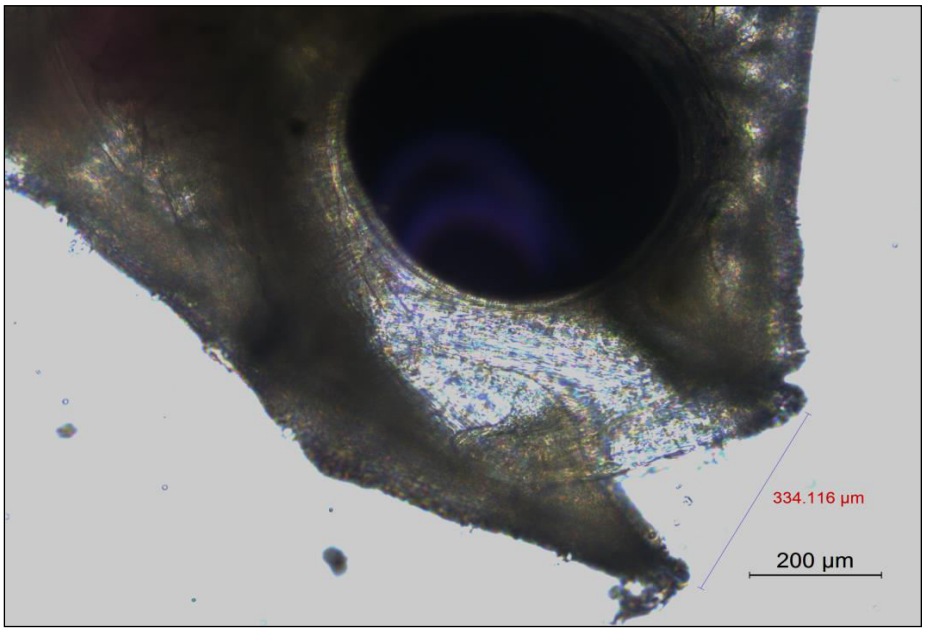


Figure 6.8. Mouth size of the fish larvae

6.2.2 Nutritional experiments

The ornamental fish larvae were transferred from the hatching facilities into the rearing tanks either using a wide bore (>5 mm) aeration tube or a beaker. The stocking density was maintained at about 50 larvae per tank. The experiments were carried out in triplicate in a 50 l tank with water level maintained at 40 l. The larvae were transferred to the tanks on the day of hatch. Feeding was initiated from the first day after post-hatch. The larvae were offered rotifers twice a day, which was enriched with the experimental diets for a period of 6 h. The salinity and pH of the system were maintained at 33-35 ‰ and 8 – 8.4, respectively. The routine rank maintenance, which proceeds feeding, included the siphoning of debris from the bottom, the skimming of surfaces, and water exchange, were carried out. The salinity and pH were recorded daily. The experiments were terminated after 10 days of the experiment. Once the water level was low enough, the fish larvae were scooped out, and counted for the final survival estimates. The dead larvae were siphoned out before being counted during the early morning hours until the experiments were terminated to estimate their survival and mortality rates.

6.2.3 Statistical analyses

A suite of statistical analyses were carried out using the statistical packages for triplicate values, and the results were expressed as mean $\pm$ standard deviation. The fatty acids were analysed in through the Microcal Origin (Version 8).

6.3 Results and discussion

6.3.1 Survival rate of larvae fed on enriched rotifers

The experiment carried out by using the rotifers enriched with the aid of six experimental media, namely, 1. Algamac 2000® (Biomarine Inc., Aquafauna, USA); 2. S. presso (Selco S. presso®, INVE Aquaculture); 3. Indigenously produced long chain PUFA enriched Oil emulsion derived from marine finfish; 4. Indigenously produced lyophilized Algal powder (*Chaetoceros calcitrans*); 5. Marine microalgae *Isochrysis galbana* and 6. Un-enriched rotifer, and their effect on the survival marine ornamental fish larvae (*A. ocellaris*). The details of their survival percentage were demonstrated in Figures 6.2. and 6.3. It was observed that the survival percentage of the fish larvae was found to be maximum (93 %) when they were fed with the rotifers enriched with the oil emulsion (C_{20-22} long chain *n*-3 PUFAs). It is of note that the survivability of the fish larvae was significantly lesser (28 %), when unenriched rotifers were used. The per cent survival of the ornamental fish larvae were found to be lesser when the rotifers were enriched with the S. presso (Selco S. presso®, INVE Aquaculture) (76 %), alga enriched (*Isochrysis galbana*) (69 %), Algamac (62 %),and algal powder (R_{40}) (53 %). Therefore it is inferred that the DHA enriched oil emulsion offers a valuable package to enrich the rotifers for their downstream use to enrich the ornamental fish larvae under aquaculture conditions.

Larval rearing of marine fish is the bottleneck for many years (Dhert *et al*., 1998; Battaglene and Cobcroft, 2007; Andrews, 2000). In India the breeding and rearing of clownfish was developed under captivity during 2001 (Ignatius *et al*., 2001). It is well known that the marine fish larvae required greater amount of essential fatty acids, particularly docosahexaenoic acid in their diet, particularly during the initial stages (Glencross, 2009). The experimental results on *Amphiprion ocellaris* larvaeshowed that the bio-encapsulated

rotifer with greater content of DHA (8.74 %TFA) fed to the larvae resulted in their better growth and survival, whilst the batch fed with un-enriched rotifer (with DHA 0.02 %TFA) exhibited lesser survival (Chapter 5). Park *et al*. (2006) observed that the diet with greater level of DHA has a dominant influence on the growth and survival of Atlantic cod larvae. This essential C_{22} fatty acid (DHA) can be delivered through the live food (rotifer, copepod, and *Artemia* nauplii) to the larvae and has been appropriate to their mouth size (Ronnestad *et al*., 1999; Ignatius *et al*., 2001). Due to the culture difficulties of copepod in mass, the rotifer were preferred as most commonly used live feed to rear the marine fish larvae (Holt, 2003; Olivotto *et al*., 2008). Rotifers are lacking the essential fatty acids, such as, eicosapentaenoic acid and docosahexaenoic acid (Maehre *et al*., 2013). Many marine fish cannot able to synthesis essential fatty acids (DHA), and usually depend on external feeding (Sargent *et al*., 1989). These fatty acids are bio-encapsulated to the rotifer through bio-enrichment technique and fed to the fish larvae. Many enrichment products are available in the market to enrich rotifer, which may enhance HUFAs. In the present study, the comparison of the commercially available enrichment products and the indigenously produced DHA rich oil emulsion were observed. The right proportion of the essential fatty acids to the marine ornamental fish larvae have not been extensively studied (Kotani *et al*., 2010).

The greater amount of long chain $C_{20\text{-}22}$ fatty acids might provide more effective lipid oxidation and larval growth (Avella *et al*., 2007). It is of note that the maximum survival of 93 % was noticed by feeding the fish larvae with a greater proportion of C_{22} fatty acid, DHA. The least survival of 28 % was observed in un-enriched rotifer fed to the fish larvae. It is apparent that the un-enriched rotifer could not satisfy the nutritional requirements of the fish larvae, and the mortality started from the 3rd day onwards when it turns from endogenous to exogenous feeding. Nutrition plays an important role during the larval stages, especially during yolk nourishment stages (Paulraj *et al*., 2009). Feeding the larvae during exogenous

feeding period of after 2 - 3rd day with DHA/EPA ratio of 3.74 showed the positive result with greater per cent larval survival. Likewise the least value of DHA/EPA ratio of 0.15 apparently resulted to a poor survival (Chapter 5). Park *et al*. (2006) demonstrated that the ratio of DHA/EPA was positively correlated with growth and survival of the fish larvae.

The U.S. retailers found that between one-third or more than half of the aquarium fish imported from Southeast Asia are died shortly after arrival according to the survey reported in 1997 (Andrew, 2000). This is because, the animal were directly collected from wild habitats, and could not adapt in the captive environment in a shorter period due to the differential environmental stress. One way to reduce the pressure of the animal and on coral reef ecosystems is to improve the ability to hatchery-based organisms for trade. Until now the maintenance and the survival rate in captivity is relatively lower. The present effort is to improve survival and growth of ornamental finfish *Amphiprion ocellaris* under captive condition. This will otherwise safeguard these valued ornamentals from human poaching along the coral reef ecosystem. The ANOVA (Table 6. 1.) showed the survival rate of the fish larvae (*A. cellaris*) fed by rotifer enriched with six experimental diets, namely, Algamac 2000; S. presso; C_{22} long chain fatty acid enriched oil emulsion; algal powder; marine microalgae *Isochrysis galbana* and un-enriched rotifer as negative control.

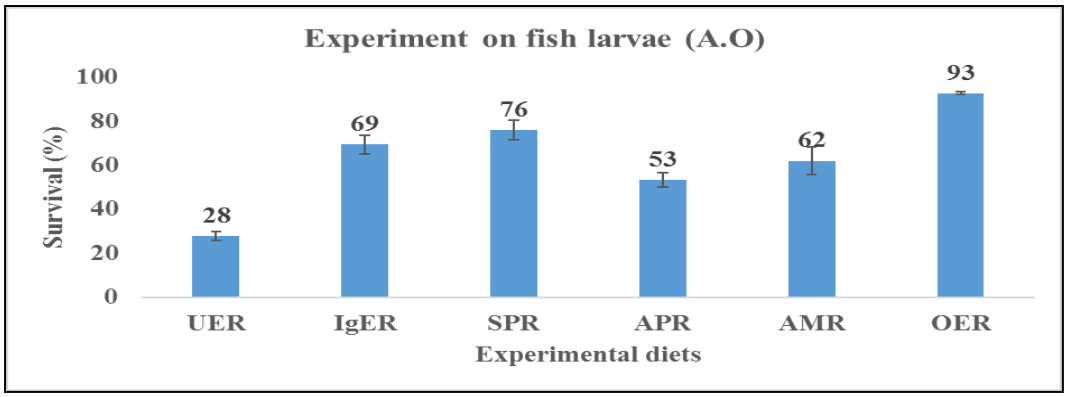


Figure 6.9. Survival percentage of experimental media and its effect on fish larvae (*Amphiprion ocellaris*) (The standard deviations in the graph have been presented as standard error bar)

Table 6.1. ANOVA for survival rate of the *A. ocellaris* fish larvae

Enrichment Diet	IGER	SPER	APER	AMER	UNER	OEER
Survival	69 ± 8.3^{c}	76 ± 9.2^{c}	53 ± 6.1^{ab}	62 ± 12.5^{bc}	43 ± 7^{a}	93 ± 1.1^{d}

Mean sample size = 3.0

6.3.2 Nutritional composition of clownfish grown with enriched rotifers

The nutritional composition of clownfish grown with enriched rotifer was shown under Table 6.1. The eggs of *A. ocellaris* were found to contain saturated fatty acids to 29% (TFA) and 31% polyunsaturated fatty acids. The initial hatch out larvae of *A. ocellaris* was found to contain 57% saturated fatty acids and 10% polyunsaturated fatty acids. The *A. ocellaris* larvae fed by rotifer enriched with *Isochrysis galbana* contain 10% saturated fatty acids and 17% polyunsaturated fatty acids were found in the larvae of *A. ocellaris* fed by rotifer enriched with the C_{22} long chain fatty acid enriched oil emulsion. However, the total content of DHA was recorded to be lesser than 2 % of the total fatty acids from the initial hatch out larvae and larvae fed by rotifer enriched with *Isochrysis galbana* during the 11^{th} day of enrichment. Ma and Qin, (2012) mentioned that the nutritional composition of fish eggs or embryos must be considered to prepare the rotifer enrichment diet for the successful hatchery operation. In the present study the DHA content of fish egg and oil emulsion were found to be 20% and 38% of the total fatty acids, respectively. It is of note that a greater content of DHA is essential for the optimum growth and development of the larval growth (Glencross, 2009). It is therefore imperative to enrich the larvae with an external source containing a greater content of C_{22} long chain fatty acid DHA. A greater content of DHA (~ 20% TFA) in the egg (5^{th} day) of the ornamental fish larvae apparently signified the importance of this C_{22} long chain fatty acid for the larval growth and development (Ma and Qin, 2012). It is therefore desirable to feed the larvae of ornamental fish with an external source, preferably a live feed (rotifer) with a greater content of DHA for the optimum growth

and development in captivity. The *n*-3 LC-PUFAs are considerably important for the growth and development of the marine fish larvae at the early stages as reported in earlier literatures (Avella *et al*., 2007; Hamre *et al*., 2013; Izquierdo *et al*., 1996; Olivotto *et al*., 2011; Sargent *et al*., 1999, 1997; Sargent, 1996; Tocher *et al*., 2008). In this context, the rotifers enriched with the indigenously developed C_{20-22} long chain *n*-3 PUFA enriched oil emulsion were used to increase the content of DHA in the ornamental fish larvae grown in captivity. The present outcome has been substantiated by the earlier works on red seabream, sea bass, yellowtail, turbot, and plaice (http://www.fao.org/docrep/003/ab412e/ab412e10.htm). The metamorphosis of the fish larvae was reported to be impaired, possibly due to a lack of DHA (Soudant *et al*., 1998). The DHA content of the larvae of *A. ocellaris* was found to increase to 6.7% of the total fatty acids from the initial value of 1.5% after feeding on rotifers enriched with the DHA enriched oil emulsion developed in the present study. No significant increase in the content of the long chain C_{20-22} long chain fatty acid was apparent when the larvae were grown in the algae alone. The indigenously developed DHA enriched oil emulsion developed in this study can therefore potentially substitute the imported products available in the market for ornamental fish aquaculture. The ANOVA (Table 6.3.) showed the fatty acid composition of *A. ocellaris* larvae enriched with the experimental diets. The enrichment effects were found to be highly significant ($P< 0.001$).

Table 6.2. Fatty acid composition of *A. ocellaris* larvae enriched with experimental diets

Fatty acids	**Egg of *A. Ocellaris***	**Initial *A. ocellaris***	***A. ocellaris* (algae enriched)**	***A. ocellaris* (oil emulsion enriched)**
14:0	7.38±0.36	6.36±0.62	5.82±0.80	2.45±0.56
15:0	0.08±0.04	3.32±0.12	4.44±0.39	3.95±0.25
16:0	21.01±0.50	28.83±0.68	29.15±0.73	24.94±0.16
18:0	0.15±0.03	12.56±0.46	14.08±0.35	11.32±0.24
20:0	0.09±0.05	2.83±0.52	2.53±0.45	2.24±0.06
22:0	0.05±0.02	2.49±0.27	1.42±0.28	1.52±0.13
24:0	0.09±0.04	1.25±0.05	0.65±0.06	0.22±0.04
∑ SFA	**28.85±0.24**	**57.65±1.89**	**58.09±1.48**	**46.65±0.92**
15:1	0.19±0.04	1.62±0.41	1.18±0.09	0.45±0.05
16:1n-7	21.89±0.41	8.71±0.55	7.81±0.12	8.31±0.17
18:1n-9	19.34±0.64	18.38±1.19	16.05±0.12	22.10±0.64
20:1	0.07±0.03	0.59±0.04	0.20±0.05	0.27±0.06
22:1	0.12±0.06	0.69±0.08	0.35±0.09	0.58±0.05
24:1	0.04±0.01	0.10±0.07	0.10±0.04	0.18±0.04
∑ MUFA	**41.66±0.90**	**30.08±0.99**	**25.69±0.19**	**31.90±0.61**
18:2n-6	0.18±0.04	1.76±0.69	1.26±0.08	1.60±0.14
18:3n-6	0.17±0.07	1.27±0.12	1.00±0.18	0.47±0.05
18:3n-3	0.12±0.04	0.24±0.06	0.28±0.06	0.46±0.10
20:2n-6	0.20±0.07	2.82±0.42	1.85±0.07	1.42±0.11
20:3n-6	0.19±0.05	1.45±0.08	1.38±0.08	0.40±0.11
20:4n-6	0.43±0.07	1.32±0.09	1.08±0.06	0.47±0.03
EPA	9.68±0.54	1.39±0.10	1.84±0.12	5.64±0.09
DHA	20.23±0.03	1.48±0.05	1.48±0.08	6.75±0.08
∑ PUFA	**31.20±0.70**	**11.74±0.87**	**10.16±0.54**	**17.20±0.26**

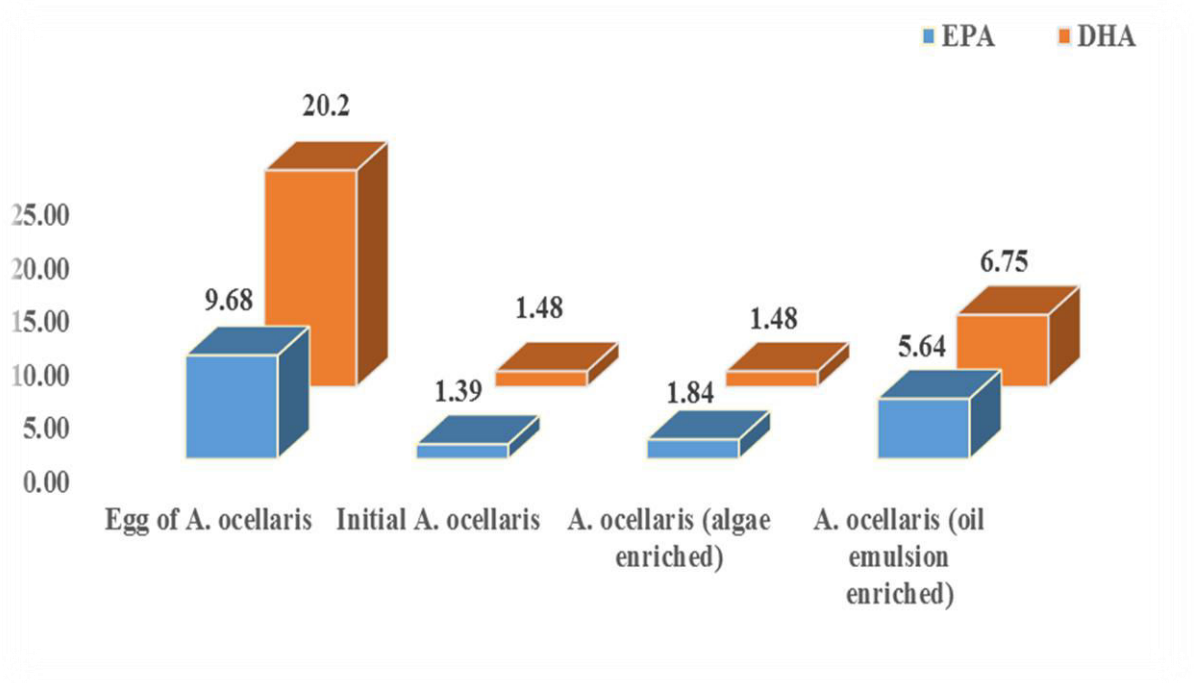


Figure 6.10. DHA and EPA contents (egg, hatchout larvae, rotifer enriched algae fed larvae, and rotifer enriced oil emulsion fed larvae) of the experimental animal (*Amphiprion ocellaris*)

Table 6.3. ANOVA for fatty acid composition of *A. ocellaris* larvae enriched with experimental diets

Fatty acids	**Egg of *A. ocellaris***	**Initial *A. ocellaris***	***A. ocellaris* (algae enriched)**	***A. ocellaris* (oil emulsion enriched)**
∑ SFA	28.85±0.24^{a}	57.65±1.89^{c}	58.09±1.48^{c}	46.65±0.92^{b}
∑ MUFA	41.66±0.90^{d}	30.08±0.99^{b}	25.69±0.19^{a}	31.90±0.61^{c}
EPA	9.68±0.54^{c}	1.39±0.10^{a}	1.84±0.12^{a}	5.64±0.09^{b}
DHA	20.23±0.03^{c}	1.48±0.05^{a}	1.48±0.08^{a}	6.75±0.08^{b}
∑ PUFA	31.20±0.70^{d}	11.74±0.87^{b}	10.16±0.54^{a}	17.20±0.26^{c}
n3	30.03±061^{d}	3.11±0.21^{a}	3.60±0.26^{b}	12.85±0.27^{c}

Mean Sample Size = 3.000

6.4. Conclusions

The rotifers enriched with five different media and one unenriched rotifer were thereafter used with an aim to enrich the C_{22} long chain *n*-3 PUFA in the *larvae of A. ocellaris*. A greater content of DHA (~ 20% TFA) in the egg (5th day) of the ornamental fish larvae signified the importance of this C_{22} long chain fatty acid for the larval growth and development. It is therefore desirable to feed the larvae of ornamental fish with an external

source, preferably a live feed (rotifer) with a greater content of DHA for the optimum growth and development in captivity. The rotifers enriched with the indigenously developed C_{20-22} long chain *n*-3 PUFA enriched oil emulsion were used to increase the content of DHA in the ornamental fish larvae grown in captivity. The survival percentage of the fish larvae was found to be maximum with (93 %) when they were fed with the rotifers enriched with the oil emulsion (C_{20-22} long chain *n*-3 PUFA).

CHAPTER 7

Summary

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Summary

Marine ornamental fishes are one of the most popular aquatic organisms due to their beauty and easy adaptability to live in the confinement of coral reef ecosystem. They are ten times more expensive than their freshwater counterparts, and hundred times costlier than the edible fishes. Due to the perpetual demand for marine ornamentals, wild collection through destructive fishing methods is increasing. To meet the demand and conservation of the coral reef ecosystems, captive breeding is necessary. Even though marine ornamental fish culture for trade is being practised globally, the early mortality of these altricial fish larvae is still unidentified. Feed is one of the vital inputs for the successful operation of any hatchery.

In the broad area of marine fish larviculture nutrition, the present study has been focussed to develop a successful larviculture protocol, and in this way, the following steps were followed: (1) eighteen marine microalgae, most commonly used were profiled for their fatty acid composition, (2) cost effective marine oil and phospholipid source, *viz*; crude sardine oil and fish roe were identified, profiled and used for development of an indigenous live feed enrichment product, (3) marine microalgae rich in *n*-3 polyunsaturated fatty acids particularly docosahexaenoic acid was shortlisted, enriched with rotifer for 48 hour, and profiled for their fatty acid composition, (4) marine microalgae rich in total polyunsaturated fatty acids was shortlisted and lyophilized for use in aquaculture, (5) the rotifer enriched with enrichment product (oil emulsion) for 24 hour time duration at two concentrations were profiled for fatty acid composition at every 3 hour, (6) rotifer enriched with experimental media at optimized enrichment time period and profiled for fatty acid composition, (7) the effect of enriched rotifer with experimental media on the growth and survival of the

clownfish larvae *Amphiprion ocellaris* Was demonstrated, (8) rotifer enriched with experimental media were fed to the experimental organism *Amphiprion ocellaris* and the following samples, (a) egg of *Amphiprion ocellaris*, (b) initial day of post hatch *A. ocellaris* larvae, (c) 11 day of post hatch of microalgae enriched rotifer fed *A. ocellaris* larvae and, (d) 11 day of post hatch of oil emulsion enriched rotifer fed *A. ocellaris* larvaewere profiled for fatty acid composition.

(1) Eighteen available marine microalgal species *Nannochloropsis* (RA_{01}, RA_{02} and RA_{05}); *Isochrysis* (RA_{06}, RA_{09}, RA_{10}); *Dicrateria* (RA_{14}); *Tetraselmis* (RA_{17}, RA_{18}, RA_{19}); *Dunaliella* (RA_{20}); *Chlorella* (RA_{24}, RA_{26}); *Chaetoceros* (RA_{35}, RA_{40}); *Pavlova* (RA_{45}, RA_{46})*; Picochlorum* (RA_{47}) have been screened for fatty acid composition to shortlist the microalgae rich in $C_{20\text{-}22}n$-3 polyunsaturated fatty acids and docosahexaenoic acid (DHA, 22:6*n*-3) as feed for mass production of rotifer. From the fatty acid composition *Isochrysis galbana* was found to contain greater concentration of $C_{20\text{-}22}n$-3 polyunsaturated fatty acids and docosahexaenoic acid (DHA, 22:6*n*-3). The same was used as feed for mass production of rotifer during the experimental study.

(2) The crude sardine oil has been subjected to various processes to obtain various proportions of long chain *n*-3 fatty acids required by the marine fish larvae. The various processes included saponification and extraction of fatty acid, formation of the fatty acid by urea inclusion complex, acid-catalyzed transesterification of fatty acid to methyl esters (FAMEs), silver ion thin-layer chromatography ($AgNO_3$ TLC), preparation of DHA concentration by *Bacillus subtilis* lipase-catalyzed hydrolysis of fatty acyl esters, separation of phospholipid fraction from the seerfish roe and preparation of DHA rich oil emulsion and an experiment was conducted to understand the effect of emulsifier to stabilise the DHA concentrate. From the results it has been demonstrated that the essential $C_{20\text{-}22}n$-3

polyunsaturated fatty acids, particularly docosahexaenoic acid (DHA, 22:6*n*-3) was maximum, which is required for the marine fish larvae for growth and survival.

(3) The shortlisted microalgae *Isochrysis galbana* was used to enrich the rotifer *Brachionus plicatilis* for 24 hour to optimize the maximum concentration of $C_{22}n$-3 polyunsaturated fatty acids. From the experiment conducted the enrichment period of 12 hour time duration was found to contain the maximum concentration of $C_{22}n$-3 polyunsaturated fatty acids, and the concentration was gradually decreased after this time duration.

(4) The microalgae *Chaetoceros calcitrans,* which is rich in total polyunsaturated fatty acids, was shortlisted and lyophilised for use as a feed ingredient in aquaculture.

(5) The indigenously prepared oil emulsion rich in $C_{22}n$-3 polyunsaturated fatty acids, particularly docosahexaenoic acid (DHA, 22:6*n*-3) from marine source, which has been used to enrich the rotifer *Brachionus plicatilis* at two different concentrations, namely, 0.1 and 1 ml^{-1} and profiled for fatty acid composition every 3 hour. From the experiment it was demonstrated that the oil emulsion used at $1ml^{-1}$ concentration after the enrichment period of 6 hour harboured maximum $C_{22}n$-3 polyunsaturated fatty acids, particularly docosahexaenoic acid (DHA, 22:6*n*-3).

(6) The PUFA – enriched experimental media used in the present study were as follows: (a) Algamac – 2000 (Biomarine Inc., Aquafauna, USA); (b) S. presso (Selco S.presso®, INVE Aquaculture); (c) marine fish derived oil emulsion; (d) *Cheatoceros calcitrans* (RA_{40}) derived microagal powder; and (e) rotifer enriched with microalgae (*Isochrysis galbana*). The rotifer was enriched with the enrichment media at an optimized enrichment time period of 6 hours, and thereafter profiled for fatty acid composition. From the results the maximum concentration of $C_{22}n$-3 polyunsaturated fatty acids, particularly

docosahexaenoic acid (DHA, 22:6*n*-3) was rich in indigenously produced oil emulsion from the crude oil of sardine.

(7) The live feed (rotifer *Brachionus plicatilis*) was enriched by utilizing the oil emulsion with greater DHA content, and the enrichment efficiency with other available enrichment media was compared. The feeding protocol of the clownfish larvae *Amphiprion ocellaris* by using polyunsaturated fatty acid enriched live feed with DHA rich oil emulsion was optimized, and the effects on larval growth and survival under captivity was demonstrated. It is of note that at the initial growth stages (10 days day post hatch), the marine ornamental fish larvae had considerable requirement of *n*-3 long chain polyunsaturated fatty acids particularly docosahexaenoic acid (DHA, 22:6*n*-3) for optimum growth and development. These fatty acids were bioenriched in the rotifer, *Brachionus plicatilis* and fed to the fish larvae. Commercially available imported rotifer enrichment products used in hatcheries are imported and costly. In the present study the procedure to produce *n*-3 long chain PUFA rich oil emulsion from locally available oil marine finfish for use in aquaculture has been demonstrated, which are cost-effective as compared to those available in market. The oil emulsion was further used to enrich live feed rotifer and its effect on marine ornamental fish larvae was demonstrated and compared with other different commercially available enrichment media. The results obtained in the present study were significant in improving the growth and survival of the most economically important marine ornamental fish (*Amphiprion ocellaris*). Similar protocol can be verified or modified to improve the growth and development of other marine finfish and shellfish.

(8) The fatty acid composition of the experimental organism *Amphiprion ocellaris* was carried out in the following samples (a) egg of *Amphiprion ocellaris*, (b) initial day of post hatch, (c) 11 day of post hatch of microalgae enriched rotifer fed *A. ocellaris* and, (d) 11

day of post hatch of docosahexaenoic acid (DHA) enriched rotifer fed *A. ocellaris*. From these results, the maximum accumulation of the long chain polyunsaturated fatty acids was found in *A. ocellaris* larvae fed by rotifer enriched with C_{22} *n*-3 long chain polyunsaturated fatty acids, particularly docosahexaenoic acid rich (DHA, 22:6*n*-3) oil emulsion.

The present study presents an innovative attempt to optimise the bio-enrichment protocol in the marine ornamental fish larvae by using various formulations enriched with long chain *n*-3 polyunsaturated fatty acids such as, indigenously produced C_{20-22} long chain polyunsaturated fatty acid, particularly C_{22} fatty acid asdocosahexaenoic acid (DHA, 22:6*n*-3), marine microalgae rich in essential *n*-3 polyunsaturated fatty acids with optimized enrichment time period, and lyophilised marine microalgal powder rich in *n*-3 polyunsaturated fatty acids. The outcome of the study revealed that the lyophilised marine microalgae powder rich in essential polyunsaturated fatty acids can be potentially used as a feed for mass production of rotifers, and that the lyophilised marine microalgae powder rich in essential polyunsaturated fatty acids can be used as a direct feed to the finfish and shellfish larvae. The indigenously produced C_{20-22} long chain polyunsaturated fatty acid, particularly docosahexaenoic acid rich (DHA, 22:6*n*-3) oil emulsion can be used as a live feed enrichment product to the finfish larvae. These C_{20-22} long chain polyunsaturated fatty acid enriched formulation might prove to be the effective alternatives to the expensive and imported enrichment products available in the market.

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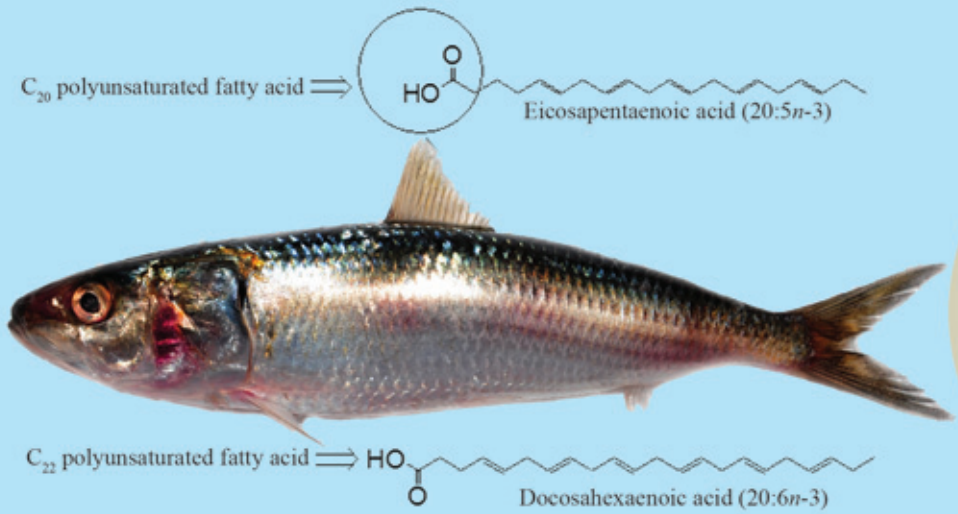

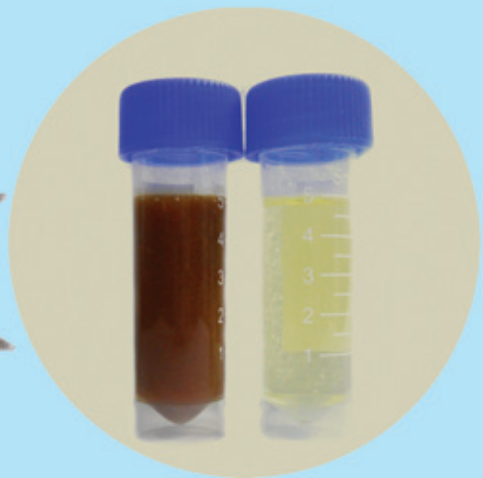

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Long chain n-3 polyunsaturated fatty acid enriched oil emulsion from sardine oil

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Dietary fats are used to build every cell in the body and cell membranes are made of a variety of individual fatty acidswhich are carboxylic acids with long hydrocarbon chains (usually C_{12-22}). The essential fatty acids from marine fish have protective mechanisms against coronary heart disease, which became apparent in the investigations of the health status of Greenland Eskimos who consumed diets very high in fat from seals,whales, fish etc, and yet had a low rate of coronary heart disease. This paradox was explained by the fact that the Eskimos diet contained large quantities of the very-long-chain and highly polyunsaturated fatty acids with C_{20-22} carbons and 5-6 olefinic bonds, which are abundant in marine fish, but scarce or absent in terrestrial animals and plants. Fatty acids with e" 2 double bonds are termed as polyunsaturated fatty acids (PUFAs) which are broadly divided into two major families, n-3 and n-6 PUFAs. The long chain C_{20-22} n-3 fatty acids are found abundantly in marine fish and phytoplankton. These affect many physiological processes including cognitive function, visual acuity, immunosuppressive and anti-thrombic activities along with a major role in glucose and lipid metabolism. Research on exploring sources for long-chain C_{20-22} PUFAs (LC-PUFAs), such as, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) for nutrition have received considerable attention (Figure 1) since these PUFAs, are usually low in abundance in humans, but regarded as essential . They have to be supplied in the diet. The importance of PUFAs in human nutrition has been extensively investigated during the past 20 years. DHA is one of the important PUFAs, which maintains structural and functional integrity in larval cell membranes in addition to the neural development and function, while arachidonic acid (AA, 20:4n-6) and EPA areinvolved in the production and modulation of eicosanoids respectively. DHA is a vital component of the phospholipids of cellular membranes, especially in the brain and retina, and necessary for their proper functioning. An imbalance in n-3/n-6 ratio can accentuate n-3 fatty acid deficiency state, as shown by earlier studies. The ratio is found to have increased in industrialized societies because of increased consumption of vegetable oils rich in n-6 fatty acids, *ie*, linoleic acid (18:2n-6) and reduced consumption of foods rich in n-3 fatty acids. Another important feature of n-3 fatty acids is their role in the prevention and modulation of certain diseases that are common. A partial list of diseases that may be prevented or ameliorated with n-3 fatty acids is given below.

- Coronary heart disease and stroke
- Diabetes
- Cancers of the breast, colon, and prostate
- Hypertension

The LC- PUFAs are also recognized to have beneficial therapeutic, physiological, and nutritional effects on human health.

Imported products (PUFA supplement) include Seven Seas by a UK healthcare company producing PUFA rich Cod Liver Oil by Ocean Gold™ technology. A value-added PUFA concentrate named "fish oil-1000 natural omega 3®" containing 100 capsules manufactured by Healtheries of New Zealand Ltd. priced at about ₹ 1150 per pack, is currently being marketed in India by Perma Healthcare, Bangalore. "EPAX 1050 TG®", a marine omega 3 formula produced from selected marine oils and marketed by

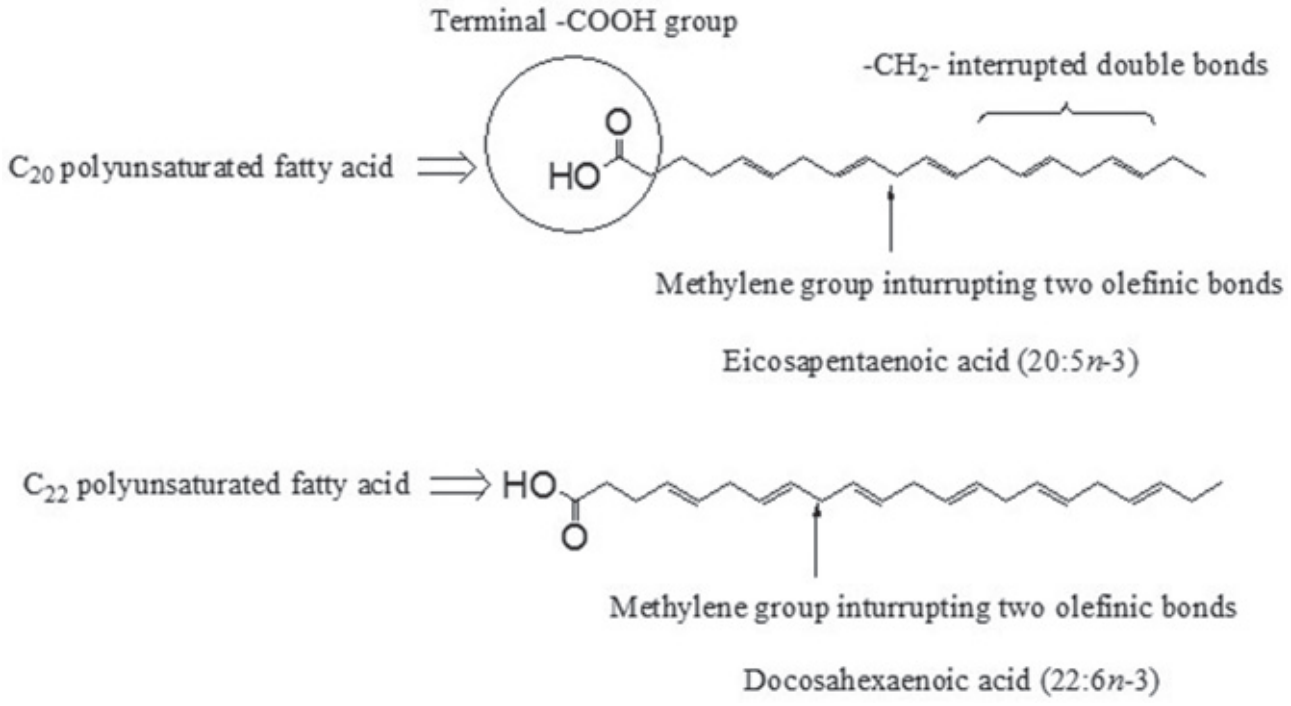


Figure 1. C_{20-22} polyunsaturated fatty acids (Eicosapentaenoic and Docosahexaenoic acids)

EPAXAS, Norway is an imported product containing 17% EPA imported to India for use as fish feed supplement. OMEGA XL® is marketed by DeColores in a bottle containing 60 capsules of refined combination of omega-3's costing about $50 per pack, as a nutraceutical. Great Health Works manufactures a concentrate of PUFA from Blue Grenadier Fish that is also a costly PUFA supplement. DSM Nutritional Products, Switzerland manufactures and sells PUFA concentrate under the trade name ROPUFA® produced from refined vegetable and marine oils. These products are currently imported to meet the domestic demand of PUFA concentrate formulation for fish feed supplements and nutraceutical purposes. This underlied the need to develop an indigenous n-3 (PUFA) supplement The Marine Bioprospecting section of Marine Biotechnology Division screened locally available low-value fish for n-3 PUFAs, concentrating these essential fatty acids therefrom by chemical and/or enzymatic process.The aim was to develop an indigenous n-3 PUFA enriched formulation(s) comprising fatty acid concentrate and individual or combination of additives with potential antioxidant properties to form a stabilized and concentrated form of long chained n-3 PUFAs which may be a cheaper alternative to the imported PUFA supplements, and will be useful as nutraceuticals and in mariculture operations for fish larval nutrition.

It is believed that the optimal formulations for first-feeding fish larvae should simulate the yolk composition and to some extent reflect the nutrient requirements and metabolic capacities of the pre-feeding fish. Dietary long chain PUFAs play an important role as vital sources of essential fatty acids, needed for normal growth and suvival. Larvae of many marine fishes are believed to require highly unsaturated fatty acids of the n-3 series, such as, DHA due to the absence of essential enzymes required for biosynthesis of C_{20-22} LC-PUFAs from their short chain analogues (Figure 2). Some investigations have shown that DHA is superior to EPA for larval fish suggesting a different physiological function.

The important natural sources of n-3 LC-PUFAs are fishes such as, mackerel, sardines, sharks; microalgae, polychaetes, etc. Among these, sardines are inexpensively available and contains a considerable amount of PUFAs, particularly 20:5n-3. Hence sardine oil was preferred as the raw material to formulate the n-3 C_{20-22} LC-PUFA concentrates based

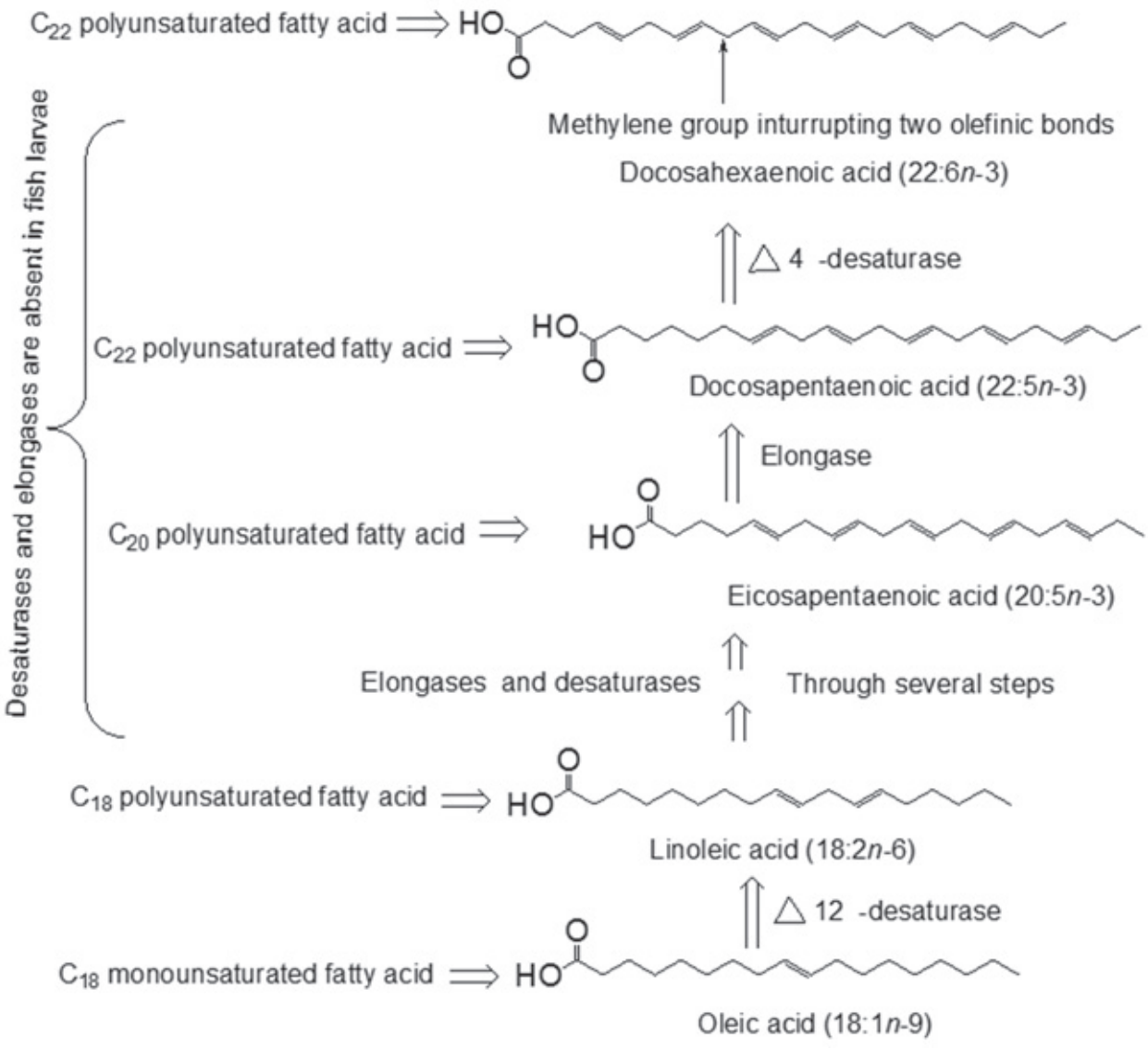


Figure 2. Biosynthetic pathwaysof the long chain polyunsaturated fatty acids (LC-PUFA)

on different physicochemical properties associated with the olefinic bonds in fatty acids and/or acyl chain length. The objective was to purify EPA and DHA from sardine oil by saponification of fish oil to derive free fatty acids, enrichment of PUFA content from the mixed fatty acid concentrate by amide fractionation and chromatography by utilizing the silica gel complexed with a d-block element.The unique substrate specificity of microbial triacylglycerol acyl hydrolases was utilized for the enhancement of PUFA content in triglycerides to further enrich the C_{20-22} LC PUFAs. Triacylglycerol acyl hydrolases specifically hydrolyse carboxyl esters of triglycerides into free fatty acids and partial acylglycerols. The unique characteristics of this group of enzymes such as positional and stereospecificity were utilized to selectively concentrate targeted fatty acids in triglycerides that can be readily absorbed into plasma triglycerides.

PUFA enrichment by different physicochemical procedures

The crude sardine oil was clarified by a sequential process of degumming, decolorization, and

deodorization, and was found to contain LC-PUFAs, particularly 20:5 n-3 or EPA (17.80 ± 1.57%) of total fatty acids, TFA) and 22:6 n-3 or DHA (7.67 ± 1.50% of TFA) along with other n-3 and n-6 PUFAs like linolenic acid (LA or 18:3 n-3; 4.47 ± 0.84% TFA), linoleic acid (18:2 n-6; 0.71 ± 0.23% TFA), and docosapentaenoic acid (DPA or 22:5 n-3; 1.14 ± 0.08% TFA) (Figure 4). The n-6 fatty acids have a minor share of the total fatty acid content of sardine oil (0.81% TFA). The PUFAs containing C_{18}-C_{22} acyl chain length contributed a major share of the total fatty acids of the sardine oil (>30% TFA). Among the saturated fatty acids (SFAs), 14:0 was found to be predominant (7.04 ± 0.22% TFA), while 16:1 n-7 contributed the major share (31.56 ± 2.59% TFA) among the monounsaturated fatty acids (MUFAs). When fatty acids are required in free form for further analyses, lipids were hydrolyzed in alkaline medium for extracting the unsaponifiable material. Sardine oil was saponified with NaOH/Na_2EDTA to yield free fatty acids. Na_2EDTA appeared to form complex with traces of metal ions (Cu, Fe), which catalyze oxidation of unsaturated fatty acids during saponification, and subsequently removed by extraction with water thus hindering the interferences of metal ions during the course of further purification process. Relatively large volumes of n-hexane were added to the aliquot of the salt of fatty acid mixtures for better phase separation, thus removing the unsaponificable materials. Among saturated fatty acids (SFAs), 14:0 was found to be predominant (7.04% TFA), while 16:1n-7 contributed the major share among all individual fatty acids in the crude sardine oil (>31% TFA). EPA and DHA were found to be the major n-3 PUFAs contributing to 17.8% and 7.67% of TFA, respectively. The n-6 fatty acids have minor share in the total fatty acid content of sardine oil. Solvent extraction resulted in marginal increase of unsaturation (0.85%) in the fatty acid profile. The PUFA exhibited an increase of 6.49%, while MUFA and SFA reduced by 2.96% and 6.91% respectively. The n-3 fatty acids exhibited an increase of 5.73% in the solvent extract of fatty acids.

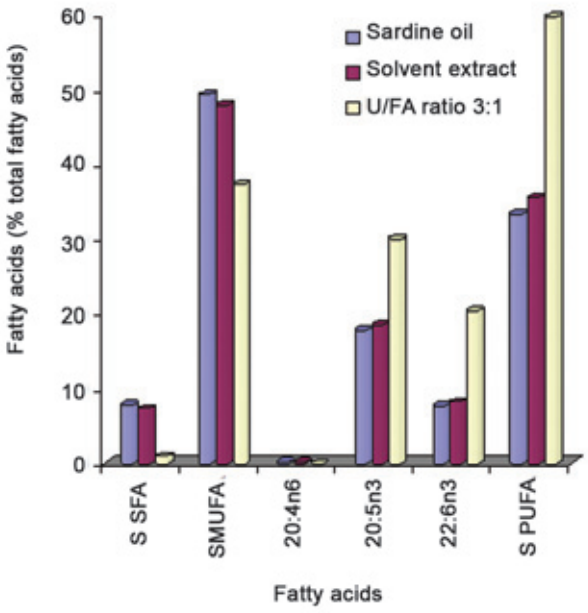


Figure 4. Fatty acid composition of crude and solvent extracted sardine oil, and PUFA concentrate obtained by the amide fractionation (U/FA: amide/fatty acid ratio)

The free fatty acids derived from sardine oil were subjected to amide fractionation using methanol as solvent at three different temperatures and urea-fatty acid ratios to obtain PUFAs of high purity. The interfering SFAs and most of the MUFAs were removed in the form of amide inclusion compound. Further, as oxidized products do not form amide adducts, the peroxidation of n-3 PUFAs could be avoided during the extraction of free acids from fish oil triglycerides. Amide fractionation resulted in total reduction of SFAs (>95%) (14:0, 16:0, and 17:0), moderate reduction of MUFAs (>65%). The U/FA ratio of 4:1 (w/w) was found to be optimal for getting high-purity EPA (47.8%) while that at 3:1 (at 2^0 C) yielded higher content of DHA (>20 %) when crystallized at 2^0C. Based on these results, DHA obtained from sardine oil at 2°C temperature of amide-crystallization by using a U/FA ratio of 3:1 was selected for subsequent purification of C22 fatty acid DHA. It is likely that at the lower temperature (2^0C), the reaction kinetics to form amide-inclusion complex with SFAs and MUFAs was relatively lower resulting in higher DHA in the extract.

Change in fatty acid composition as a function of microbial triacylglycerol acyl hydrolase-catalyzed hydrolysis of fatty acids from sardine oil

The free fatty acids were esterified by using a mixture of ethyl alcohol and 0.1 (N) H_2SO_4 resulting in the ethyl ester of the fatty acids. An extracellular triacylglycerol acyl hydrolase derived from *Bacillus subtilis* isolated from marine macroalga, *Turbinaria conoides*, was used to prepare C_{22} n-3 polyunsaturated fatty acid concentrates from the ester fraction. The enzyme was purified 132-fold with specific activity of 386 triacylglycerol acyl hydrolase units/mg. The urea fractionated fatty acyl esters were hydrolyzed with enzyme purified from the bacterium *Bacillus subtilis* and the TFA content of fatty acyl esters after lipase hydrolysis were analyzed. SFA levels showed a reduction to 0.05% after 3 hours (h) of hydrolysis. The decrease in the content of SFAs and MUFAs in the fatty acyl ester mixture with the progress of hydrolysis suggested that SFAs and MUFAs were more easily hydrolyzed by the lipase than those in esterified fatty acids that contain DHA, resulting in the enrichment of the latter in the ester fraction.

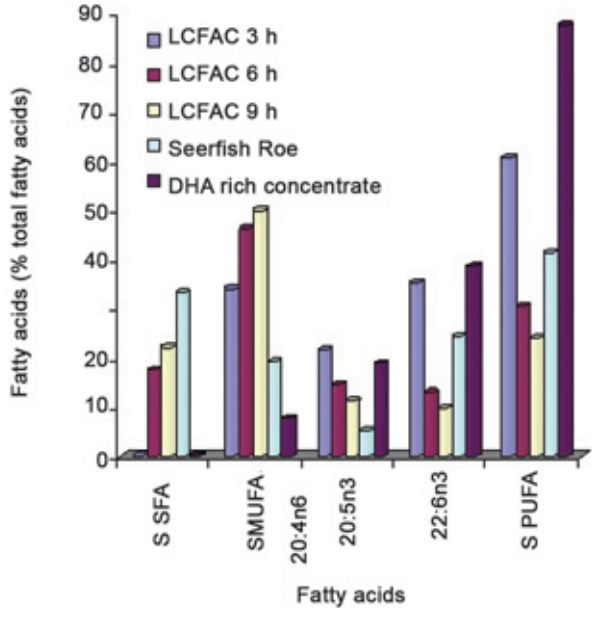


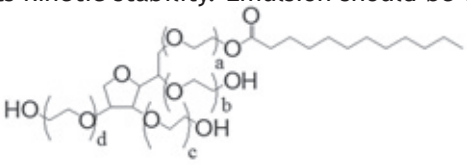
Figure 5. Fatty acid composition of urea concentrated fatty acids, triacylglycerol acyl hydrolase-catalyzed fatty acid concentrate, seerfish roe, and DHA rich concentrate

The variations of PUFA content of sardine oil triglycerides as a function of time during the microbial triacylglycerol acyl hydrolase-catalyzed hydrolysis are illustrated in Figure 5. The total DHA of fatty acyl ester fraction increased with time up to 3 h of enzyme-catalyzed hydrolysis (35.27% TFA), beyond which it slowly decreased (9.81% TFA after 9 h). The purified triacylglycerol acyl hydrolase was able to enrich DHA with 35.27% 22:6n-3 after 3 h of hydrolysis. The results also suggested that the esteritic bonds of C_{22} acyl chain lengthened n-3 PUFAs are resistant to hydrolysis by the lipase. However, after prolonged hydrolysis (>9 h), when only a few target fatty acid ester bonds (n-6 fatty acyl ester bonds and esters other than C_{22} n-3 fatty acids) were available in the enzyme hydrolysate that were susceptible to hydrolysis by the bacterial hydrolase, the microbial triacylglycerol acyl hydrolase could cleave bonds highly resistant to hydrolysis, i.e., DHA. It can therefore be concluded that it is possible to separate and concentrate C_{22} PUFAs with n-3 double bonds like DHA using lipase from seaweed associated bacteria like *Bacillus subtilis*.

Separation of phospholipid fraction from the seerfish roe and preparation of DHA rich oil emulsion was also done.The total lipids of seerfish roe were separated into different lipid classes by silicic acid column chromatography. The lipid fractions were qualitatively analyzed by Thin Layer Chromatography (TLC) for identifying triglycerides, glycolipids and phospholipid components. Fatty acid methyl esters (FAMEs) of the total lipid and the individual lipid classes were prepared by transesterification process. The DHA enriched fatty acid concentrate was enriched through biochemical and microbiological procedures to formulate enrichment emulsions which contained roughly, 90% DHA enriched fatty acid concentrate and 10% phospholipids fraction extracted from seer fish roe. The aggregate content of DHA in fish roe phospholipidic fraction was recorded as 24.3% DHA along with 5.2% EPA. The polyunsaturated fatty acid concentrate of the fish body oil after adding fish roe was found to contain greater than 35% DHA with significantly lesser content of saturated fatty acids (0.22%) and monounsaturated fatty acids (~7% of total fatty acids).

Use of emulsifiers to increase the stability of enriched PUFA emulsion

An emulsion is a mixture of two or more liquids that are normally immiscible and an emulsifier is a substance that stabilizes an emulsion by increasing its kinetic stability. Emulsion should be used when both the dispersed and the continuous phase are liquids. An experiment was conducted to understand the effect of emulsifier to stabilize the DHA concentrate. The emulsifier used was Tween 20 (or Polysorbate 20), which was able to contain the stability of the preparation for an extended time period (Fig.7). Polysorbate 20 is a polyoxyethylene derivative of sorbitan monolaurate, and is distinguished from the other members in the polysorbate range by the length of the polyoxyethylene chain and the fatty acid ester moiety (Figure 6). Longer chain TAGs such as polysorbate 20 are more hydrophobic and therefore have higher oil/water interfacial tension than shorter chain ones. The stability of the PUFA concentrate (as such without Polysorbate 20) decreased after 15 minutes whereas the same appended with Polysorbate 20 was able to maintain the stability for an extended period of time due to increased kinetics (increased Brownian movement)



Tween 20: Cn (a, b, c, and d) = a + b + c + d = 20

Figure 6. Polyoxyethylene derivative of sorbitan monolaurate (or Polysorbate 20)

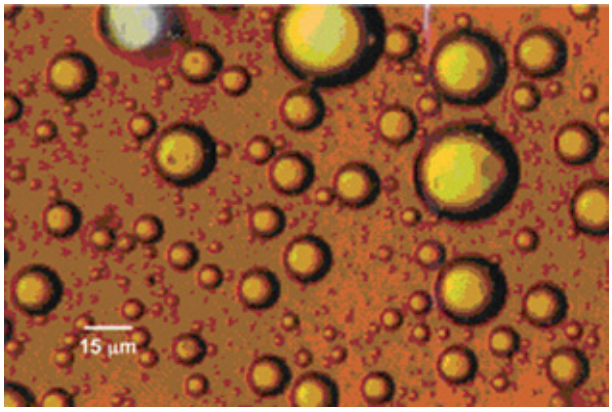


Figure 7. Photomicrograph of a water-in-oil emulsion by using Tween 20

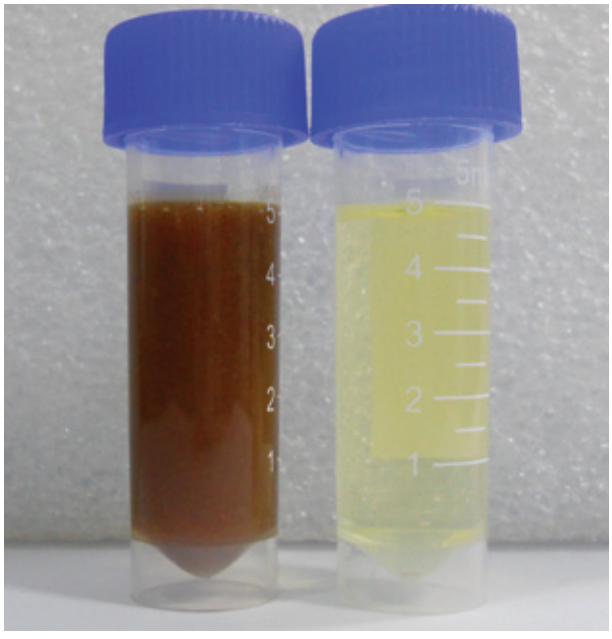


Figure 8. C20-22 n-3 polyunsaturated fatty acid concentrate prepared from sardine oil. (A) Crude sardine oil (B) PUFA concentrate

Conclusions

A fatty oil from the livers of various fishes (as cod, halibut or sharks) used chiefly as a source of fatty acids, vitamin A and also of vitamin D is called fish liver oil. Fish oil is short for "fish body oil". It is not the same thing as "cod liver oil" or other liver oils available in market. Cod liver oil contains greater concentrations of vitamin A. Taking cod liver oil in the same amounts that are recommended for fish oil can be toxic, and even more so in people who have chronic renal failure (because vitamin A can build up to toxic levels). The $C_{20\text{-}22}$ n-3 polyunsaturated fatty acid concentrate prepared from the inexpensively available marine sources, such as sardine oil can be a potential substitute of the imported PUFA supplements as functional food product.The PUFA enriched formulation (Figure 8) from low value "fish body oils" will also overcome the risks associated with hypervitaminosis (A, D) and exposure to environmental toxins (mercury, PCBs, dioxins etc.) associated with "liver oils" (cod liver oil) available in market.The indigenous n-3 polyunsaturatated fatty acids emulsion developed from the locally available low-value fish may also serve as a cheaper alternative to the imported fatty acid emulsions for use in fish larval nutrition during mariculture operations.

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Development of live feed enrichment product for marine fish larviculture

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ABSTRACT

Phytoplankton cultures of *Nanochloropsis oculata*, *Isochrysis galbana*, *Pavlova viridis* and *Dicrateria inornata*, were scaled up and analysed for fatty acids. *I. galbana* proved the richest source of docosahexaenoic acid (DHA). *P. viridis* and *D. inornata* are rich in eicosapentaenoic acid (EPA). *N. oculata* is rich in EPA and arachidonic acid (ARA). When rotifers were enriched with *I. galbana* and analysed for fatty acids at specific time intervals, DHA content increased till 30 h with a maximum DHA level of 1.13% obtained in enriched rotifers. Subsequently, development of enriched emulsions using sardine oil (90%) and fish roe (10%) as the major ingredients yielded a DHA content of 39% and EPA content of 17% and was used to enrich rotifers. The resultant enrichment level was 8.76% DHA and 2.35% EPA at six hours after which, a decline in the polyunsaturated fatty acid (PUFA) content was observed. The enrichment formulation holds promise as an import substitute.

Keywords: *Dicrateria inornata*, Enrichment, *Isochrysis galbana, Nanochloropsis oculata, Pavlova viridis,* Phytoplankton

Introduction

Live feed enrichment *per se* is an established practice in fish hatcheries keeping in mind the fact that the most popular zooplankton *Artemia* nauplii and rotifers are naturally deficient in polyunsaturated fatty acids (Sargent *et al.,* 1999). Most fish hatcheries follow a protocol of hatching in green water, followed by feeding with rotifers and *Artemia* nauplii. Co-feeding with artificial microdiets and then weaning on to a dry diet completes the hatchery phase. Protocols by and large remaining the same, variations are in the nutritional composition of the feeds provided either live, inert or bio-encapsulated. These variations reflect upon the ultimate larval survival which is the driver of the economic viability of the hatcheries and the availability of seed for growout of the fish species targeted. In such a situation, differences are noticed in the nutrient content of the primary producers, the phytoplankton (Ogata *et al.,* 2004) which in turn influences the content and ratios of fatty acids, the nutrients looked into critically. The work reported here is a compilation of the level of fatty acid enrichment posible with rotifers, the common phytoplankton used in the marine sector in south India. Results of an attempt to develop an indigenous enrichment emulsion and assess its propensity to enrich live feeds especially, rotifers and *Artemia* nauplii for use in the initial nutrition of marine fish larvae are presented.

Materials and methods

Phytoplankton culture and enrichment

Phytoplankton cultures of *Nanochloropsis oculata*, *Isochrysis galbana*, *Pavlova viridis* and *Dicrateria inornata*, obtained from the stock cultures maintained at the Central Marine Fisheries Research Institute, Cochin, were developed at a density of 1.2 x 10^6 cell ml^{-1} with a salinity of 30 ± 5‰, temperature, 28 ± 1 °C and pH 7.8 - 8.1 following standard protocols.

Initially, the levels of fatty acids in these were assessed along with baker's yeast (*Saccharomyces cerivisiae*) which is also a conventionally used medium to grow rotifers. Subsequently, rotifers were grown on *N. oculata* and *I. galbana* and sampled at intervals and then profiled for their fatty acid composition to ascertain the time taken and the maximum level of enrichment obtained. The nutritional enrichment experiments with rotifers were conducted in duplicate at a final volume of 10 l. Cultures of rotifers were maintained and fed with *Saccharomyces cerevisiae* (Baker's yeast) and constantly checked for water quality. Initial mean rotifer density at start of the (10 l) culture was

5×10^{-4} cells l^{-1}. Biomass was harvested at intervals as shown in Table 3, from each replicate with 20 µ filters, right from the start of the experiment. Initial microalgal cell densities inoculated were 1.2×10^{-6} cells l^{-1}. Samples for biochemical analysis were washed with double distilled water to remove salts, and stored at -20 °C until analysis.

Development of live feed enrichment emulsions and their evaluation

Sardine oil enriched through biochemical and microbiological procedures according to Kajal and Paul Raj (2009) and Kajal *et al*. (2010), were used to formulate enrichment emulsions which contained grossly 90% PUFA enriched fish oil and 10% lecithin extracted from roe of seer fish *Scomberomorus guttatus* based on the report of Sargent *et al*. (1999) that, fish roe and milt are the richest source of phospholipids having the most appropriate ratio of docosahexaenoic acid (DHA): eicosapentaenoic acid (EPA). Seer fish roe, sourced from the local fish market @ Rs.80 kg^{-1} was also profiled for its fatty acid composition. Other than the stability and emulsification ability imparted by lecithin, tocopherol acetate (vitamin E) at 0.5% was also included as a stabilizer. Two types of emulsions, -EPA rich and DHA rich were developed.

Fatty acid analysis

Total lipid in the samples was extracted by the cold extraction method using a chloroform-methanol mixture in a 2:1 ratio (Folch, *et al.,* 1957). After isolating the lipid phase, the solvent was evaporated. About 2 ml of sample was refluxed with 5 ml of 0.5 N alcoholic potassium hydroxide solution for 30 min and subsequently with 5 ml of BF_3-MeOH (Sigma-Aldrich) for another 5 min. The whole process of refluxing was carried out in an atmosphere of nitrogen. The dry fatty acid methyl esters in the flask were quantitatively extracted with petroleum ether (10 x 3). The extract was further washed twice with 25 ml of saturated sodium chloride and filtered over anhydrous sodium sulphate (10 g) to remove any moisture. The solvent was then evaporated under a stream of nitrogen gas. About 2.0 µl each of the prepared fatty acid methyl ester samples was injected into the Perkin Elmer Auto-System XL Gas chromatograph (Perkin Elmer, Waltham, MA, USA). The gas analyses was accomplished with a SP 2560 (crossbond 5% diphenyl – 95% dimethyl polsiloxane) capillary column (100 m x 0.25 mm i.d., 0.50 µm film thickness, Supelco, Belfonte, PA) using a flame ionization detector (FID) equipped with a split/splitless injector, which was used in the spit (1:15) mode. The oven temperature ramp programme: 140 °C for I min. rising at 30 °C min^{-1}. to 250 °C where it was held for 1.0 min. followed by an increase of 25 °C min^{-1} to 285 °C, where it was held for 2 min. until all peaks appeared. The injector and detector were held at 285 and 290 °C, respectively, The injection volume of 0.04 µl, FAMEs were identified by comparison of retention times with known standards (37 component FAME Mix, Supelco). Results were expressed as percentage weight of total fatty acids. Nitrogen (ultra high purity >99% at 25 cm s^{-1} flow rate) was used as the carrier gas with a pressure of 5.6×10^3 kg m^{-2}. The flow rate of hydrogen and air were maintained at a pressure of 3.5×10^4 kg m^{-2}. All the samples were injected in triplicate and the data acquisition was carried out with TOTAL-CHROME 6.X.X software (Perkin Elmer). The total run time per sample was set at 52 min.

Results and discussion

PUFAs derived from microalgae, *i.e*., DHA, EPA and arachidonic acid (ARA) are known to be essential for fish larvae (Langdon and Waldock, 1981; Sargent *et al.,* 1997). Most microalgal species have moderate to high percentages of EPA (7 to 34%). Prymnesiophytes (*eg. Pavlova* spp. and *Isochrysis* sp. and cryptomonads are relatively rich in DHA (0.2 to 11%), whereas eustigmatophytes (*Nannochloropsis* spp.) and diatoms have the highest percentages of ARA (0 to 4%). Chlorophytes (*Dunaliella* spp. and *Chlorella* spp.) are deficient in both C20 and C22 PUFAs, although some species have small amounts of EPA (up to 3.2%). Because of this PUFA deficiency, chlorophytes generally have low nutritional value and are not suitable as a single species diet (Brown *et al.,* 1997). An initial profiling of fatty acids (Table 1) was thus carried out to ascertain whether the classification of microalgae into DHA rich, EPA rich and ARA rich holds good for the cultures available from South India. Baker's yeast was deficient both in DHA and EPA. *N. oculata* was rich in EPA and ARA. *I. galbana* was rich in DHA and *P. viridis* and *D. inornata* were both rich in EPA which conforms to the report by Brown *et al*.(1997). Similar results have been reported previously (Lubzens *et al*., 1985; Olsen, 1990; Whyte and Nagata, 1990; Reitan *et al*., 1993).

Subsequently, rotifers grown on baker's yeast were starved for a day and enriched with *I. galbana* and *N. oculata* and profiled periodically as shown in Tables 2 and 3 respectively to ascertain the optimum time required to obtain maximum enrichment. Maximum PUFA enrichment was observed within six to twelve hours. Even though increases in individual fatty acids occurred up to 30 h, the overall trend was increase in saturated fatty acids (SFA) and decline in monounsaturated fatty acids (MUFA) and PUFA beyond 30 h. The DHA: EPA ratio congenial for fish larval growth is 2: 1 (Sargent *et al*., 1997; 1999) which is not attainable with *I. galbana* as the sole source of feed. As opined by Reitan *et al.* (1993), microalgae

Table 1. Fatty acid composition of baker's yeast (*Saccharomyces cerivisiae*) and microalgae (Total fatty acids %)

Fatty acids	*S. cerivisiae*	*N. occulata*	*I. galbana*	*P. viridis*	*D. inornata*
14:0	4.88 ± 0.04	5.23 ± 0.05	6.28 ± 0.10	11.11 ± 0.21	1.37 ± 0.02
15:0	0.80 ± 0.06	1.03 ± 0.06	0.79 ± 0.03	0.86 ± 0.01	0.61 ± 0.02
16:0	23.78 ± 0.42	21.80 ± 0.30	14.46 ± 0.06	19.29 ± 0.12	18.88 ± 0.04
18:0	3.61 ± 0.03	8.79 ± 0.04	5.89 ± 0.06	1.93 ± 0.01	0.09 ± 0.01
24:0	0.32 ± 0.06	0.55 ± 0.01	0.63 ± 0.01	1.15 ± 0.00	0.76 ± 0.01
ΣSFA	33.39 ± 0.24	37.39 ± 0.34	28.05 ± 0.25	34.32 ± 0.33	21.70 ± 0.01
16:1	15.13 ± 0.09	16.91 ± 0.04	3.53 ± 0.09	27.88 ± 0.04	0.58 ± 0.08
18:1	24.97 ± 0.17	19.54 ± 0.06	20.67 ± 0.59	7.35 ± 0.09	25.18 ± 0.28
24:1	0.87 ± 0.01	1.24 ± 0.01	0.34 ± 0.08	0.83 ± 0.05	0.35 ± 0.01
ΣMUFA	40.97 ± 0.09	37.69 ± 0.11	24.53 ± 0.57	36.05 ± 0.18	26.11 ± 0.35
Cis-18:2n6	12.37 ± 0.05	4.66 ± 0.04	8.30 ± 0.26	3.44 ± 0.06	6.33 ± 0.04
Cis-18:3n6	0.62 ± 0.04	0.38 ± 0.04	0.17 ± 0.02	1.06 ± 0.01	0.39 ± 0.01
18:3n3	3.56 ± 0.05	0.63 ± 0.06	5.74 ± 0.06	1.48 ± 0.01	12.82 ± 0.04
18:4n3	0.31 ± 0.08	0.13 ± 0.03	15.26 ± 0.08	2.86 ± 0.06	8.60 ± 0.08
C20:2n6	0.81 ± 0.06	0.84 ± 0.02	0.07 ± 0.02	0.22 ± 0.03	0.21 ± 0.02
C20:3n6	0.20 ± 0.02	0.51 ± 0.05	0.23 ± 0.05	0.17 ± 0.02	1.07 ± 0.01
C20:4n6	0.59 ± 0.02	2.17 ± 0.02	0.49 ± 0.01	1.34 ± 0.02	0.99 ± 0.01
20:5n3	0.34 ± 0.01	9.71 ± 0.02	2.63 ± 0.04	9.51 ± 0.05	6.81 ± 0.02
22:5n3	0.01 ± 0.01	0.14 ± 0.01	0.46 ± 0.03	0.16 ± 0.01	0.37 ± 0.02
22:6n3	0.05 ± 0.06	0.62 ± 0.03	9.76 ± 0.01	1.79 ± 0.04	1.26 ± 0.01
ΣPUFA	18.83 ± 0.30	19.76 ± 0.01	43.07 ± 0.28	21.99 ± 0.27	38.81 ± 0.09

Table 2. Fatty acid composition of rotifers enriched with *I. galbana* (Total fatty acids %)

Fatty acids	3h	6h	12h	30h	48h
14:0	6.39	6.62	6.94	11.09	15.34
15:0	0.93	0.98	1.06	2.39	2.76
16:0	23.02	22.18	23.52	25.71	27.86
18:0	3.15	3.82	3.95	5.36	7.49
24:0	0.19	0.21	0.32	0.5	1.24
ΣSFA	33.68	33.81	35.79	45.05	54.69
16:1	14.85	14.06	13.53	11.48	9.17
18:1	24.17	23.52	22.25	17.06	16.94
24:1	0.91	1.06	1.85	2.11	3.28
ΣMUFA	39.93	38.64	37.63	30.65	29.39
Cis-18:2n6	12.09	11.45	9.2	8.16	7.14
Cis-18:3n6	0.61	0.72	0.75	0.54	0.32
18:3n3	3.78	3.85	4.27	3.04	1.85
18:4n3	0.46	0.51	0.75	0.89	0.62
20:2n6	1.36	1.38	1.65	1.72	0.78
20:3n6	0.26	0.39	0.63	0.65	0.20
20:4n6	0.37	0.42	0.45	0.28	0.19
20:5n3	0.38	0.59	1.64	1.75	1.93
22:5n3	0.00	0.09	0.13	0.15	0.09
22:6n3	0.23	0.39	0.92	1.13	0.51
ΣPUFA	19.54	19.79	20.39	18.31	13.63

Table 3. Fatty acid composition of rotifers enriched with *N. oculata* (% Total fatty acids)

Fatty acids	3h	6h	12h	30h	48h
14:0	8.08	9.29	10.41	12.8	16.11
15:0	0.73	0.79	0.85	1.62	2.15
16:0	21.53	22.05	22.43	25.67	28.14
18:0	4.18	4.26	4.5	6.32	7.45
24:0	0.21	0.28	0.39	0.87	0.86
Σ SFA	34.73	36.67	38.58	47.28	54.71
16:1	14.52	13.69	10.25	10.03	9.37
18:1	24.86	23.14	18.08	19.37	16.75
24:1	1.13	1.58	2.16	2.37	2.91
Σ MUFA	40.51	38.41	30.49	31.77	29.03
Cis-18:2n6	11.57	11.39	14.58	12.06	7.26
Cis-18:3n6	0.58	0.62	0.66	0.68	0.38
18:3n3	3.05	2.62	3.48	1.03	1.24
18:4n3	0.38	0.42	0.58	0.58	0.43
20:2n6	0.82	1.28	1.26	0.64	0.31
20:3n6	0.18	0.2	0.28	0.31	0.13
20:4n6	0.52	0.56	0.84	0.91	0.82
20:5n3	0.97	1.28	2.51	2.58	2.63
22:5n3	0.00	0.06	0.09	0.11	0.12
22:6n3	0.12	0.11	0.15	0.15	0.05
Σ PUFA	18.19	18.54	24.43	19.05	13.37

commonly used in the hatcheries in general cannot meet the PUFA requirements of larval fish; however the extent to which PUFA enrichment is possible with *I. galbana* is not possible with other microalgae. In the preset study, we found DHA: EPA to be less than one with both *I. galbana* and *N. oculata*. The DHA content in *I. galbana* in our study was 9.76 % which was similar to that of *I. galbana* clone T-ISO reported by Ferreira *et al.* (2008). The most abundant PUFA was 18: 4 n-3 (Linolenic acid) followed by DHA as in the aforementioned report. DHA enrichment in rotifers with *I. galbana* increased to a level of 1.13% at 30 h beyond which decline was noticed. This was lower than the enrichment level obtained by Faulk and Holt (2005), which was 3.4 % for DHA for enrichment duration of 24 h.

The development of an indigenous fish oil based enrichment emulsion for live feeds was thus attempted. Crude sardine oil is available locally at a cost of Rs. 45 kg^{-1} ($1 kg^{-1}). Fish roe of quality fishes collected and sold by the fish vendors is available for Rs. 80 kg^{-1} (<$2 kg^{-1}). Fish oil was enriched as described earlier (Kajal and Paul Raj, 2009; Kajal *et al.,* 2010). Blended fish roe phospholipids (Table 4) were emulsified and stabilized with enriched sardine oil, to have two blends; one rich in DHA and another rich in EPA. The DHA rich emulsion, with a DHA content of 39% and EPA content of 19% was used to enrich rotifers, which resulted in an enrichment level of 8.76% DHA and 3.17% EPA in six hours after which decline was seen (Table 5). Enrichment levels of 27% DHA and 15% EPA is reported with a commercial product, High DHA Super Selco, INVE, SA, Belgium (Lie *et al.,* 1998). More than three to four fold higher level of enrichment reported by Lie *et al.* (1998) was not achievable with the emulsion developed in the present work. The ratio of DHA: EPA reported to be most appropriate (2:1) for marine fish could be obtained by enriching rotifers in the DHA rich emulsion (Sargent *et al.,* 1999). The ratio of DHA: EPA in rotifers after enrichment duration of 6 h was 2.76: 1 (Table 6). With another set of enrichment products Algamac 2000 and Algamac 3050 which are spray dried forms of a marine protist *Schizochytrium* rich in DHA (Faulk and Holt, 2005), DHA levels of 20% and 30% respectively is reported. This is because these products are spray dried powder (Algamac 2000) and flakes (Algamac 3050) with a content of 27% and 47% DHA respectively (www.aquafauna.com). Moreover, such high enrichment levels were achieved after growing the rotifers in *I. galbana* and then enriching them with these products.

Table 4. Fatty acid composition of seerfish roe phospholipids and triglycerides (% Total fatty acids, means ± SE)

Fatty acids	Phospholipids	Triglycerides
12:0	0.04 ± 0.01	1.70±0.06
14:0	1.80 ± 0.06	2.09±0.08
15:0	0.59 ± 0.10	0.87±0.03
17:0	1.10 ± 0.01	3.78±0.05
16:0	22.69 ± 0.57	19.87±0.29
18:0	7.34 ± 0.08	8.20±0.27
Total SFA	33.55 ± 0.35	36.50±0.56
16:1n7	3.43 ± 0.23	0.88±0.05
18:1n9	11.71 ± 0.90	14.07±0.02
17:1	0.40 ± 0.01	0.75±0.04
24:1	2.80 ± 0.11	2.88±0.16
Total MUFA	18.34 ± 1.25	18.57±0.27
18:2ω6	1.18 ± 0.03	1.29±0.01
18:3ω6	0.39 ± 0.01	0.56±0.06
18:3ω3	0.43 ± 0.05	0.41±0.02
GLA	0.57 ± 0.14	0.41±0.04
20:4ω6	6.09 ± 0.08	4.16±0.13
20:5ω3	5.17 ± 0.03	4.15±0.04
22:5ω3	3.28 ± 0.09	4.09±0.23
22:6ω3	24.02 ± 0.37	19.85±0.25
Total PUFA	41.11 ± 0.21	34.91±0.74

Table 5. Fatty acid composition of EPA rich and DHA rich oil emulsions (% Total fatty acids, means ± SE)

Fatty acids	EPA rich concentrate	DHA rich concentrate
SFA		
12:0	0.04 ± 0.01	0.03 ± 0.00
14:0	0.11 ± 0.01	0.03 ± 0.01
16:0	0.27 ± 0.04	0.14 ± 0.01
18:0	0.06 ± 0.01	0.03 ± 0.01
Σ SFA	0.47 ± 0.47	0.23 ± 0.01
MUFA		
16:1n7	11.3 ± 0.07	3.17 ± 0.03
18:1n9	1.17 ± 0.11	4.27 ± 0.02
17:1	0.15 ± 0.04	0.10 ± 0.03
20:1n11	0.12 ± 0.04	0.12 ± 0.02
Σ MUFA	12.73 ± 0.19	7.65 ± 0.01
PUFA		
18:2n6	2.26 ± 0.04	6.44 ± 0.21
18:3n3	8.19 ± 0.21	11.39 ± 0.02
18:4n3	1.31 ± 0.03	3.28 ± 0.13
20:4n6	0.14 ± 0.01	4.15 ± 0.05
20:5n3	47.70 ± 0.11	19.08 ± 0.17
22:5n3	2.88 ± 0.04	4.56 ± 0.11
22:6n3	17.11 ± 0.01	38.77 ± 0.17
Σ PUFA	79.57 ± 0.28	87.65 ± 0.18

Table 6. Fatty acid composition of rotifers enriched with DHA rich oil emulsion (% Total fatty acids)

Fatty acids	3h	6h	9h	21h	24h
14:0	3.98	3.20	4.50	5.60	5.82
15:0	1.38	0.43	3.20	3.69	4.11
16:0	18.96	16.42	22.04	22.24	25.09
18:0	9.64	6.18	7.12	7.50	8.10
24:0	0.92	0.69	2.11	2.23	1.89
Σ SFA	34.88	26.93	38.97	41.26	45.01
16:1	2.91	2.54	5.27	5.78	4.05
18:1	14.85	21.11	24.41	30.54	30.58
24:1	4.75	2.87	1.13	1.39	1.47
Σ MUFA	22.50	26.53	30.80	37.71	36.10
Cis-18:2n6	9.45	6.96	4.32	1.16	1.20
Cis-18:3n6	2.45	0.59	2.22	4.81	3.15
18:3n3	6.12	5.00	2.54	1.53	1.06
18:4n3	1.38	1.69	0.73	0.32	0.19
C20:2n6	0.46	0.17	1.31	2.45	2.34
C20:3n6	1.53	1.69	1.74	1.67	0.98
20:4n6ARA	6.12	9.84	8.93	4.53	4.28
20:5n3EPA	2.57	2.35	2.07	1.48	1.20
22:5n3	1.53	3.17	0.47	0.51	0.23
22:6n3DHA	4.29	8.76	1.43	1.23	1.14
Σ PUFA	35.90	40.21	25.76	19.70	15.77

With this product profile, our efforts to standardise live feed enrichment protocols to improve larval survival in marine ornamental fish (clowns and damsels) and food fish (*Rachycentron canadum*) are under way. The study proved that *I. galbana* has the most suitable fatty acid profile for marine fish larviculture in terms of DHA and EPA as reported from other parts of the world. Fish roe and fatty acid enriched sardine oil are good start material available locally to develop cost effective live feed enrichment products for domestic markets which can aid as a an import substitute. Refining the product to the extent of achieving the optimum fatty acid levels for translating into improved larval survival cycles and healthy fry for farming and grow out is the challenge ahead.

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