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Unraveling the effects of live microalgal enrichment on *Artemia* nauplii

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ABSTRACT

Artemia nauplii, though deficient in many essential nutrients, are used extensively in fish/shellfish larviculture. Enrichment using various diets can enhance their nutrient profile to the required level. The present study examines the effects of enrichment of *Artemia* nauplii with live microalgae *viz*., *Pavlova viridis, Isochrysis galbana, Nannochloropsis oculata* and *Dicrateria inornata*. Total length and width, survival percentage and the fatty acid profile of the microalgae enriched and unenriched nuaplii were estimated at 0, 1, 3, 5, 7 and 9 h time intervals. No significant increase in total length and width was observed between the enriched and unenriched *Artemia* nauplii during the study, indicating the absence of any enrichment diet induced growth rate of the nauplii. Salinity stress study revealed that the microalgae enriched nauplii can live long in low saline conditions than the unenriched nauplii. The total PUFA content of the live microalgae enriched nauplii reached maximum at 7 h post-enrichment followed by a significant drop after 9 h. The results of the study indicated that live microalgae can be used as excellent enrichment dietary sources for *Artemia* nauplii, which in turn can provide many of the vital nutrients essential for fish larviculture.

Keywords: *Artemia* nauplii, Fatty acid, Microalgal enrichment, Salinity stress, Survival rate

Introduction

Aquaculture is one of the fastest growing animal food producing sectors in the world, accounting for almost half of the total food fish supply (FAO, 2010). In the present scenario, aquaculture industry depends on hatchery produced larvae and fingerlings than wild caught seeds for farming. In marine hatchery production, availability of ideal starter diets for the larvae is the main concern. Most of the marine fish larvae have small mouth gape and hence the larval feed should be sufficiently small during the early phase of their development (Sargent *et al*., 1997). Among the different live feeds used in marine larviculture, *Artemia* nauplii have an important role to play. The convenience in storage of the cysts, easiness to hatch on demand and their soft texture make them quite essential in marine larviculture (Amat *et al*., 2005; 2007; Abatzopoulos *et al*., 2006; Beck and Turingan, 2007). To reduce the dependency on *Artemia* and to reduce the larval rearing expenses, sevral studies have been undertaken to use formulated diets as alternatives, but due to reduced preference/acceptability by the larvae, water quality deterioration *etc*. these diets did not get much acceptance in fish larviculture (Srivastava *et al*., 2006).

Artemia nauplii are not the natural prey for marine fish larvae and lack certain nutrients essential for the larvae. Among these, polyunsaturated fatty acids (PUFAs) *viz*., eicosapentaenoic acid (EPA, 20:5n3), docosahexaenoic acid (DHA, 22:6n3) and arachidonic acid (AA, 20:4n6) play major role in deciding the growth and survivability of the larvae (Sorgeloos *et al*., 1991). In nature, finfish and shellfish larvae have limited ability to synthesise the long chain PUFAs from shorter carbon chain precursors using the desaturase and elongase enzyme mediated pathway. These are to be supplied through diets because lack of these essential nutrients in the diet may adversely affect the physiological functioning, survival and growth of the larvae (Anger, 1998). Except *Artemia tibetiana*, almost all the *Artemia* species lack the long chain PUFAs, especially the 22:6n3 in nauplii (Narciso and Morais, 2001). So, it is necessary to meet the essential PUFA requirement, through enrichment of the these live feed organims. Commercial enrichment diets such as ALG, DHA Protein Selco, A1 Selco, Selco® (INVE Aquaculture, Belgium) *etc*. are widely used for this purpose (Sorgeloos *et al*., 1991; Biswas *et al*., 2006; Figueiredo *et al*., 2009; Chakraborty *et al*., 2010;). However, the high PUFA content in these enrichment diets produce harmful *trans* fatty acids when exposed to light, high temperature and air which may cause larval mortalities (McEvoy *et al*., 1995; Woollard and Indyk, 2003; Chakraborty *et al*., 2007).

As an alternative to the commercial enrichment diets, there is a growing interest in aquaculture industry to use marine microalgae as enrichment diet for the live feeds (Chakraborty *et al*., 2007). Microalgae make up the basis of food chain and serve as the renewable reservoir of PUFAs in nature (Ferreira *et al*., 2008). Studies on the fatty acid composition of many marine microalgae revealed that PUFAs present in them are much more stable than many commercially available enrichment diets (Volkman *et al*., 1989). The 22:6n3: 20:5n3: 20:4n6 ratios in live microalgal cells closely resemble natural larval diets and further, PUFAs in microalgae are better protected against oxidation by the natural antioxidants present in them. Earlier studies on microalgae revealed the importance of *Nannochloropsis, Chaetoceros* and *Chlorella* as suitable enrichment diets for live feeds (Vazhappilly and Chen, 1998). The nutritional value of microalgae can vary significantly depending on the species and their culture conditions. They are amenable to mass culture and biomass scale-up through photobioreactor and/or fermentation technology, and the nutrient profile of these organisms can be optimised to a great extent by manipulating the culture conditions (Martinez-Fernandez, 2006).

The major problems associated with *Artemia* nauplii enrichment are the incidence of naupliar mortality and rapid growth during enrichment. Though the enrichment process can increase the nutrient content of the *Artemia* nauplii, larger size prevents them from being ingested by the small mouthed fish larvae (Sorgeloos *et al*., 2001).

Studies on the viability of nauplii following enrichment in different media are scanty. Incidence of higher naupliar mortality before being ingested by the fish larvae remains another issue since dead nauplii are seldom preferred by fish larvae. According to Sastry (1983), larval survival rate is usually high under optimal environmental conditions, and gets reduced and finally stops when these conditions are not conducive. Since the optimum environmental conditions required for higher survival varies with the species of fish larvae, an enriched live feed which is able to withstand a wide range of environmental conditions will perform better. Most of the enrichment studies on *Artemia* nauplii evaluated the diet induced changes in the enriched nauplii, its nutrient content and growth rate (Narciso, 2000; Han *et al*., 2001; Ritar *et al*., 2004; Figueiredo *et al*., 2009). Investigations on salinity stress tolerance in microalgae enriched *Artemia* nauplii can unravel the role of exogenous diets in regulating the salinity induced mortality. However, studies to evaluate the salinity stress tolerance of *Artemia* nauplii after enrichment with microalgae are scanty.

The present study was aimed to evaluate the effect of the microalgae, *Pavlova viridis, Isochrysis galbana, Nannochloropsis oculata* and *Dicrateria inornata* on the survival percentage, nauplii length and width as well as fatty acid content of *Artemia* nauplii at different time intervals following enrichment and also to test the salinity stress tolerance of the microalgae enriched nauplii..

Materials and methods

Preparation of microalgal culture for enrichment

Microalgal cultures of *P. viridis, I. galbana, N. oculata* and *D. inornata* were obtained from the marine microalgal culture facility of Central Marine Fisheries Research Institute (CMFRI), India. Microalgae were cultured in Walne's medium (Walne, 1970) in Haffkine glass flasks (3 l) under circadian light: dark cycle (12 h: 12 h) with a light intensity of 2000 lx at a temperature of 24 ± 1 °C. Microalgae were inoculated from a stock culture (7×10^6 cells ml^{-1}) and grown up to stationary phase and were further mass cultured at similar culture conditions. Microalgal cell density was estimated using a Neubauer haemocytometer under a microscope (Leica, Wetzlar, Germany). The cultures were maintained for one week before being used as enrichment diet for *Artemia* nauplii and further biochemical analysis.

Artemia cyst hatching

Allochthonous *Artemia franciscana* cysts obtained from the Indian *Artemia* Reference facility of the Central Marine Fisheries Research Institute, India were used for the study. *A. franciscana* cysts (1 g l^{-1}) were incubated (24 h) in cylindrical culture flasks (5 l) holding autoclaved seawater (35‰ and 27 ± 1 °C) with strong bottom aeration and optimum light (2000 lx) for hatching (Sorgeloos, 1986). Freshly hatched *Artemia* nauplii were harvested and stocked in glass beakers (5 l) and the density was estimated from the subsamples (10 replicates of 1 ml subsamples).

Enrichment experiment

Enrichment experiment was carried out using the four species of microalgae *viz*., *P. viridis, I. galbana, N. oculata* and *D. inornata* as diet. *Artemia* metanuaplii were stocked (@50 nauplii ml^{-1}) in 15 cylindrical enrichment tanks (5 l), each having one of the microalgal diet *i.e*., *P. viridis, I. galbana, N. oculata* or *D. inornata* (in triplicates) at a density of 45±5 X 10^4 cells ml^{-1}along with control group without microalgal diet, in triplicate . Optimum temperature (23 ± 1°C) and strong bottom aeration was maintained in all the nauplii enrichment tanks.

Evaluation of survival rates of the enriched nauplii

Enrichment induced naupliar mortality were computed in all the enrichment tanks (microalgae and control group) at five different time intervals (0, 1, 3, 5, 7 and 9 h). Nauplii survival rates were computed following the subsampling method. The number of live and dead nauplii in 1 ml subsamples (20 times) in all the tanks was counted and the percentage survival was computed [Survival percentage = Number of live nauplii / (Number of live nauplii + Number of dead nauplii)*100]. Thereafter the live nauplii were restocked in the respective enrichment tanks.

Evaluation of nauplii length and width

The total length and width of the microalgae enriched (*P. viridis, I. galbana, N. oculata* and *D. inornata)* and unenriched nauplii (control groups) at 0, 1, 3, 5, 7 and 9 h were measured. In short, *Artemia* nauplii were sampled (200 individuals) from the top, middle and bottom of the enrichment containers at different time intervals and fixed in Lugol's iodine to arrest further growth. Total length (top of the head to the end of the caudal furca) and width of the nauplii were measured under a sterozoom microscope (Leica, Wetzlar, Germany) attached with digital camera (Leica, DFC 290) and image analysis software.

Evaluation of salinity stress tolerance of the enriched nauplii

Salinity stress tolerance of the *Artemia* nauplii were estimated at 9 h post-enrichment.. For this, water having salinity of 0, 5, 10, 20 and 30 ppt was prepared by adding double distilled freshwater to aged seawater (35 ppt). Microalgae enriched nauplii were transferred to cylindrical culture flasks (15 ± 4 nauplii ml^{-1}) containing autoclaved water of salinities 0, 5, 10, 20, 30 and 35 ppt. The nauplii cultures were maintained with continuous aeration at optimum temperature (23 ± 1°C) and light (2000 lx). Survival rates of the enriched nauplii at different time intervals (3, 6 and 12 h) were estimated by counting the live and dead nauplii from 1 ml subsamples (twenty replicates) and the mortality percentage was computed [Mortality % = (Total number of dead individuals/Total live individuals + Total dead individuals)*100]. The results were compared with that of unenriched nauplii.

Estimation of fatty acid content

Fatty acid content of the microalgal enrichment diets (*P. viridis, I. galbana, N. oculata* and *D. inornata*) as well as that of enriched and unenriched nauplii at different time intervals was estimated. The four microalgal enrichment diets used for the experiments were harvested by centrifugation (10,000 rpm for 5 min at 4 °C) at the middle of stationary phase and stored at -80 ºC until used for analysis prior to the experiment.

Microalgae enriched and unenriched nauplii samples (1.5 to 1.8 g in triplicate) from each treatment tanks were harvested at 0, 1,3,5,7 and 9 h time intervals during the experiment and rinsed with freshwater. The harvested nauplii were homogenised and the total lipid was extracted following Bligh and Dyer (1959). Fatty acid composition of all the samples were determined as per Metcalf *et al.* (1966). Triglycerides were extracted using $CHCl_3$/MeOH/H_2O (2:4:1, v/v/v), and saponified with alkaline reagent (3 ml, 0.5 N KOH/MeOH). The saponified materials were allowed to react 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 the removal of the aqueous layer, the *n*-hexane layer was passed through Na_2SO_4, concentrated in vacuum, reconstituted in petroleum ether (40-60 °C) and stored at -20 °C until analysis. The esterified fatty acid content of the samples was analysed by gas liquid chromatography (GLC) with FID detector using fatty acid methyl ester standard (Supelco FAME 37 standard).

The data were subjected to analysis of variance (ANOVA) and the means of all parameters were examined for significance ($p<0.05$) using the Duncan multiple range tests using SPSS programme 13.0 (SPSS Inc, Chicago, USA).

Results

Survival percentage of Artemia nauplii during enrichment

Survival rate of *Artemia* nauplii during the enrichment process showed variations at 3, 5, 7 and 9 h enrichments while no mortality was observed after the first one hour of enrichment. The overall mortality rate at 9 h post-enrichment was significantly high ($p<0.05$) in the unenriched nauplii group (5.2 ± 1.8%) when compared to the microalgae enriched nauplii (0.5 ± 1.5 to 3.2 ± 2.2%). Among the different microalgal diets, lowest mortality percentage was observed in *N. oculata* and *D. inornata* diets (0.5 ± 1.5 and 0.6 ± 1.8% respectively) while *P. viridis* enriched nauplii showed the highest mortality percentage (3.2 ± 2.2 %) (Table 1).

Table 1. Mortality percentage of *Artemia* nauplii in different enrichment diets at different time intervals

Time (h)	3	5	7	9
Control	2.3 ± 4.6^{ab}	4.1 ± 4.3^{a}	4.6 ± 1.6^{a}	5.2 ± 1.8^{a}
I. galbana	0.6 ± 1.8^{b}	0.6 ± 1.8^{b}	0.6 ± 1.8^{b}	1.6 ± 2.2^{b}
P. viridis	1.4 ± 3.0^{ab}	1.9 ± 1.4^{ab}	2.2 ± 1.0^{ab}	3.2 ± 2.2^{ab}
N. oculata	0.0^{b}	0.5 ± 1.5^{b}	0.5 ± 1.5^{b}	0.5 ± 1.5^{b}
D. inornata	0.0^{b}	0.6 ± 1.8^{b}	0.6 ± 1.8^{b}	0.6 ± 1.8^{b}

Values are represented as mean ± SD
Column-wise values with different superscripts indicate significant difference ($p<0.05$)

Effect of enrichment time on the total length and width of the nauplii

No significant difference in total length (TL) was observed between the microalgae (*P. viridis, I. galbana, N. oculata* and *D. inornata*) enriched and unenriched nauplii at different post-enrichment time intervals. Total length (TL) of the *Artemia* nauplii in all the experiments increased significantly ($p<0.05$) after 1st hour of enrichment (560.4 ± 75.6 to 582.1 ± 69.5 µm) (Table 2) . *I. galbana* enriched group had the smallest nauplii (600.2 ± 72.0 µm) when compared to the other microalgae enriched nauplii (628.3 ± 50.7 to 614.9 ± 47.3 µm) at 9 h post-enrichment.

Total width of the nauplii significantly reduced ($p<0.05$) at 9 h in all microalgae enriched groups as well as control group (142.5 ± 17.0 to 156.0 ± 20.1 µm) as compared to 0 h (164.6 ± 18.4 µm) (Table 3). No significant difference in nauplii width was apparent between the various microalgae enriched nauplii and unenriched nauplii at different time intervals.

Salinity stress tolerance of the enriched nauplii

No significant mortality percentage was observed after 3 and 6 h of incubation in 0, 5, 10, 20 and 35 ppt salinities in microalgae enriched and unenriched *Artemia* nauplii. At 12 h post-enrichment, nauplii mortality percentage significantly ($p<0.05$) increased in unenriched *Artemia* nauplii (21.1%) when compared to the microalgae enriched nauplii (6.22 to 11.76%) (Table 4). *P. viridis* enriched *Artemia* nauplii showed high salinity stress tolerance compared to the all other microalgae enriched nauplii (Table 4).

Effect of enrichment time on the fatty acid profile of the nauplii

Fatty acid profile of the microalgal diets showed notable differences in the levels of major fatty acids. Saturated fatty acids (SFAs) were found to be low in the microalgae *D. inornata* and *I. galbana* (23.81 and 28.95%, respectively) as compared to *P. viridis* and *N. oculata* (35.16 and 38.64 %, respectively) diets (Table 5a, 5b, 5c and Fig. 1). Impact of enrichment time and microalgal species on the fatty acid profile of the *Artemia* nauplii are illustrated in Table 5a and 5b. Except in *N. oculata* enriched nauplii, a gradual decline in SFAs was obvious in all the microalgae enriched nauplii up to 7 h post-enrichment. After 9th h of enrichment, the SFA content of the nauplii showed a significant ($p<0.05$) increase. Among the different SFAs, fatty acid 14:0 and 16:0 contributed the major share of the total SFA in the nauplii. Interestingly, the 9 h enriched nauplii showed significant positive correlation between the microalgal SFA and enriched nauplii SFA, while no correlation was observed in others. In the unenriched *Artemia* nauplii a gradual increase in total SFA was apparent with time (Table 5c).

The monounsaturated fatty acids (MUFAs) showed a significant decline in all the enriched nauplii when compared to the unenriched nauplii (42.4%). No correlation was observed between the MUFA content in the microalgal

Table 2. Total length (µm) of the *Artemia* nauplii in different enrichment diets at different time intervals

Time (h)	Control	*I. galbana*	*P. viridis*	*N. oculata*	*D. inornata*
0	516.6 ± 61.4^{a}	516.6 ± 61.4^{a}	516.6 ± 61.4^{a}	516.6 ± 61.4^{a}	516.6 ± 61.4^{a}
1	576.6 ± 76.9^{b}	560.4 ± 75.6^{a}	578.4 ± 96.3^{b}	582.1 ± 69.5^{b}	560.0 ± 68.4^{b}
3	593.2 ± 83.8^{b}	575.2 ± 94.6^{b}	609.0 ± 86.2^{bc}	600.0 ± 65.5^{b}	602.5 ± 59.3^{c}
5	614.3 ± 55.3^{b}	593.7 ± 71.8^{b}	612.2 ± 54.7^{bc}	604 ± 45.5^{b}	604.3 ± 40.0^{c}
7	617.7 ± 43.5^{b}	599.2 ± 48.7^{b}	617.7 ± 37.4^{bc}	606.9 ± 44.0^{b}	612.8 ± 40.3^{c}
9	618.6 ± 58.6^{b}	600.2 ± 72.0^{b}	628.3 ± 50.7^{c}	614.9 ± 47.3^{b}	623.1 ± 55.1^{c}

Values are represented as mean ± SD (n = 200)
Column-wise values with different superscripts indicate significant difference ($p<0.05$)

Table 3. Total width (µm) of the *Artemia* nauplii in different enrichment diets at different time intervals

Time (h)	Control	*I. galbana*	*P. viridis*	*N. oculata*	*D. inornata*
0	164.6 ± 18.4^{a}	164.6 ± 18.4^{a}	164.6 ± 18.4^{a}	164.6 ± 18.4^{a}	164.6 ± 18.4^{a}
1	176.8 ± 11.8^{b}	165.3 ± 22.1^{a}	158.3 ± 20.7^{a}	164.0 ± 16.9^{a}	159.6 ± 19.5^{a}
3	157.4 ± 17.5^{a}	157.8 ± 15.8^{a}	156.9 ± 14.0^{a}	153.9 ± 20.3^{ab}	165.8 ± 18.3^{a}
5	153.9 ± 17.3^{a}	155.2 ± 9.07^{a}	156.9 ± 13.3^{a}	147.0 ± 9.75^{b}	161.9 ± 19.1^{a}
7	162.1 ± 17.3^{ac}	161.0 ± 24.6^{a}	163.4 ± 14.9^{a}	151.2 ± 13.7^{ab}	153.9 ± 12.9^{a}
9	143.5 ± 12.7^{c}	151.9 ± 28.1^{a}	156.0 ± 20.1^{a}	142.5 ± 17.0^{b}	151.8 ± 23.0^{a}

Values are represented as mean ± SD (n = 200)
Column-wise values with different superscripts indicate significant difference ($p<0.05$)

Table 5a. Percentage fatty acid composition of microalgae (*P. viridis* and *D. inornata*) *vis-à-vis Artemia* nauplii enriched with microalgae for up to 9 h post-enrichment.

Fatty acids		*Artemia* nauplii enriched with *P. viridis*							*Artemia* nauplii enriched with *D. inornata*					
	P. viridis	0h	1h	3h	5h	7h	9 h	*D. inornata*	0h	1h	3h	5h	7h	9 h
Saturated fatty acids (SFAs)														
12:0	0.29	0.08	0.06	0.05	0.03	0.03	1.38	0.93	0.08	0.06	0.06	0.05	0.07	0.88
13:0	0.04	0.03	ND[a]	ND	ND	ND	0.27	0.62	0.03	0.03	0.01	ND	ND	0.25
14:0	11.25	3.53	3.49	3.38	2.74	2.65	5.09	1.35	3.53	3.26	2.59	3.15	3.8	6.92
15:0	0.86	1.39	1.34	1.22	0.41	0.38	0.85	0.59	1.39	1.28	0.57	0.52	0.48	1.24
16:0	19.37	19.3	19.2	18.7	18.3	17.6	22.5	18.91	19.3	19.1	18.6	18.2	18.2	23.4
17:0	0.14	1.52	1.48	1.35	1.28	1.19	1.53	0.10	1.52	1.38	1.29	1.25	0.51	0.84
18:0	1.92	5.1	5.04	4.85	4.24	4.18	7.27	0.48	5.1	5.29	5.64	5.87	4.55	5.06
20:0	0.11	0.04	ND	ND	ND	ND	0.08	0.08	0.04	0.29	0.55	0.73	0.56	0.82
22:0	0.03	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.08
24:0	1.15	0.12	0.09	0.06	0.05	0.03	0.15	0.75	0.12	0.64	1.07	1.38	0.98	1.21
ΣSFA	35.16	31.1	30.7	29.7	27.1	26	39.1	23.81	31.1	31.3	30.4	31.1	29.2	40.7
Monounsaturated fatty acids (MUFAs)														
14:1n9	0.38	0.03	ND	ND	0.03	0.05	0.06	0.23	0.03	0.03	ND	ND	ND	ND
16:1n7	27.9	13.6	13.5	12	12	10.1	9.32	0.64	13.6	12.6	11.5	8.82	7.5	7.19
16:1n9	0.52	0.06	0.04	ND	ND	ND	ND	8.16	0.06	0.05	0.03	0.03	0.03	0.03
18:1n7	1.64	0.12	0.06	0.03	0.03	ND	ND	0.13	0.12	0.1	0.08	0.06	0.05	0.05
18:1n9	7.41	28.5	28.4	27.5	26.3	24.6	21.5	23.57	28.5	27.7	25.1	24.1	22.5	22.1
24:0	0.86	0.08	0.05	0.03	ND	ND	ND	0.34	0.08	0.06	0.05	0.03	0.03	0.12
ΣMUFA	38.71	42.4	42.1	39.6	38.3	34.8	30.9	33.07	42.4	40.6	36.8	33.1	30.2	29.4
Polyunsaturated fatty acids (PUFAs)														
18:2n6 cis	3.48	7.52	8.2	8.61	8.74	8.92	8.82	6.3	7.52	9.83	10.4	10.6	10.8	8.56
18:3n6	1.05	1.18	1.29	1.67	2.24	2.68	0.53	0.39	1.18	1.35	1.42	1.54	1.68	1.19
18:4n6	0.72	0.03	0.09	0.16	0.22	0.62	0.46	0.14	0.03	ND	ND	ND	ND	ND
18:3n3	1.47	3.21	3.82	3.91	4.19	4.26	3.09	12.84	3.21	3.53	5.92	5.89	6.72	5.26
18:4n3	2.9	0.08	0.35	0.49	0.58	0.87	0.73	8.65	0.08	0.2	1.17	1.35	2.26	1.93
C20:2n6	0.24	0.15	0.19	0.21	0.16	0.25	0.18	0.19	0.15	0.23	0.45	0.57	0.65	0.41
C20:3n6	0.18	1.1	1.23	1.34	1.48	1.70	1.15	1.06	1.1	1.35	1.37	1.51	1.93	1.28
C20:4n6	1.86	1.35	1.14	1.62	2.07	2.31	1.82	0.98	1.35	1.42	1.61	1.73	1.93	0.85
20:5n3	9.54	3.08	3.26	3.79	4.15	7.58	4.12	6.82	3.08	3.12	3.19	4.18	5.98	3.06
22:5n3	0.16	0.02	0.04	0.06	0.09	0.48	0.32	0.38	0.02	ND	0.03	0.12	0.08	0.06
22:6n3	1.81	0.32	0.43	1.48	2.72	1.37	0.85	1.26	0.32	0.36	1.09	1.95	1.85	0.82
ΣPUFA	23.41	18	20	23.3	26.6	31	22.1	39.01	18	21.4	26.6	29.4	33.9	23.4
Σ n3	15.88	6.71	7.9	9.73	11.7	14.6	9.11	29.95	6.71	7.21	11.4	13.5	16.9	11.1
Σ n6	7.53	11.3	12.1	13.6	14.9	16.5	13	9.06	11.3	14.2	15.2	15.9	17	12.3
n3/n6	2.11	0.59	0.65	0.71	0.79	0.88	0.7	3.31	0.59	0.51	0.75	0.85	0.99	0.91
Σ PUFA/Σ SFA	0.67	0.58	0.65	0.79	0.98	1.19	0.56	1.64	0.58	0.68	0.88	0.94	1.16	0.58
DHA/EPA	0.19	0.10	0.13	0.39	0.65	0.18	0.21	0.18	0.10	0.12	0.34	0.47	0.31	0.27

The individual fatty acid is expressed as the percentage of total fatty acids.
[a]ND, fatty acid identified on GC trace but not integrated by the instrument.
ΣSFA : total SFAs; ΣMUFA; total MUFAs and ΣPUFA: total PUFAs

diet and the enriched nauplii. Total MUFA of the enriched nauplii is mainly contributed by 18:1n9 (60%) followed by 16:1n 7 (30%). Compared to SFA, MUFA content was low in all the enriched nauplii throughout the enrichment period except in *I. galbana* enriched nauplii at 9 h post-enrichment (41.7%). Among the microalgae sources evaluated, *I. galbana* (43.31%) showed the highest PUFA content followed by *D. inornata* (39.01%), *P. viridis* (23.41%) and *N. oculata* (19.81%). Variation in the total PUFA was clear in the microalgae enriched nauplii at different durations of enrichment (Table 5a and b). Total PUFA in the nauplii increased slightly up to 7 h post-enrichment in *I. galbana, P. viridis* and *D. inornata* diets (29.3, 31 and 33. 9% respectively) and at 9 h post-enrichment the PUFA was reduced considerably to 18.5, 22.1 and 23.4% respectively. But, the total PUFA of the *N. oculata* enriched nauplii reached a maximum at 5 h post-enrichment (27.6 %) and later showed a decline after the 7 (25.7%) and 9 h post-enrichment (12.9 %) (Table 5a and 5b).

Among the different PUFAs observed in the enriched nauplii, 18:2n6 cis, C20:4n6 and 20:5n3 contributed the major share irrespective of the specific microalgal diet used. 18:3n3 and 20:5n3 was high at 7th h of enrichment with *D. inornata* (6.72% and 5.98%), *I. galbana* (4.54 and 4.11%) and *P. viridis* (4.26 and 7.58%) while in *N. oculata* enriched nauplii, it reached the maximum level (3.69 and 7.81%) at 5th h of enrichment. However, 20:5n3 was high (7.81%) at 5 h post-enrichment in *N. oculata* enriched nauplii and was 7.58% in *P. viridis* enriched nauplii at 7th h post-enrichment. *N. oculata* enriched nauplii showed an incremental trend in 20:4n6 and 18:3n6 content up to the 5th h of enrichment (2.15 and 1.56% TFA, respectively),

Table 5b. Percentage fatty acid composition of microalgae (*N. oculata* and *I. galbana*) *vis-à-vis Artemia* nauplii enriched with microalgae for up to 9 h post-enrichment

Fatty acids		*Artemia* nauplii enriched with *N. oculaa*							*Artemia* nauplii enriched with *I. galbana*					
	N. oculata	0h	1h	3h	5h	7h	9 h	*I. galbana*	0h	1h	3h	5h	7h	9 h
Saturated fatty acids (SFAs)														
12:0	0.94	0.08	0.06	0.05	0.05	0.03	2.6	0.56	0.08	0.03	ND	ND	ND	0.13
13:0	0.29	0.03	ND	ND	ND	ND	0.12	0.08	0.03	ND	ND	ND	ND	0.05
14:0	5.19	3.53	3.32	3.2	3.15	3.2	5.14	6.35	3.53	3.41	3.34	3.35	2.68	5.14
15:0	1.07	1.39	1.33	1.2	1.18	1.73	2.16	0.81	1.39	1.36	1.27	1.23	0.91	1.73
16:0	21.59	19.3	19.2	19.1	18.8	20.6	27.2	14.5	19.3	19.2	18.3	18.1	17.8	26.2
17:0	0.09	1.52	1.48	1.48	1.42	1.36	3.94	0.03	1.52	1.5	1.48	1.47	1.25	1.86
18:0	8.76	5.1	4.47	4.51	4.43	3.65	9.84	5.93	5.1	5.06	4.89	4.74	4.5	6.38
20:0	0.11	0.04	0.05	0.03	ND	ND	0.63	0.05	0.04	ND	ND	ND	0.03	0.08
22:0	0.06	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
24:0	0.54	0.12	0.1	0.08	0.06	0.05	0.17	0.64	0.12	0.08	0.05	0.05	0.06	0.18
ΣSFA	38.64	31.1	30	29.7	29.1	30.7	51.8	28.95	31.1	30.7	29.3	28.9	27.2	41.7
Monounsaturated fatty acids (MUFAs)														
14:1n9	0.08	0.03	0.03	0.02	ND	ND	0.03	0.19	0.03	ND	ND	ND	0.05	ND
16:1n7	16.88	13.6	13.2	12.7	12	12	8.3	3.59	13.6	13.5	13.2	11.5	10.9	9.24
16:1n9	0.13	0.06	0.04	0.03	ND	ND	ND	0.15	0.06	0.04	0.04	0.03	ND	ND
18:1n7	0.14	0.12	0.09	0.06	0.04	0.04	0.11	0.08	0.12	0.08	0.05	0.05	0.03	0.03
18:1n9	19.5	28.5	27.9	27.3	25.1	24.3	21.2	20.25	28.5	28.3	28.2	26.1	23.6	21.6
17:01	0.05	ND	ND	0.08	0.05	0.03	0.07	ND	ND	ND	ND	ND	ND	ND
24:00	1.23	0.08	0.06	0.04	0.09	0.06	0.19	0.28	0.08	0.06	0.08	0.05	0.03	0.03
ΣMUFA	38.01	42.4	41.3	40.2	37.3	36.4	29.9	24.54	42.4	42	41.6	37.7	34.6	30.9
Polyunsaturated fatty acids (PUFAs)														
18:2n6 cis	4.63	7.52	7.69	8.13	8.75	8.83	4.15	8.48	7.52	8.14	9.09	10	10.6	6.27
18:3n6	0.35	1.18	1.23	1.38	1.56	1.43	0.64	0.15	1.18	1.15	1.21	1.39	1.45	0.51
18:4n6	0.04	0.03	ND	ND	0.05	0.02	ND	0.04	0.03	0	0.15	0.18	0.64	0.47
18:3n3	0.67	3.21	3.38	3.42	3.69	3.5	1.92	5.78	3.21	3.25	3.56	4.18	4.54	4.05
18:4n3	0.15	0.08	0.15	0.18	0.23	0.14	0.09	15.32	0.08	0.13	0.21	0.26	0.34	0.28
C20:2n6	0.82	0.15	0.21	0.29	0.36	0.25	0.18	0.08	0.15	0.18	0.21	0.2	0.39	0.25
C20:3n6	0.54	1.1	1.24	1.72	1.76	1.72	0.18	0.19	1.1	0.98	1.15	1.24	1.32	0.86
C20:4n6	2.15	1.35	1.42	1.86	2.15	1.72	1.51	0.48	1.35	1.42	1.59	1.68	2.21	1.39
20:5n3	9.69	3.08	4.87	5.09	7.81	6.93	3.63	2.60	3.08	3.27	3.58	4.05	4.11	3.03
22:5n3	0.13	0.02	ND	0.06	0.06	0.03	ND	0.44	0.02	ND	ND	0.03	0.05	ND
22:6n3	0.64	0.32	0.12	0.11	1.22	1.15	0.62	9.75	0.32	0.28	0.69	1.38	3.69	1.41
ΣPUFA	19.81	18	20.3	22.2	27.6	25.7	12.9	43.31	18	18.8	21.4	24.6	29.3	18.5
Σ n3	11.28	6.71	8.52	8.86	13	11.8	6.26	33.89	6.71	6.93	8.04	9.9	12.7	8.77
Σ n6	8.53	11.3	11.8	13.4	14.6	14	6.66	9.42	11.3	11.9	13.4	14.7	16.6	9.75
n3/n6	1.32	0.59	0.72	0.66	0.89	0.84	0.94	3.60	0.59	0.58	0.6	0.67	0.77	0.90
Σ PUFA/Σ SFA	0.51	0.58	0.68	0.75	0.95	0.84	0.25	1.50	0.58	0.61	0.73	0.85	1.08	0.44
DHA/EPA	0.07	0.1	0.02	0.02	0.16	0.17	0.17	3.75	0.1	0.09	0.19	0.34	0.9	0.47

while in all others it reached maximum after 7th h of enrichment. Total n3 PUFAs were high in *N. oculata* enriched *Artemia* nauplii which ranged from 8.52 (at 1 h post-enrichment) to 13% (at 5 h post-enrichment) (Fig. 1). Total PUFA in control gradually decreased with time and lowest percentage was observed at 9 h post-enrichment (Table 5c).

Table 5c. Percentage fatty acid composition of unenriched *Artemia* nauplii at different time intervals

Fatty acids	Incubation time (h)					
	0	1	3	5	7	9
Saturated fatty acids (SFAs)						
12:0	0.08	0.06	0.06	0.09	0.07	1.01
13:0	0.03	ND	ND	ND	ND	0.27
14:0	3.53	4.5	4.7	5.2	6.99	7.87
15:0	1.39	1.4	1.5	1.7	1.87	1.41
16:0	19.3	22	24.5	26.8	27.9	28.8
17:0	1.52	1.8	1.78	2.1	1.89	1.6
18:0	5.1	6	5.99	6.44	7.87	8.2
20:0	0.04	ND	ND	ND	ND	0.08
24:0	0.12	0.09	0.09	0.09	0.08	0.15
ΣSFA	31.11	35.85	38.62	42.42	46.67	49.39
Monounsaturated fatty acids (MUFAs)						
14:1n9	0.03	0.02	0.08	0.03	0.05	0.06
16:1n7	13.6	12.1	11	10.8	9.8	8.8
16:1n9	0.06	0.07	0.02	0.04	0.03	0.02
18:1n7	0.12	0.21	0.03	0.19	0.16	0.13
18:1n9	28.5	27.6	25.6	23.4	21.5	19.8
24:0	0.08	0.1	0.03	0.01	0.07	0.05
ΣMUFA	42.39	40.1	36.76	34.47	31.61	28.86
Polyunsaturated fatty acids (PUFAs)						
18:2n6 cis	7.52	7.5	7.3	6.89	6.01	4.38
18:3n6	1.18	1.29	1.67	1.54	1.14	0.99
18:4n6	0.03	0.09	0.16	0.22	0.62	0.02
18:3n3	3.21	3.11	2.98	2.55	2.01	1.98
18:4n3	0.08	0.12	0.14	0.1	0.2	0.34
C20:2n6	0.15	0.13	0.26	0.16	0.25	0.18
C20:3n6	1.1	1.23	1.01	0.98	0.88	1.15
C20:4n6	1.35	1.14	1.15	0.93	0.67	1.87
20:5n3	3.08	2.98	2.88	2.12	1.98	1.6
22:5n3	0.02	0.04	0.06	0.09	0.04	0.05
22:6n3	0.32	0.28	0.2	0.19	0.11	0.1
ΣPUFA	18.04	17.91	17.81	15.77	13.91	12.66
Σ n3	6.71	6.53	6.26	5.05	4.34	4.07
Σ n6	11.33	11.38	11.55	10.72	9.57	8.59
n3/n6	0.59	0.57	0.54	0.47	0.45	0.47
Σ PUFA/Σ SFA	0.58	0.50	0.46	0.37	0.30	0.26
DHA/EPA	0.10	0.09	0.07	0.09	0.06	0.06

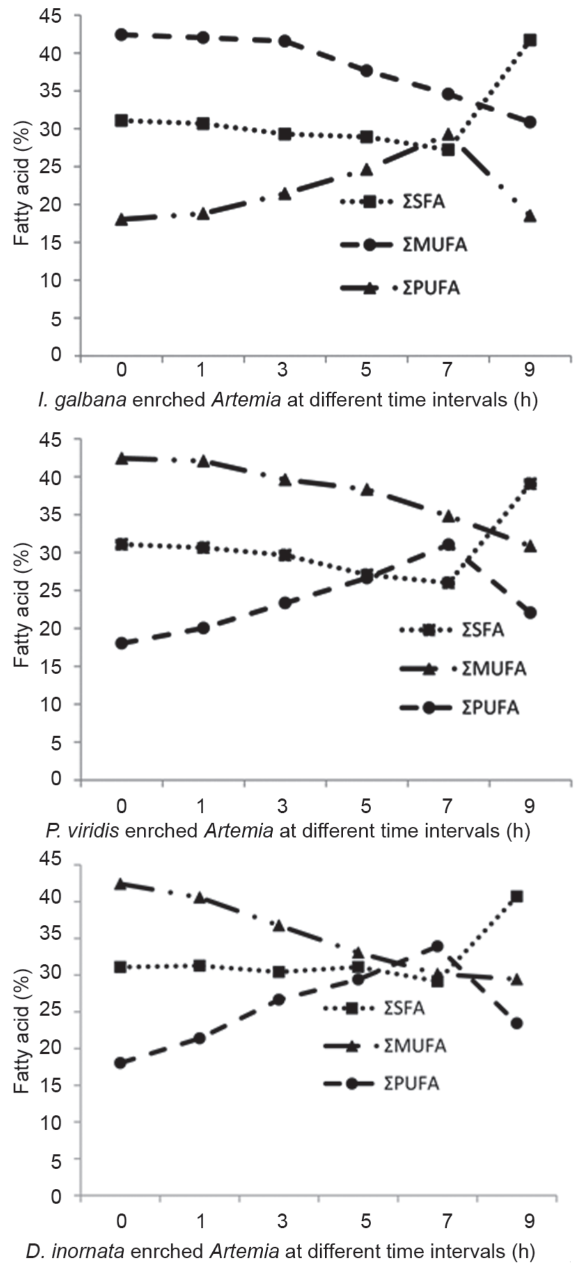

Fig. 1. The differential composition of ΣPUFA (total polyunsaturated fatty acid), ΣSFA (total monounsaturated fatty acid) and, ΣMUFA (total monounsaturated fatty acids) in *Artemia* nauplii enriched with microalgae at different time intervals

Discussion

Prey size has always been a key limiting factor influencing the feeding efficiency of the predatory larvae. Barros and Valenti (2003) suggested the most favorable relationship of prey size to predator length as 0.2, indicating the importance of small size prey in larviculture. In the present study, no significant increase in total length and width was apparent between the microalgae enriched and

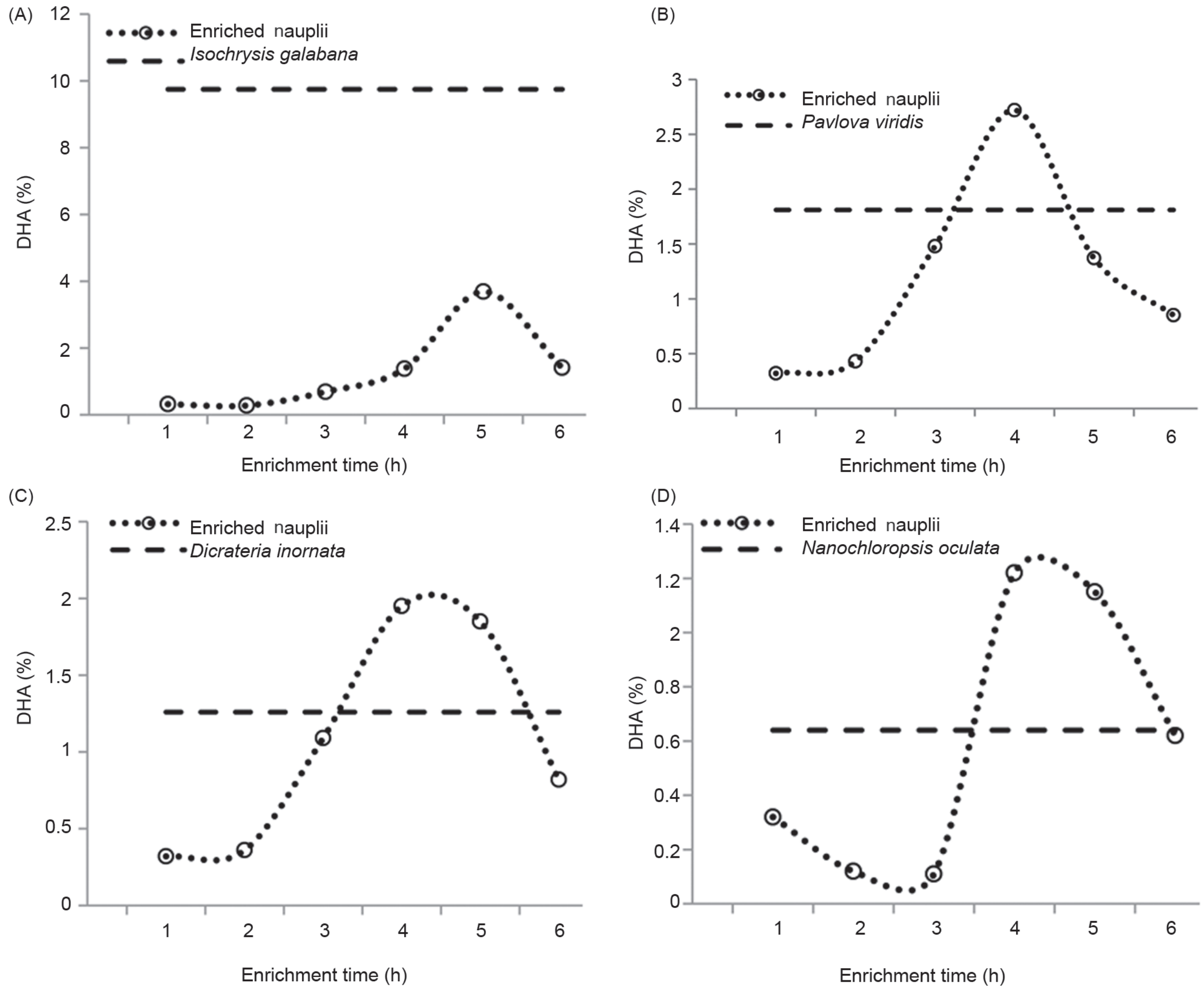

Fig. 2. 22:6n 3 content of the microalgal diets and the enriched *Artemia* nauplii

unenriched *Artemia* nauplii during the 9 h enrichment study, indicating the absence of any enrichment diet induced overgrowth in length and width of the nauplii.

One of the major drawbacks associated with the enrichment of *Artemia* nauplii using different diets is the reduced naupliar survival rates. Low mortality rate observed in the microalgae enriched nauplii when compared to the unenriched nauplii could be attributed to the presence of biomolecules such as betaine, inosine 5-monophosphate and free amino acids in the microalgal diet (Muller-feuga, 2000; Kolkovski *et al*., 1997). Figueiredo *et al*. (2009) while studying the enrichment of *Artemia* nauplii with Algamac 2000, observed that naupliar mortality increased with enrichment time and temperature and suggested that it could be due to elevated bacterial load and enhanced metabolism at higher temperatures.

Major environmental parameters which limit the development and survival of larvae in aquaculture include salinity, temperature, pH, *etc*., their specific requirement varying with the species reared (Paul and Paul, 1999). Among these, except in the case of euryhaline species, salinity stands out as the most important factor affecting the culture conditions. Superiority of a live feed in larviculture is primarily based on their versatility to acclimatise to sudden environmental changes when added to the larval rearing tanks.

The present observations show that nauplii enriched with microalgae have higher tolerance to variations in salinity especially at 0 ppt, indicating their better osmotic stress tolerance when compared to the unenriched nauplii. Fish larvae always prefer to ingest moving or swimming nauplii and the percentage of dead nauplii in larval culture tanks will result in reduced feeding and lead to water quality problems and disease outbreaks. Thus tolerance to varying salinity conditions is always an advantage while using nauplii as live feed in larval rearing. The very long-chain fatty acids (VLCFA) assimilated from the microalgae may have played a role in protecting the membrane integrity thereby raising the salinity tolerance while facing the osmotic changes associated with the various salinity regimes. Prasitchoke *et al*. (2007) observed that VLCFAs are important for membrane construction and stabilisation in eukaryotic cells. Though the commercial enrichment diets are also rich in VLCFAs, it has been observed earlier that the nauplii enriched with the commercial enrichment diets are already weakened/stressed which might have further reduced their salinity tolerance. The high salinity stress tolerance of the microalgae enriched nauplii can improve the feeding efficiency and the water quality conditions in the larval rearing systems, especially in low saline conditions.

Fatty acids, especially the polyunsaturated fatty acids (PUFAs) have a vital role in deciding the survival, growth and development of finfish larvae. The long chain PUFAs *viz.,* 20:5n3 and 20:4n6 are mostly involved in the production and modulation of eicosanoids (Brown, 1994), while the 22:6n3 function to keep the structural and functional integrity in larval cell membranes, including neural function (Bell *et al.,* 2003; Chakraborty *et al*., 2007). However, finfish larvae have a very limited ability to synthesise PUFAs in required quantity to meet their demand. Freshly hatched nauplii of *A. franciscana* showed high 20:5n3 (3.08%) with rather low 22:6n3 (0.32%) and 20:4n6 (1.35%), which is in agreement with the reports by several authors (Han *et al*., 2001; Narciso and Morais, 2001, Kara, *et al*., 2004). This demands the supplementation of essential fatty acids in the nauplii before feeding the larvae (Sargent *et al*., 2002; Bell *et al.,* 2003; Monroig *et al.,* 2006).

Though only small amounts of C_{20} fatty acids were present in *I. galbana* and *D. inornata* , the precursors for the biosynthesis of C_{20} PUFAs namely C_{18} PUFAs 18:2n6 cis and 18:4n3 were present in large amounts. Fatty acid content in the enriched nauplii varied as a function of microalgal dietary treatment and enrichment time. Essential fatty acids *viz.,* 20:5n3 and 22:6n3 increased with the progress of enrichment up to 5-7 h post-enrichment. Among all the microalgal dietary sources studied, *I. galbana* had high 22:6n3, C_{18} fatty acids and total PUFA followed by *D. inornata* and *P. viridis*. Nauplii enriched with *I. galbana* for 7 h had the highest 22:6n3 content (3.69%), followed by *P. viridis* (2.7%) and *D. inornata* (2%) after 5 h of enrichment (Fig. 2). The 22:6n3 content of *Artemia* nauplii during the present study was far higher than that of *Artemia* nauplii enriched with a mixture of *Nannochloropsis salina, Chaetoceros calcitrans* and *Chlorella salina* (Chakraborty *et al*., 2007). Figueiredo *et al*. (2009) reported 6.45% of 22:6n3 in *Artemia* nauplii enriched with ALG which has a DHA content of 27% showing an assimilation of 23.88%. The present study shows that *Artemia* nauplii enriched with live microalgae with an average DHA content of 3.37% has succeeded in assimilating an average 2.01% of 22:6n3, indicating a far higher percentage (59.88%) of 22:6n3 assimilation.

The results of the present study shows that among the microalgae studied, *N. oculata* had the highest 20:5n3 content. *N. oculata* and *I. galbana* enriched nauplii yielded notable 20:5n3 at 5th (7.81%) and 7th h (7.58%) respectively. This was found to be higher than the baker's yeast and microalgae enriched *Artemia salina* nauplii (4.2%) and *Odonus niger* liver oil enriched *A. franciscana* nauplii (2.5 to 5.1%) suggesting their superiority as enrichment diets (Chakraborty *et al*., 2007; Immanuel *et al*., 2007). The drop in 20:5n3 after the 7th h of enrichment may be due to inadequate rate of desaturation (by Δ5-fatty acid desaturase) and elongation of 18:3n3 or 18:4n3 (Chakraborty *et al*., 2007). The higher 20:5n3 (2.6%) and 22:6n3 (9.75%) content observed in *I. galbana* enriched nauplii points out their ability to effectively amass enough PUFAs from the microalgae.

The total PUFA content of the live microalgae enriched nauplii reached maximum by the 7th h of enrichment (except in *N. oculata* enriched nauplii) followed by a significant drop after the 9th h. Except in *D. inornata,* 18:3n"3 contributed a very low percent of the total PUFA in all the microalgae (3.38 and 13.34%) suggesting the nutritional superiority of live microalgae as an enrichment diet. Low linolenic acid (18:3n"3) in enrichment diets can increase the bioconversion of 20:5n3 to 22:6n3 (Buzzi *et al*., 1996). This study has proved that short time enrichment with *N. oculata* (5 h) can improve the PUFA profile of the nauplii while a minimum of 7 h is required for enrichment with *I. galbana, D. inornata* and *P. viridis* .

Selected live microalgae or their combinations can be used as excellent enrichment dietary sources for *Artemia* nauplii, which in turn can provide many of the vital nutrients essential for the fish larvae in larviculture. The present study reveals that 5 to 7 h of enrichment with live microalgal diets can significantly improve the essential PUFA content while keeping the nauplii size at minimum. The high survival rate of the live algae enriched nauplii will enhance feeding of the fish larvae and reduce the deterioration of water quality in rearing tanks. Further, the high salinity stress tolerance observed in the microalgae enriched nauplii makes them suitable live feed for a variety of fish species under different salinity regimes.

Present study explored the suitability of using live microalgae as enrichment diets for *Artemia* nauplii. Microalgae were found to be superior enrichment dietary sources for *Artemia* nauplii due to their higher survival rate, nutritional content and salinity tolerance when compared to the commercial enrichment diets. Further, it was observed that short term enrichment of *Artemia* nauplii with live microalgae can enhance the nutritive value of the nauplii while keeping their size at minimum.

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