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Laboratory Culture and Population Growth of Brackish water Harpacticoid Copepod, *Nitokra affinis* (Gurney, 1927) under Different Temperatures, Salinities and Diets

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Abstract: The present work pertains to study the effects of temperature, salinity and diet on population growth of brackish water harpacticoid copepod, *Nitokra affinis*. The highest population growth recorded in our study at 30±1°C was significantly higher (P<0.001) than the rest of the temperature treatments tested except at 25±1°C, (P>0.05). The population growth observed in 35±1 ppt salinity showed higher when compared to other different salinities (P<0.001). In different monoalgal diets, the highest population recorded in *Chlorella marina* was significantly superior to the other monoalgae tested (P<0.001). In regard to formulated diets, cow dung (juice form) showed the better production when compared to carrot juice (P<0.001) and fish extract (juice form) (P>0.05). The present study suggested that *Nitokra affinis* could be a potential copepod for mass cultured at a temperature of 30°C, salinity of 35 ppt and by fed with *Chlorella marina* and cow dung (juice) appropriately.

Key words: *Nitokra affinis* · Harpacticoid · Monoalgal · *Chlorella marina* · Cow Dung

INTRODUCTION

Intensive marine finfish larviculture largely depends on the regular availability of rotifer and *Artemia* as live feeds. However, a range of studies have shown that both live feeds have inadequate nutritional profiles especially long chain fatty acids that are essential for larval development [1-5]. In aquaculture, copepods are the most preferable feed for marine fish larvae [6, 7]. The marine copepods are the commercially cultivable species rich in protein, lipid (especially HUFA, 20:5 *n*-3 and 22:6 *n*-3), carbohydrates and enzymes and hence they are referred to be "nutritionally superior live feed" in aquaculture [8-10]. Copepods are naturally exposed to fluctuation in temperature, salinity, food quality and their developmental stages were affected especially in estuarine habitats [11]. The successful culture systems mainly depends upon environmental parameters especially temperature, salinity and diet which influences the population density of marine copepods (wild). Temperature has been shown to regulate the growth and reproductive potential of marine copepods [5, 12-17]. Harpacticoid copepods are emerging to be well-suited food organisms for higher marine fauna as they can able to survive at a broad range of temperature and salinit y regimes [18-20]. Species of flatfish, gobies, salmonids, ciaenids and blennies requires harpacticoid copepod as live feed during their early life cycle [21]. Several works have declared that harpacticoid copepods particularly; species of the genera *Tisbe* or *Tigriopus* were the ideal candidates for large-scale culture [19, 22]. Some experiments showed good improvement in the growth and survival of fish larvae using harpacticoid copepods as a live feed [18, 23]. Food quality and quantity are also most important factors, which affect the growth and fecundity of copepods [5, 24]. Copepods require n-3 HUFA essential fatty acids for egg production [25] especially, the highly unsaturated fatty acids (HUFA) such as eicosapentaenoic acid, 20: 5n-3 (EPA) and docosahexaenoic acid, 22:6n-3 (DHA) which is the major essential nutrients needed for the harpacticoid copepods [26-28]. Algal feeds are necessary for the copepods, which allow sustained and maximum biomass for the production in aquaculture [29, 30]. A mixture of algae and bacteria are also considered one of the superior diets for

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the growth of marine harpacticoid copepods. A mixed diet (algae and inert feed) may provide the best nutritional value for the copepods [31]. Drillet *et al*. [32] have recommended harpacticoid copepods in industries to yield large mass, which would form better live feed in aquaculture. However, in India such kind of study is so meagre particularly on harpacticoid copepods. The present study examined the laboratory culture and population growth of brackish water harpacticoid copepod, *Nitokra affinis* under different temperatures, salinity and diets.

MATERIALS AND METHODS

Algal Culture: Pure strains of the marine microalgae *Isochrysis galbana (Prymnesiophyceae), Nannochloropsis salina (Chlorophyceae)*, *Chlorella marina (Chlorophyceae)* and freshwater alga *Botryococcus* sp. *(Botryococcaceae)* were obtained from the Central Institute of Brackish water Aquaculture (CIBA), Chennai. They were grown at 25 °C temperature, 30 ppt salinity and 12 L: 12 D light regimes and fertilized with Conway's medium. The algae were harvested during the log phase (approx. 30,000 cells/ ml) for feeding the copepods.

Copepod Culture (*Nitokra affinis*): Zooplankton samples were collected during early morning of full-moon time, using 158 µm plankton mesh (0.35m mouth diameter) from the Vellar estuary (Lat.11°29' Nand Long. 79°46' E). The collected samples were immediately transported to the laboratory and vigorously aerated using aerator and thoroughly rinsed to reduce contamination of other zooplankter. Then the zooplankter was screened to isolate the size fractions containing predominantly adult copepods and later-stage copepodites of *Nitokra affinis.* This was achieved by a first crude screening through a 500-µm mesh to eliminate the fish and prawn larvae. Then the samples were rinsed for 2hr in a zooplankton washer [33] fitted with a 190-µm mesh screen to remove rotifers and nauplii of copepods and barnacles. After rinsing, the remaining adult copepods and larger copepodites were used to start the culture. A stock culture of *Nitokra affinis* was maintained in a rectangular, flat-bottomed fibreglass tank (550 mm dia., 850 mm height) filled with 100 litre- filtered seawater with vigorous aeration. Rearing containers were covered with nylon cloth to prevent excessive evaporation. Seawater was filtered through a membrane filter (pore size 100µm). Contamination of the rearing tank was reduced by daily water changes. Temperature (°C), salinity (ppt), pH and dissolved oxygen (ml L^{-1}) were maintained in the ranges of: 28-32, 30-34, 7-8.5 and 5-6.8 respectively. During culture, the copepods were fed with equal quantities of *Isochrysis galbana, Nannochloropsis salina* and *Chlorella marina* in the ratio of 3:3:3 (30,000: 30,000: 30,000 cells/ ml of each algal species). The stock cultures were harvested every 13th day by gentle siphoning and the components of the culture tank were transferred to a zooplankton washer and then rinsed for 1h with seawater (from the reservoir). Adult and copepodites collected in the zooplankton washer were used to restart the stock culture.

Experimental design and Setup

Temperature Experiments: Five different temperature treatments *viz.* 15±1, 20±1, 25±1, 30±1 and 35±1 °C were made for population density with triplicates for each treatment. Great care was taken during the experiment to ensure the temperature maintenance by using air conditioner and water baths. For population experiment, initially 20 healthy gravid females were inoculated in 1000 ml glass bowl. At the beginning of the experiments, adults of *Nitokra affinis* were siphoned from the stock cultures onto a 158µm sieve and were then placed in a Petri dish with a small amount of seawater. Individuals were randomly captured using a fine-tipped pipette and were then transferred to the experimental bowl. Throughout the experimental period, *Nitokra affinis* was fed daily with mixed algal diet of *I. galbana, N. salina* and *C. marina* in a ratio of 3:3:3 daily with gentle aeration provided to each bowl. The photoperiod was 12:12h light: dark cycle with light intensity at 1000 Lx and salinity at 35±1 ppt. 50-75% water was exchanged daily using a siphon (38µm mesh) attached to the end and new seawater that had been pre-adjusted to desired temperatures. The population experiment lasted for 8 days, after which all the contents of each triplicate bottle was drained onto a 38µm mesh and the number of nauplii, copepodites and adults retained on the mesh were counted and recorded. All the counts were made using a Sedgewick - Rafter counter under binocular microscope (Olympus CH20i).

Salinity Experiments: Five salinity treatments, i.e., 15±1, 20±1, 30±1, 35±1 and 45±1 ppt were made for population density experiments. The copepods used in these experiments were gradually acclimatized to various salinities by changing salinity at the rate of 2.5 ppt every 12h until the required salinity level was reached.

The experimental procedure followed was similar to temperature treatment experiments. The temperature was maintained constant at 30±1 °C for salinity treatments.

Diet Experiments: Seven different diets were used to estimate the population growth of *Nitokra affinis* for 12 days. For all seven experiments, 4-Monoalgal and 3-Formulated diets were used.

Monoalgal Diets:
Feed 1: *Chlorella marina*
Feed 2: *Isochrysis galbana*
Feed 3: *Nannochloropsis salina*
Feed 4: *Botryococcus* sp. (freshwater alga)
Formulated diets
Feed 5: Cow dung (juice form)
Feed 6: Fish extract (juice form)
Feed 7: Carrot juice

The experiments were carried out in similar condition as described for the stock culture (i.e., 30±1 °C; 35±1 ppt). Throughout the experimental period, *Nitokra affinis* cultures were fed with three different monoalgal and one freshwater alga separately in a ratio of 30,000 cells/ml and three formulated feeds in the form of juice separately for a period of 12 days.

Population Growth Experiments: For population experiments, an initial number of 20 healthy gravid females were inoculated in 1000ml glass bowl. At the beginning of experiments, adult *Nitokra affinis* from the stock culture was siphoned onto a 158µm sieve and then placed in a Petri dish with 2 ml of seawater. Individuals were randomly captured using a fine-tipped pipette and transferred to the experimental bowl. Throughout the experimental period, *Nitokra affinis* was fed with designed diet daily. The experiment lasted for 12 days. After 12 days, each triplicate bottle was drained onto a 38µm mesh and the number of nauplii, copepodites and adults (retained on the mesh) were counted and recorded. All counts were made using a Sedgewick-rafter chamber using binocular microscope (Olympus CH20i).

Statistical Analysis: Data for population density of *Nitokra affinis* from different temperature, salinity and diets were analyzed using one-way ANOVA. If significant differences ($P<0.05$) were found, Tukey's multiple comparisons test was used to determine specific difference among treatments. Data are presented as Mean±SE.

RESULTS AND DISCUSSION

Effect of Temperature on Population Growth of *Nitokra affinis*: The result showed that temperature had a significant effect on the population growth of *Nitokra affinis* ($P<0.001$) (Fig. 1). The highest recorded mean population was at 30±1°C (2190.28±6.41 org.l^{-1}), which was significantly higher ($P<0.001$) than all the other temperature regimes except at 25±1°C (2105.52±71 org.l^{-1}), though they were not differed significantly ($P>0.05$), while 25±1°C was significantly higher ($P<0.001$) than 15±1°C (1126.33±28.70), 20±1°C, (1623.23±47.76) and 35±1°C (1462.99±42.02) treatments respectively. There was no significant difference found in population among the temperature treatment 20±1 °C and 35±1°C ($P>0.05$) practiced. The lowest mean total population was recorded at 15±1°C (1126.33±28.70) which was significantly lower when compared to 20±1°C ($P<0.001$), 25±1 °C ($P<0.001$), 30±1°C ($P<0.001$) and 35±1°C ($P<0.01$). The mean total population decreased when the temperature rose above 30±1°C as the population suddenly declined 1/3 after the temperature rose to 35±1 °C (Fig. 1). The distribution of various life-stages, i.e. nauplii, copepodites and adults of *Nitokra affinis* cultured at different temperatures are shown in Fig. 2. At all the temperatures, high number of the population was comprised by nauplii, followed by copepodites and adults (Fig. 2).

Estuarine copepods adapt themselves to salinity and temperature at various life stages and survival capacity vary within several species [34]. Harpacticoid copepods are naturally adapted to rapid changes in temperature and salinity [19] and also have higher productivity than calanoids and cyclopoids. In our findings, the *Nitokra affinis* a tropical estuarine species, naturally adapts to various environmental conditions and 30±1 °C was found to be the optimum condition for the maximum production. Our results were similar to the findings of Milione and Zeng [35], who have reported that maximum population of *Acartia sinjiensis* (calanoid copepod) was observed at warm temperature (25-30°C). Zaleha and Jamaludin [36] have also found that 25±1 °C was the optimum condition for the maximum production of a tropical *Pararobertsonia* sp. under laboratory condition. Rhyne [17], recently reported in *Pseudodiaptomus pelagicus* (calanoid copepod), 26-30°C was the optimal temperature to achieve high survival and nauplii production. Nagaraj [37] found that there were increase in copepodites and nauplii production with the increase in temperature but there was shortage in population at 20 °C. Chinnery and Williams [38] have reported that production of nauplii was higher

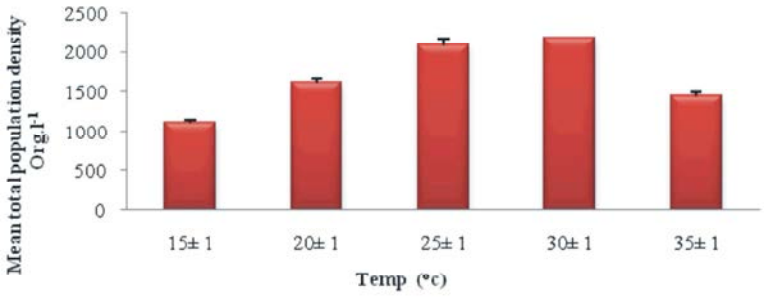

Fig. 1: Total mean population density (±SE) of *Nitokra affinis* cultured for 8 days at different temperatures (P<0.05)

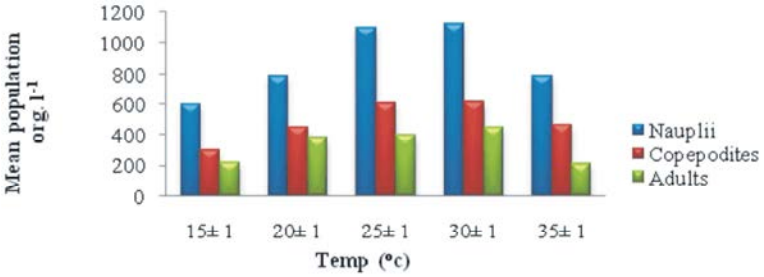

Fig. 2: Mean populations of three life-stages (nauplii, copepodites and adults) of *Nitokra affinis* cultured for 8 days at different temperatures

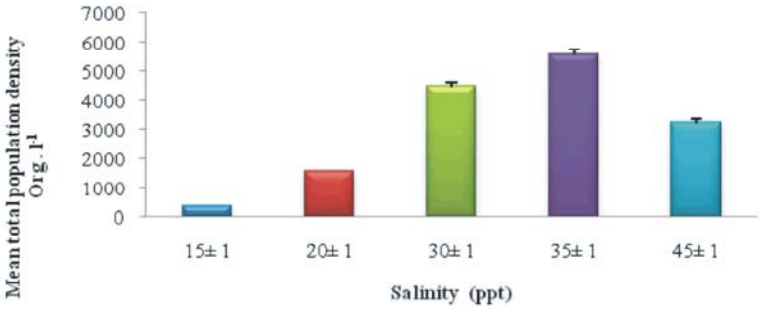

Fig. 3: Total mean population density (±SE) of *Nitokra affinis* cultured for 8 days at different salinities (P<0.05)

at warm temperature. Takahashi and Ohno [39] reported similar trend in *Acartia tsuensis* and McKinnon [40] in *Acrocalanus gibber*. Santhanam and Perumal [5] also reported the maximum production of cyclopoid copepod, *Oithona rigida* at 28±2 °C temperature. Thus, all the previous studies were showed that warm temperature (25-30°C) is required for the better production of copepods similar to our study also (30±1°C).

Effect of Salinity on Population Growth of *Nitokra affinis*: The population growth of *Nitokra affinis* was also significantly affected by changes in salinity (P<0.001) (Fig. 3). Mean final population density of *Nitokra affinis* after 8 days at 35±1 ppt salinity showed 5578.04±167.56 org.l^{-1} which was significantly higher (P<0.001) when compared to other different salinities (Fig. 3). The population growth observed at 15±1 ppt salinity was significantly lower than all the other salinities (P<0.001). The second, third and fourth mean total population densities were recorded at 30±1 ppt (4495.693±113.20 org.l^{-1}), 45±1 ppt (3236.98±122.30 org.l^{-1}) and 20±1 ppt (1557.433±28.40 org.l^{-1}) and they were significantly differed from each other (P<0.001). Like temperature, the peak production rose to 35±1 ppt salinity after then there was decreased in the population above or below 35±1 ppt (Fig. 3). The distributions of various life-stages (nauplii, copepodite and adult) of *Nitokra affinis* within the populations cultured at different salinities are shown in Fig. 4. At all salinities, high ratio of nauplii was obtained as shown in Fig. 4. The various life-stages (nauplii, copepodites and adults) of *Nitokra affinis* were most evenly distributed at 30±1 ppt, 35±1 ppt and 45±1 ppt respectively (Fig. 4).

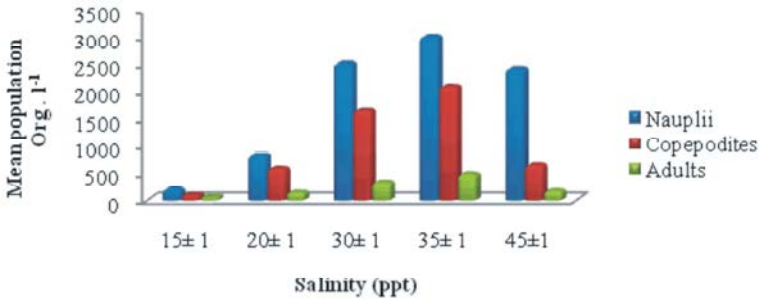

Fig. 4: Mean populations of three life-stages (nauplii, copepodites and adults) of *Nitokra affinis* at different salinities

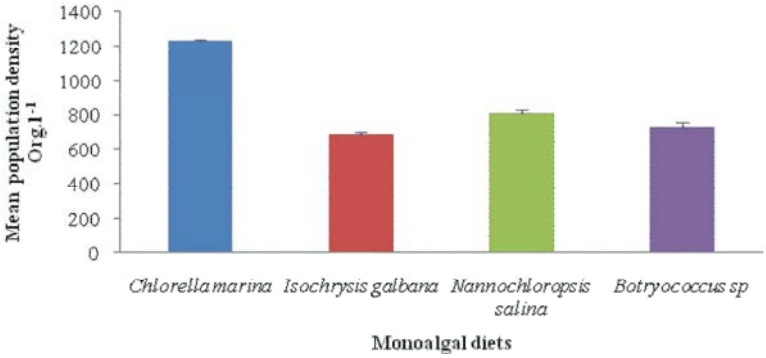

Fig. 5: Total mean population density (±SE) of *Nitokra affinis* cultured for 12 days fed with different algal diets (P<0.05)

Salinity is considered an important environmental factor which can influence nauplii survival. Presently, the better population of *Nitokra affinis* was achieved through 35ppt salinity, as it is a fact that 35ppt is generally the constant salinity recorded in Indian waters (particularly estuary) during most parts of the year. There are various reports confirming that reduction in salinity affects the nauplii survival in copepods [41-43]. Milione and Zeng [36], have reported that population in *Acartia sinjiensis* was significantly higher at 30ppt. Better production was reported earlier by Zaleha and Jamaludin [37] in *Pararobertsonia* sp. at 35 ppt for nauplii to adults, Matias-Peralta *et al*. [44] in tropical harpacticoid copepod *Nitocra affinis*, Sun and Fleeger [45] in harpacticoid copepod *Amphiascoides atopus.* However, there was no significant change in nauplii production through the salinity range of 15.5-33.3 ppt [39, 46]. Rhodes [47] reported that the highest production of nauplii was observed in *Nitokra lacustris* under different salinity regimes. It was confirmed from our study that at different salinity levels, there were increased in production of nauplii when compared to copepodites and adults. Thus, we can promote nauplii of *Nitokra affinis* as a live feed for aquaculture because nauplii are the most preferable food for early marine fish larvae as reported earlier [48]. The present study showed that 35 ppt is the best optimum condition for the culture of *Nitokra affinis* in the laboratory condition. *Nitokra affinis* being a brackish water copepod it could tolerate to the sudden changes in the salinity [49].

Effect of Different Diets on Population Growth of *Nitokra affinis*: After 12 days of culture under different algae and formulated diets the average final mean of copepod population productions are illustrated in Figs. 5, 6, 7 and 8. The distribution of various life-stages (nauplii, copepodites and adults) of *Nitokra affinis* within the populations cultured at different algal and formulated diets was shown in Figs. 6 and 8. The results revealed that the different diets showed a significant difference in the population density of *Nitokra affinis* (P<0.001). Under monoalgal diets (Fig. 5), when all the stages included (nauplii, copepodites and adults) the final number of *Nitokra affinis* population was reached peak at *C. marina* (1229.713 ± 7.141 org.l^{-1}) was significantly higher than the other tested diets (P<0.001). The *I. galbana* produced the lowest population density of 691.72±11.32 org.l^{-1} which was significantly lower (P< 0.001) than the *C. marina*, *N. salina* (P<0.01) except at *Botryococcus* sp. (735.94±25.92 org.l^{-1}) which showed no significant difference (P>0.05) (Fig. 5). The population recorded at *N. salina* (810.10±20.03 org.l^{-1}) was higher compared to *Botryococcus* sp. (735.94±25.92 org.l^{-1}) though they were not statistically significant (P>0.05). High number of

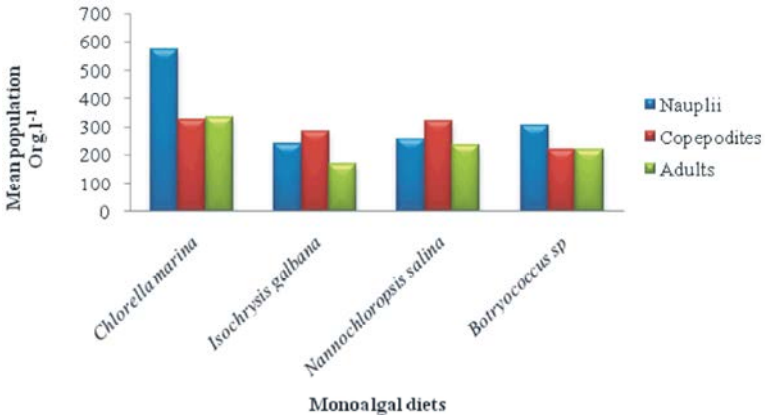

Fig. 6: Mean population of three life-stages (nauplii, copepodites and adults) of *Nitokra affinis* fed with different algal diets

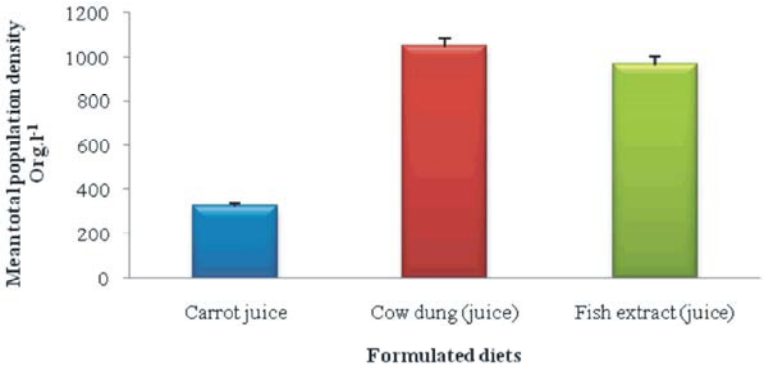

Fig. 7: Total mean population density (±SE) of *Nitokr*a *affinis* cultured for 12 days with different formulated diets (P<0.05)

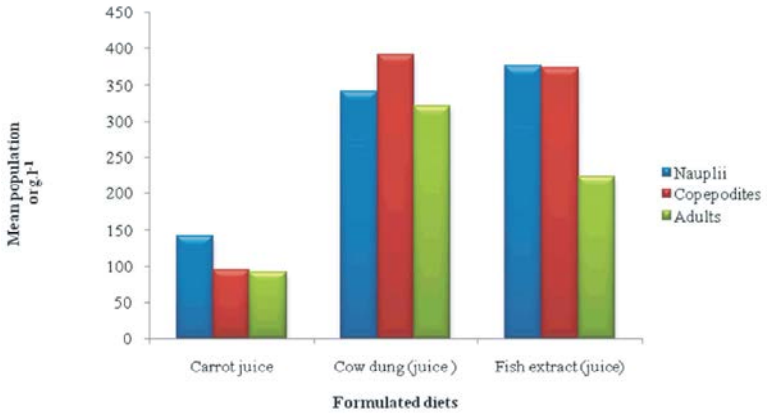

Fig. 8: Mean population of three life-stages (nauplii, copepodites and adults) of *Nitokra affinis* fed with different formulated diets

nauplii was noted in *C. marina* and *Botryococcus* sp. respectively (Fig. 6). The *I. galbana* and *N. salina* diet produced high number of copepodites compared to nauplii and adults (Fig. 6).

In the formulated diets treatment (Fig.7), the highest population was recorded in cow dung (juice form) (1053.53± 33.991 org.l^{-1}) which was significantly higher (P<0.001) when compared to carrot juice treatment (326.75±13.59 org.l^{-1}). The population observed in carrot juice was significantly lower (P<0.001) when compared to fish extract (juice form) and cow dung (juice form). However, the mean final population of cow dung

treatment (1053.53±33.991 org.l^{-1}) was higher than fish extract (juice) treatment (972.35±31.641 org.l^{-1}), though they were not statistically significant (P>0.05) (Fig. 7). The various life-stages (nauplii, copepodites and adults) of *Nitokra affinis* were most evenly distributed in cow dung (juice) and fish extract (juice) treatment respectively (Fig. 8).

For aquaculture purpose, focusing on temperature and salinity alone cannot provide complete study and to evaluate the significance of diets which are responsible for improving copepod production. Many reports were available on the effect of algal and artificial diets that influence the population density of harpacticoid copepods [50-53]. Based on the above studies, it was suggested that food quality affects development and fecundity in the harpacticoid copepods. Nauplii are the most preferable live feed for larval fish in aquaculture [54-56]. Therefore, it is necessary to culture nauplii by using different algal feed. Algal diets have significantly affected the population growth of *Nitokra affinis* in our study. Among the four algal tested diets, the highest copepod and nauplii production was achieved by *C. marina* fed culture. The reason could be due to the small size and easy digestibility of *C. marina*, which fulfils all the nutritional requirements needed for harpacticoid copepod. Similar study carried out by Vijayaragavan and Vivek Raja [57] who found that *C. marina* stimulates more growth in the rearing of microzooplankton, *Tintinnopsis cylindrical.* Similar findings were reported by Kamiyama and Aizawa [58], Santhanam and Perumal [5] and Vengadeshperumal *et al*. [4]. Presently observed lower growth of copepod in *I. galbana* fed culture was because *I. galbana* contains high docosahexaenoic acid (DHA) but low levels of eicosapentaenoic acid EPA as reported earlier by Milione and Zeng [59]. Low number of population was also observed during copepod culture when fed with *I. galbana* as a feed [60-63]. Several studies suggested that bacterial biomass and detritus were the superior source of food for the population in the growth of harpacticoid copepods [64-65]. In our study, cow dung was found to be an appropriate diet for the culture of *Nitokra affinis* where the population rose to 1060.55±74.099 org.l^{-1}. The waste products and manure has rich source of organic materials and has several type of microbes, which converts them into carbohydrates, proteins, pigments, oils, alcohol, aldehydes, etc. utilized by the plankton for their higher population rate [66-67]. Atay and Demir [68] reported that soluble organic manures are responsible for the better growth of zooplankton in the pond. Our findings are in agreement with the results of Orji and Agunwa [69] who have reported that the cow dung could be able to produce desirable nutrients for the culture of zooplankton. Presently, the fish extract (juice) also had shown considerable influence on copepod production next to cow dung. Because, they are organic biomass and have major amount of proteins, which are required for the harpacticoid copepods. Only low copepod production was observed using carrot juice as a feed. The reason may be because carrot juice was given as a monodiet only but the expected population outcomes will be more by giving blends of various juices as reported earlier by Rhodes [70].

CONCLUSION

Our experimental results indicate that *Nitokra affinis* could be mass cultured at a temperature of 30°C, salinity of 35 ppt by feeding with *C. marina* and cow dung (juice) and the cultured live-feed would be suitable for fish larviculture in the aquaculture industry. Further, in case of shortage of algal diets, *Nitokra affinis* could be cultured readily by using formulated diets. Thus, production of copepod population involves various stages and the suitability of diet must require the better nutrients. Diet is one of the important factors for the culture of marine copepods. Presently we have cultured the copepods by applying monodiet only but the production rate would be more if binary or mixed diets are supplied.

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