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Feeding Rotifers *Brachionus plicatilis* with microalgae cultivated in Tunisia

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Abstract: *In this study we investigated the feeding of rotifers Brachionus plicatilis with different diets. We used different foods: powder spirulina, concentrated Chlorella, commercial food named "culture selco" diluted in various volumes and each of these food modes associated to microalgae. We determined the rotifers density, the daily growth rate and the percentages of eggs for the different diets. The food on live spirulina did not give concluding results. The use of inert food only shows a maximum of eggs rotifers percentage and specific growth rate with concentrated Chlorella and a comparable result with powdered spirulina and selco culture (P ≥0, 05). The maximum of the density of rotifers and eggs percentage were obtained with culture selco associated with microalgae, followed by the food mode using super concentrated Chlorella and lastly the food using the powder spirulina.*

Key words: *Arthrospira platensis, Chlorella, culture of zooplanktons, nutrition, rotifers*

I. Introduction

Production of zooplankton, a key component of the productive process in a modern hatchery, remains one of the most important obstacles for the development of marine aquaculture. Zooplankton, such us the rotifer "*Brachionus plicatilis*", was used for several years as a source of animal proteins in larval breeding (Hirata, 1974). Several works were realized to investigate the possibilities of production of these rotifers using various food substrates, in particular algae [1] and yeasts [2]. These types of food permit to obtain relatively appreciable production rates but the rotifers present variable nutritional qualities (Awaïss, 1992). The production of the rotifer "*Brachionus plicatilis*" on a natural nutritive substrate like spirulina "*Arthrospira platensis"* presents several advantages due to the fact that it is a natural source concentrated in nutrients for all animals [3]. In addition, this microalgae is used in several fields, such as the larval breeding with a total value of 1 to 10% of the food, in order to increase the immunological resistance of the larvae [4] . The spirulina is also used in the production of preys like Artemias and Daphnis, in its live form and out of dry powder. This species is known by its high content in protein containing the totality of the amino-acids [5] essentially fatty-acids [6]. It is now cultivated in an industrial scale in several countries such as U.S.A., India, China and Thailand and this because of its highly interesting qualities as a food and its interest for health and wellbeing of human and animal [7].
The aim of this work is to conduct a comparative study of the production of the rotifers using inert food and the spirulina. Various food modes were tested; the spirulina powders, the inert food "culture selco", the Chlorella super-concentrated. Each of these three modes were also investigated with addition of microalgae such as *Chlorella minutissima* and *Picochlorum sp.*, in order to identify the food model which provide the best rotifers growth rate and eggs percentages.

II. Material and methods

Biological material

Rotifers

The specie *Brachionus plicatilis* used in this study is obtained from a strain of rotifers cultivated in the National Institute of Marine Science and Technology (INSTM, appendix of Monastir, Unit of the live preys). This specie presents 2 different sizes. The small ones (S) measure from 120 to 160 µm and the large ones (L) between 250 to 300 µm (Barnabé, 1991). They are used in the larval food at a rate of 5 to 30 invidious/ml.

The microalgae

Three microalgae were used in this study they were as follow:

 *Arthrospira platensis***:**, it is obtained from the Tunisian farm "Ferme Marine Bio Algues". It was used as a food for rotifers in Erlenmeyer and as an inert food (dry powder form) for rotifers in tanks at the rate of 1g of spirulina powder for 1million of rotifers.

 Picochlorum sp: belongs to the class of Chlorophycea. It was isolated from a sample taken from the bay of Monastir. In this study, it was used as a food for rotifers in Erlenmeyer (2L), in a plastic bag (60L) and as a supplement in a rotifer tank at the rate of 5 L/day.
 Chlorella minutissima: belongs to the class of Chlorophycea. It is characterized by an exceptional concentration in chlorophyll, and was used as a food for rotifers reared in a plastic bag (60L) and as a supplement in a rotifer tank (5L/day every morning)

Inert food
 Culture selco: fine particles, which must be solubilized in the water before distribution. The daily ration was calculated according to the concentration of rotifers in the tank using the following formula:

The daily ration of culture selco = 0.4g C.S * 1million R.

Where; C.S: Culture selco and R: Rotifer.
 Powder spirulina: fine dry particles, which were micronized, filtered, and solubilized in the water before distribution. The daily ration is calculated according to the following formula:

The daily ration of powder spirulina = 1g P.S * 1million R.

Where; P.S: Powder Spirulina and R: Rotifer.

 Concentrated *Chlorella*: It a fresh water specie, imported from Japan. Its concentration was 13 billion cells by milliliter. The daily ration is given according to the following formula:

The daily ration of concentrated *Chlorella* = 1ml C.C / 1million R.

Where; C.C: Concentrated *Chlorella* and R: Rotifer.

Experimental protocol
Culture of the rotifer

First, we maintained the strains of rotifers in Erlenmeyer flasks with a volume of 50 ml which contained mature microalgae. Then we passed through intermediate breeding in Erlenmeyer 2L. After the breeding flasks we moved to the production of rotifers in a plastic bag of 60L, containing microalgae in exponential growth phase. When the density of rotifers reached its maximum, they were then filtered to pass in small tanks (180L).

Breeding took place in a controlled environment with a temperature of 24 ± 1 ° C, salinity was kept at 20 ‰ and the light at 1000 lux. The sea water used to fill the tanks was simultaneously filtered through filters in 50µm, 5µm and 1µm. It then passed through a UV sterilizer. This water was also chlorinated at the rate of 5ppm then de-chlorinated by a molar solution of thiosulfate sodium. The tanks were supplied by bubbling air to ensure agitation and to maintain rotifers suspended.

Culture of the microalgae

In this study, the cultures were maintained under the growth algae optimal conditions to ensure the multiplication of the algae cells and their growths (table 1).

Preparation of diets

First, various diets were used such as inert food like culture selco, micro-fine spirulina, or concentrated Chlorella. Second, we added micro-algae to each inert food. For each type of food, three tanks were prepared to determine the assimilation of this food by the rotifers (table 2).

To recapitulate the diets tested are:

1- Alive Spirulina
2- Spirulina powder
3- Spirulina powder with microalgae
4- Culture selco
5- Culture selco with microalgae
6- Microalgae
7- Concentrated *Chlorella*
8- Concentrated *Chlorella* with microalgae

Follow up of growth
Microalgae growth

A sample was taken every day to monitor of the *Picochlorum sp.* culture growth. A cellular enumeration was done by optical microscope using the Malassez cell,. Cellular concentrations were calculated according to the following formula:

$$C = N^* 10^5 \text{cells/ml.}$$

With: C: concentration and N: average number of cells
To monitor the growth of Spirulina cultures, we measured the optical density at a 680 nm wavelength.

Rotifers growth

A sample of 250 µl was removed from the flasks and another from the tanks for counting rotifers under a dissecting microscope. Then we multiplied by the tank volume to determine the rotifer population density.
The percentage of eggs was determined by dividing the number of females carrying eggs by the total number of rotifers found in the volume of 250 µl then multiply by 100.
The daily Specific Growth Rate was calculated using the following equation described by [8]:

$$SGR = (\ln Nt - \ln N0)/t$$

where: SGR = specific growth rate; Nt = rotifer density after culture period t (individuals ml-1); N0 = initial rotifer density (individuals ml^1); t = culture period (day).

Data analysis

The StatView statistical program (version 5.0) was used for data analysis. Analysis of Variance (ANOVA) was used to determinate any significant differences between tested variables of populations densities, daily specific growth rate and of rotifer eggs percentages. All significance tests were performed at P = 0.05.

III. Results

Growth of rotifers nourished with live food in low volumes (*Erlenmeyer****)***

Culture of rotifers with microalgae

The results of the growth of the rotifers in 3 Erlenmeyer fed by the microalgae, *Picochlorum sp.*, were represented in figure 1. Evolution of the microalgae growth in the Erlenmeyer showed an increasing curve with a maximum at the tenth day followed by a phase of reduction. The rotifers were introduced when the micro-algae were in full growth phase. The growth of the latter followed an exponential curve and corresponded to the phase of increase in rotifers. Indeed the density of the rotifers exceeded the 400 individuals/ml towards D17.

Culture of rotifers with a live spirulina

It was noted that the density of rotifers cultivated in the 3 Erlenmeyer containing the spirulina decreased from 16 to 5 rotifers /ml after eight days (figure 2). This decrease was likely due to the fact that the food with spirulina proposed was not accepted by the rotifers. The bacteriological analysis of the distributed sample showed a contamination.

Growth of rotifers fed by composed aliment in big volumes (tanks 180 L)

Rotifers fed with microalgae + inert food:

Density of the rotifers

For the three food modes tested (microalgae + culture selco; microalgae+ spirulina powder and microalgae+ concentrated *Chlorella*) (figure 3), we noted an increasing evolution of the density from D0 to D4. The best growth was obtained for the first food mode (microalgae + culture selco) with a maximum density of 208 rotifers /ml, followed by the diet of microalgae + concentrated *Chlorella*, where the density reached 150 rotifers /ml. The growth of the rotifers, obtained with a food containing microalgae + powder spirulina, also presented an increasing evolution but less than the others diets. Indeed, the maximum density obtained was about 103 rotifers/ml.

Specific Growth Rate of rotifers

Variation of the average daily Specific Growth Rate of the rotifers according to composed food modes (figure 4) shows that the rotifers fed with the second food mode (microalgae + spirulina powder) had a growth rate of 15.21 %. It was slightly lower than that obtained with the other diets with 20.08 % for the mode microalgae + concentrated *Chlorella* and 17.43 % for that of the microalgae + selco culture. However these differences remain non significant ($P \geq 0.05$).

Eggs percentages

Concerning the percentage of rotifers eggs (figure 5), the best results were obtained with the mode containing the microalgae + selco culture with a percentage of 30%, which was significantly different compared to the others percentages ($P<0.05$).The percentage of eggs of rotifers fed with microalgae + spirulina powder was of 21%. It was close that obtained with microalgae + concentrated *Chlorella* (22%) ($P \geq 0.05$).

Rotifers fed with inert food only

Density of rotifers

The culture of rotifers fed on inert food only on (selco culture, powder spirulina and concentrated *Chlorella*) showed a slightly increasing density at the beginning of the culture which declined rapidly with time for the three tested modes (Figure 6). Indeed, for rotifers fed with selco culture only, their density varied from 63 at D0, to reach a maximum of 77 rotifers /ml at D2. At D3, it decreased to 49 rotifers /ml. The same result was obtained for the second food mode (powder spirulina) where the density of rotifers fluctuated from 63 at D0 to 90 at D2, it decreased to 55 at D3. Rotifers fed with the concentrated Chlorella followed the same shape curve, but the density was higher and increased during the first three days of breeding from 55 to 138 and started to decrease on D4, where the density reached 122 rotifers /ml.

Specific Growth Rate of rotifers

Rotifers fed with the concentrated *Chlorella* presented a better specific growth rate (17.59%), which was significantly different ($P<0.05$) for these fed on spirulina mode (with a rate about - 4.51%) and on selco culture mode (with a rate of -10.97) (Table 3).

The specific growth rate of rotifers using powdered spirulina or selco culture mode didn't show a significant difference ($P \geq 0, 05$).

Eggs percentages

The best result was obtained using a food mode containing concentrated *Chlorella* where the percentage of eggs was 26%, which was significantly different from feeding on selco culture (19%) ($P<0.05$) and on powdered spirulina (20%) ($P\geq0.05$). But the powdered spirulina compared to selco culture did not show a significant difference ($P\geq0.05$).

IV. Discussion

Traditionally, algae species like *Dunaliella*, *Isochrysis*, *Monochrysis*, and *Chlorella* were often used as a food for the rotifers because of their small size which is lower than 20 µm [9]. [10] showed that these algae have also a high nutritional effect.

Several diets were experimented in different studies to perform the growth of rotifers. [11] showed significant effects on growth rate and viability of rotifers using other microalgae. [12] showed that yeast (*Saccharomyces cerevisiae*) when mixed with green algae (*Chlorella vulgaris* and *Scenedesmus acutus*), was appropriate for growing freshwater rotifers.

The xenic cultures of marine rotifer *Brachionus "Cayman"* fed on microalgae (*Tetraselmis suecica*) performed significantly better than those fed on baker's yeast (*Saccharomyces cerevisiae*), when comparing population density, growth rate and egg ratio [13]. A significant effect of L-carnitine addition was only found in yeast-fed cultures. [14] developed a test system with genotobiotically grown *Brachionus plicatilis* for the evaluation of the microbial functions and the nutritional value of different feed types. The authors found that all of the tested microbial communities were able to increase the rotifer growth rate when yeasts were used as a major food source.

The study of [15] showed that a single addition of a mixture of three probionts (*Phenylobacterium sp.*, *Gluconobacter sp.*, and *Paracoccus denitrificans*) had no significant effect on rotifer growth in batch culture, but a rotifer growth rates was significantly higher when *Nannochloropsis oculata* paste was provided instead of a yeast-based diet.

The inert food like the powdered spirulina, which has the advantage to be available in the market, is also used for the mass culture of zooplanktons. Indeed, the spirulina is employed in the production of live preys like Artemias and Daphnis. [16] showed that for the culture of *Artemia salina*, the use of powder atomized of the spirulina *Spirulina maxima* optimizes the growth and the survival. A satisfactory rotifer reproduction was demonstrated by using spirulina as a supplement in alimentation with microalgae [17].

In this study, we tested the assimilation of the spirulina by the rotifers cultivated in small volumes (Erlenmeyer of 2L) compared to the other species of microalgae (*Picochlorum sp.*, *Chlorella minutissima*). The results showed that the rotifers could not incorporate the spirulina as a live food. Indeed, Spirulina is more difficult to be filtered than the mono-cellular algae [16]. The size of the algae is a considerable factor which affects the development of this type of zooplankton. According to [18], the rotifers (from the group of the Brachionides) cannot filter particles with a size higher than 18 µm. However the tested spirulina, in spite of its fine crushing, has probably a higher size. In addition, microbiological analyses carried out on spirulina, culture showed the presence of pathogenic germs like total coliforms, fecal coliforms and anaerobic sulfato-reducing. These kinds of bacteria could also contribute to the decrease of rotifers density.

For the culture of the rotifers in great volumes (vats of 200 L), the use of a food mode composed of the microalgae and powdered spirulina, contributed to the rotifers growth rate in a similar way than obtained with microalgae enriched by culture selco or by the *Chlorella* concentrated.
Moreover, the use of the powdered spirulina alone as a food for the rotifers showed an increase of the rotifers density showing that the latter could assimilate them. In addition, this density was slightly better than that obtained with culture selco but lower than the one using the concentrated *Chlorella.*
[16] showed that freeze-dried of the *Tetraselmis* algae has food effectiveness higher than that of Spirulina. But for *Artemia salina* the use of powder atomized algae of *Spirulina maxima*, optimizes the growth and the survival.

The curve presenting the variation of the density of rotifers according to the food mode based on the powdered spirulina shows a decrease only at D3. This could be explained by biotic factors presented mainly by microbial interactions. Indeed, in the vats of mass cultures, various species of microbes including the protozoa and the bacteria coexist and are able to have harmful effects on the growth of the rotifers. Among these micro-organisms, Ciliophora was the frequent protozoa met in this culture [19].

Concerning the percentage of eggs of the rotifers, we did not observe a significant difference for the various tested food modes. In fact, throughout this work, environmental parameters, particularly the quality of the culture was maintained constant. Their variation could influence the percentage of eggs in the rotifers [20].
Although daily growth rates of rotifers nourished with powdered spirulina were satisfactory compared to those obtained with the other diets, its use as an inert food, presents a pollution problem of the breeding vat. This was observed especially on the level of the wall of the vat with fast sedimentation and an unpleasant odor. Thus, a frequent cleaning of the wall is recommended several times per day and following each distribution. Moreover, the mixing of the culture is not sufficient to ensure the maintenance in suspension of the algae powder. The only found solution was a progressive uninterrupted distribution of food [16].

V. Conclusion

The rotifers constitute the most used zooplanktonic preys in feeding larvae in aquaculture. However, the use of the phytoplankton as a source of food for these rotifers causes problems each in their costs and in their biochemical composition. Within this work, we followed the density of rotifers, their daily specific growth rate and the percentage of eggs of the rotifers *Brachionus plicatilis* cultivated on spirulina *Arthrospira platensis* in its live and powdered forms compared with other food modes.

The spirulina, which the production is increasing worldwide and in Tunisia, has several uses particularly as a food source and as pharmaceutical products. This is due to its high content in easily assimilating protein. In aquaculture this species could be used in the culture of the rotifers which is the basic food for the fish larvae.
This study shows that the powdered spirulina alone or added with microalgae, could be used as an inert food in feeding rotifers. But, its use in a live form did not contribute to their growth. Nevertheless, an improvement of the microbiological quality of spirulina could lead to better results.

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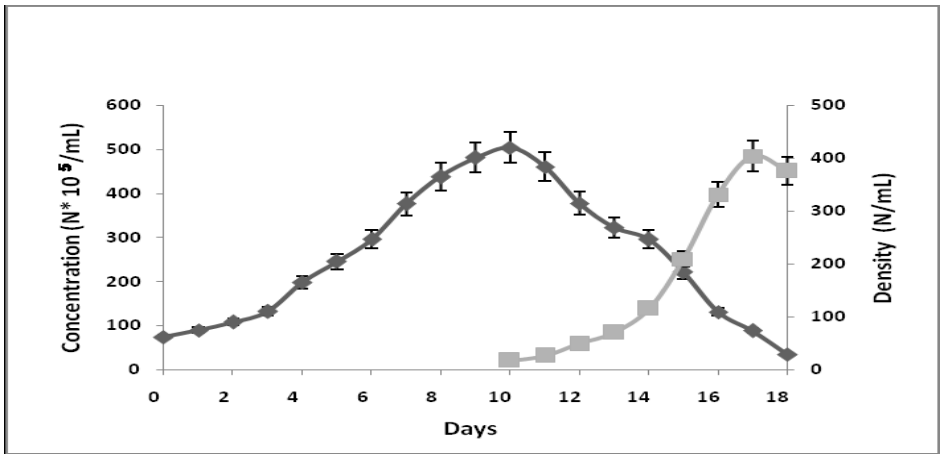

Fig.1: Change in microalgae cellular concentration and rotifer density through time.

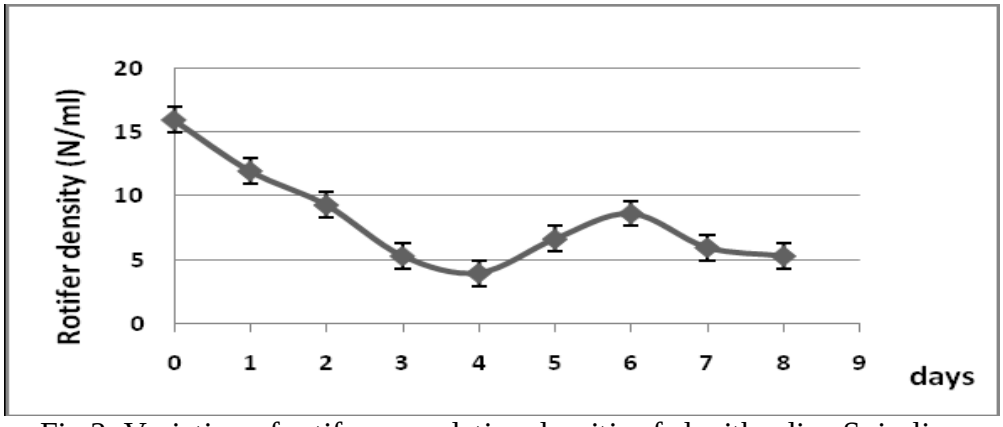

Fig.2: Variation of rotifers population densities fed with a live Spirulina

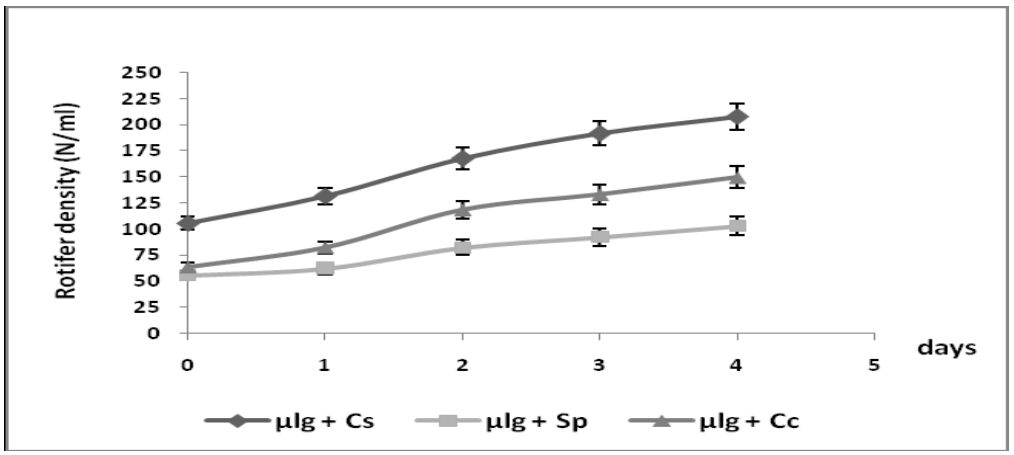

Fig.3: Variation of rotifer densities according to the various diets. µlg : microalgae. Cs: Culture selco, Sp: Micro-fine Spirulina, Cc: concentrated Chlorella

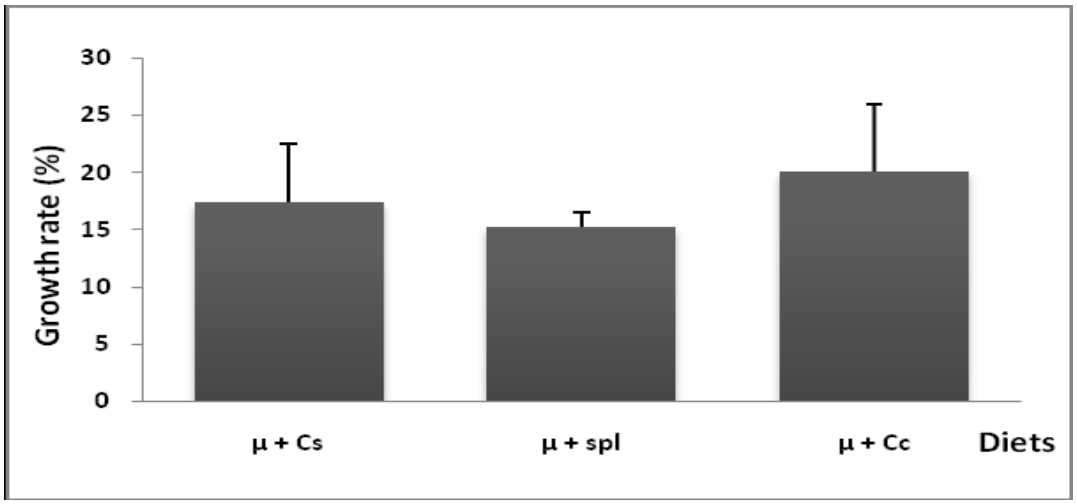

Fig. 4: Daily average Specific growth rate of rotifers according to diets. µ : microalgae. Cs: Culture selco, Sp: microfine Spirulina, Cc: concentrated Chlorella

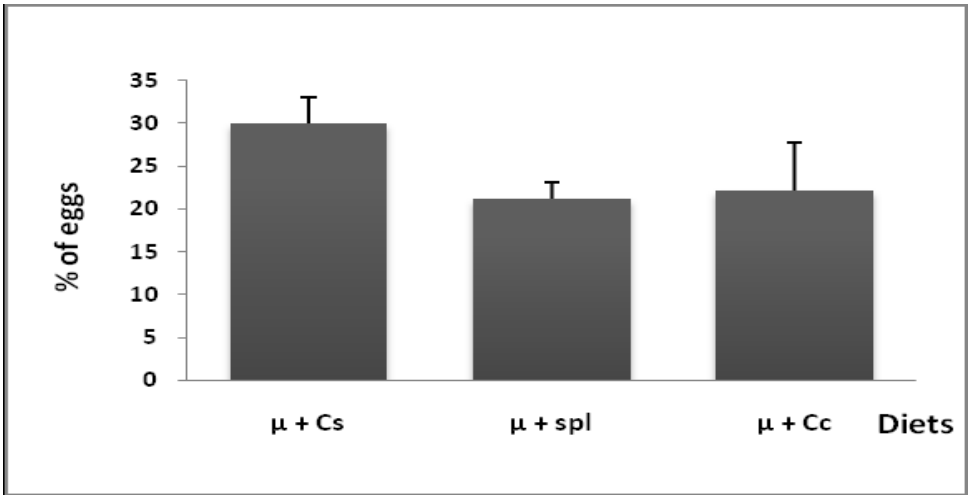

Fig. 5: Variation of rotifers eggs percentages according to different diets. µ : microalgae. Cs: Culture selco, Sp: micro-fine Spirulina, Cc: concentrated Chlorella

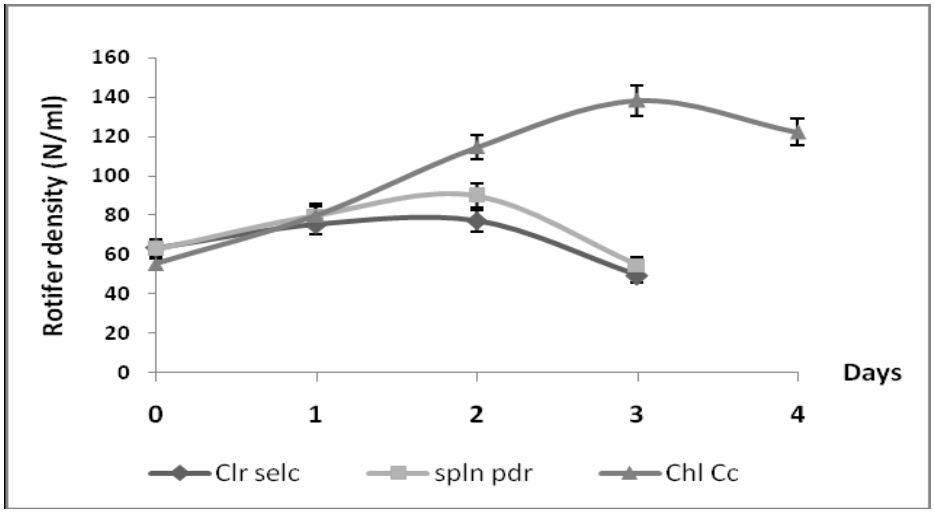

Fig.6: Variation of rotifer density according to different diets.
Clr selc : Culture Selco, Spln pdr : Spirulina powder, Ch Cc : concentrated Chlorella

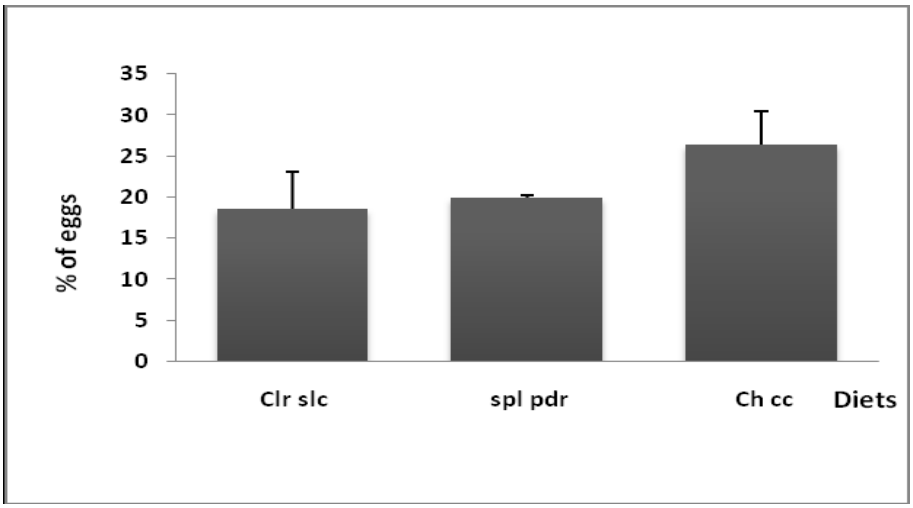

Fig. 7: Variation of rotifers eggs percentages according to different diets. Cs: Culture selco, Sp: Micro-fine Spirulina, Cc: concentrated Chlorella

Table 1: Representation of different production parameters for the microalgae species

	Picochlorum sp. and *Chlorella minitissima*	*Arthrospira plathensis*
Culture conditions	Temperature: 18-20 pH: 6.5-8 Salinity: 30‰ Light: 2000-8000 Lux	Temperature: 27-3O pH: 8.5-11 Salinity: 15‰ Light: 4000-9000 Lux
Culture media	Conway culture middle (Walne, 1966)	Zarrouk culture middle (Zarrouk, 1966)
Culture procedures	200 ml of algae inoculate into 2l sterile culture middle which contains 2ml of Conway solution	1 volume Spirulina introduced into 10 volumes of Zarrouk culture middle

Table 2: Composition of different diets distributed to rotifers in tanks

Diets	tank
Microalgae + Culture Selco	3 tanks
Microalgae + microfine Spirulina	3 tanks
Microalgae + concentrated *Chlorella*	3 tanks
Culture Selco	3 tanks
Microfine Spirulina	3 tanks
Concentrated *Chlorella*	3 tanks

Table 3: Daily Specific growth rate of the cultures (R in %) of D0 until D4 (± standard deviation, N = 3)

	Rotifer with selco	***Culture Rotifer with powdered Spiruline***	***Rotifer with concentrated Chlorella***
R (%)	-10,97 ± 12,82	-4,51 ±2,10	17,59 ± 12,95