CHAPTER THREE

III. MATERIALS AND METHODS

The present study was carried out at the experimental fish farm El-Max Research Station, National Institute of Oceanography and Fisheries (NIOF), Alexandria Governorate, Egypt. Two experiments were designed to investigate the effect of C/N ratio on biofloc technique (BFT), growth performance, survival rate, water quality, and economical evaluation parameters of flathead grey mullet *(Mugil cephalus*) fingerlings.

3.1. Experimental fish and culture techniques:

3.1.1. Frist experiment: Effect of C/N ratio levels on biofloc, water quality, survival rate, growth performance, quality and economic feasibility on *Mugil Cephalus* fingerlings.

3.1.1.1. Location and duration:

This experiment started on Tuesday 28th May 2013 and continued for 70 days using 3x3 factorial design of treatments to study the effect of C/N ratio on biofloc (BFT) system and flathead grey mullet growth performance. Three dietary protein levels (16%, 20%, and 24%) under biofloc system (no water exchange), and three carbon levels using starch (0, 30%, and 60%) of the produced daily diet .Zero starch was used as a control with water exchange 12000 liter /day . Table (3) was also employed.

Table (3). Experimental design showing (0% starch) representing control ponds, and (30%, 60% starch) of daily diet representing BFT ponds.

Dietary protein %	Starch Levels%	Culture System
16% CP	0	Control
	30	BFT
	60	BFT
20%CP	0	Control
	30	BFT
	60	BFT
24%CP	0	Control
	30	BFT
	60	BFT

3.1.1.2. Ponds:

Twenty seven Concrete ponds 6m^3 each under tent to act as a cover, filled with underground marine water. The micro biota used to inoculate BFT ponds was obtained from a tilapia fish farm (Vitafish, Dottignies, Belgium), using part of the soil, and some of drainage water from draining canal as a source of inoculation, in addition to 50gm Urea as a source of nitrogen, while the control ponds were designed under open circulation. The ponds represented nine experimental treatments in triplicate. Experimental ponds were supplied with ground water (30 m depth well). (Fig7).

Fig. (7).Tri-angle area ponds inside El-Max Research Station, National Institute of Oceanography and Fisheries, Alexandria, Egypt.

3.1.1.3. Rearing techniques:

Flathead grey mullet *Mugil cephalus* fingerlings with an initial body weight of 5.05±0.10 g/fish, were obtained from Rashid region, Elbehira Governorate, Egypt. Prior to the start of the experiment, fish was acclimatized to the new water conditions for two weeks and fed on a basal diet of (25%CP).The fingerlings were stocked in twenty seven Concrete ponds, 42 fish / pond (7

fish/ m^3 water), representing nine experimental treatments in triplicate. Fish were held under natural light (12:12 h, light: dark schedule). The water level was maintained at approximately $6m^{3,}$ and loss of water due to evaporation and leakage was replaced whenever necessary according to water size in BFT ponds. Water in ponds representing control treatments (change water system) were changed twice daily. Aeration was continuously provided using an air blower. Also, agitation was kept at biofloc ponds by continuously strong aeration.

Fig.(8).Condition of biofloc ponds during the first experiment with supplying of Aeration and strong Agitation Technique, at Almothalath area inside El-Max Research Station, National Institute of Oceanography and Fisheries, Alexandria, Egypt.

3.1.1.4. Diets formation and preparation:

The three experimental diets were formulated from fish meal, soybean meal, yellow corn, wheat bran, wheat flour, carboxy methyl cellulose (CMC), ascorbic acid, fish oil, vitamins and minerals mixture. Ingredients were obtained from the local market in Egypt. The dry ingredients were mixed thoroughly at first and with oil thereafter. The experimental diets were pelleted, all diets were put into sacks after samples had been taken and stored at -20ºC in deep freezer until use. The composition (%) and Chemical analysis (% dry matter bases) of experimental diets are presented in (Table 4).

Table (4). Formula and chemical analysis (%) of the experimental diets.

Ingredients	16% CP	20% CP	24% CP
wheat flour	28.00	25.80	20.00
wheat bran	26.40	23.40	21.20
Soya bean meal	4.00	7.00	10.00
Yellow corn	25.00	21.40	20.70
fish meal	8.00	14.00	20.00
Fish oil	5.00	4.80	4.50
CMC1	3.00	3.00	3.00
Vit, Min, Mix 2	0.40	0.40	0.40
Ascorbic acid	0.20	0.20	0.20
Proximate composition (%)			
Dry matter (%)	88.80	88.40	88.40
Protein (%)	16.13	20.05	24.05
Lipid (%)	9.01	10.47	9.66
Total carbohydrate (%) 3	67.26	61.06	55.25
Ash (%)	5.21	5.55	7.91
Gross energy(kj/kg) 4	454.83	464.7	455.93
Protein energy ratio(mgCP/kcal)	35.42	43.14	52.75

(1) CMC: Carboxy methyl cellulose

(2)Vitamins and minerals mixture : Each 1 kg contains Vit A (400000 i.u.), Vit D (100000 i.u.), Vit E (250 mg,) Vit K3 (200 mg,) Vit B1 (200 mg), Vit B2 70mg, Vit B6 (200mg), Vit B12 (1mg), Vit C 450mg, Niacin 1000mg, Methionine1000mg, Cholin chloride 10000mg, Folic acid 100mg, Biotin 2mg, Panthonic acid 220mg, Magnesium sulphate 1000mg, Copper sulphate 1000mg, Iron sulphate 3000mg, Zinc sulphate , 600mg, Cobalt sulphate 100mg, Carrier upto 1000mg.

(3) Total carbohydrate =100-(CP+EE+Ash)

(4)Gross energy (GE) was calculated as 5.64, 9.44 and 4.11 kcal/g for protein, lipid and NFE, respectively NRC, (1993).

3.1.1.5. Feeding regime:

The experiment established for 70 days, all fish were fed the experimental diets (16, 20, and 24%) using daily ration 5% of the total stocked biomass. The daily ration was divided into two equal amounts and offered at (9.00 AM and 2.00 PM) daily.

3.1.1.6. Water samples:

Water quality parameters were monitored during the study to follow the changes under biofloc system compared to control treatments. Temperature and pH values of the water samples were measured in the field, using graduating thermometer and portable digital pH meter Martini Instruments (Model 201/digital). Water salinity and total dissolved solid (TDS) were measured using Salinometer Y.S.I (Bekman, Model RS-10). Dissolved oxygen was measured using oxygen meter model Hanna oxy check. Organic phosphors were measured by seal AA3 auto analyzer. Ammonia, Nitrite, and Nitrate were measured every week calorimetrically by kites according to the Animal Health Research Institute (AHRI) Biomedical Chemistry Unite.

3.1.1.7. Fish sampling:

Fish in each replicate pond were weighted every 15 days to the nearest0.00 to adjust the feed quantity, fish were transferred to tank containing water from the experimental ponds, and then returned back to ponds after measuring their weights. Fig (9).

Fig.(9). Fish weighting process to adjust the feed quantity during the first and the second experiments at El-Max Research Station, National Institute of Oceanography and Fisheries, Alexandria, Egypt.

3.1.1.8. Carbon levels for biofloc system:

Three levels of starch (0, 30%, and 60%), of the amount of feeding ration were added to maintain the optimal C/N ratio,(>10: 1) to activate heterotrophic bacteria growth (Avnimelech,1999). Starch had been completely dissolved in water at plastic tanks, and spread over the pond surfaces at (10.AM). Adding starch as a carbohydrate source, shading ponds, and strong aeration condition are the main circumstances that cause floc growth and development (Azim and Little 2008) .

3.1.2. Second experiment: Effect of biofloc system at different salinities, and crude protein levels on water quality, growth performance, and survival rate of *Mugil cephalus*.

3.1.2.1. Location and duration:

This experiment started on Tuesday 20th August 2013 and continued for 70 days, in a (2x3) factorial arrangement. Two crude protein levels (20%, and 24%), and three water salinities (underground marine water 33 ppt (part per thousand), brackish water with salinity 15 ppt, and fresh water (tab water)), had been used to study the effect of biofloc system on flathead grey mullet performance, and water quality, in El-Max Station for applied research.

3.1.2.2. Ponds:

Eighteen Concrete ponds 6m^3 each under tent to act as a cover, filled with water. All ponds were inoculated by clay from another tilapia fish pond, and some of water from draining canal as a source of inoculation. The ponds were representing six experimental treatments in triplicate.

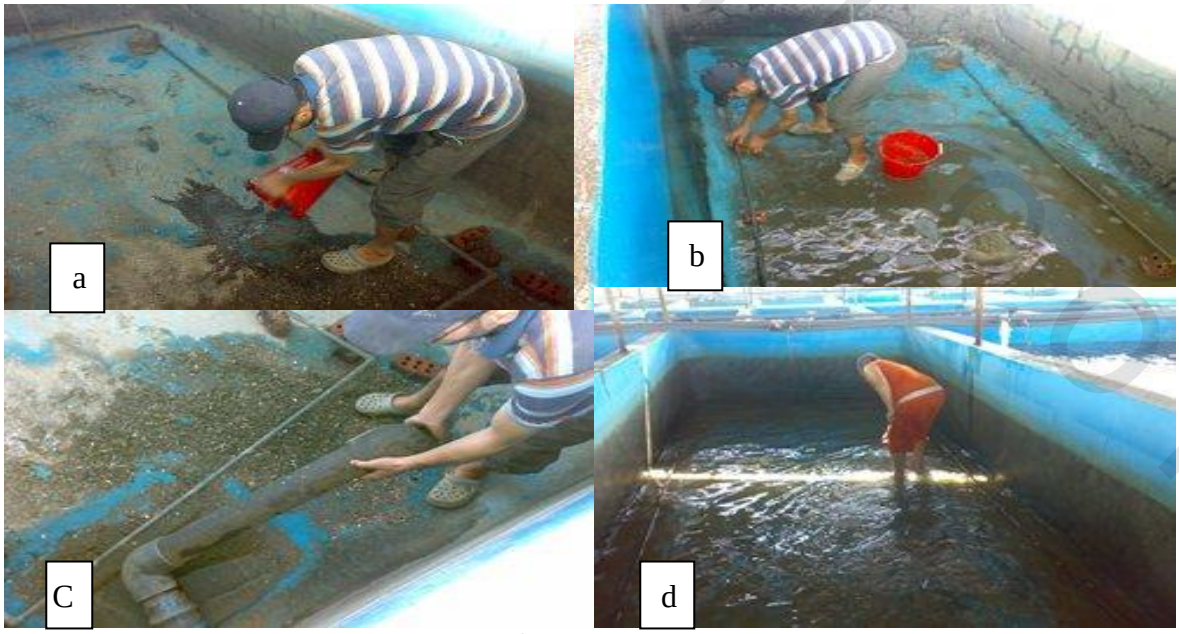

Fig. (10). ponds preparation process before the second experiment at El-Max Research Station, National Institute of Oceanography and Fisheries, Alexandria, Egypt.

3.1.2.3. Rearing techniques:

Flathead grey mullet *Mugil cephalus* fingerlings with an initial body weight of 10.89 ±0.12 g/fish, were obtained from Rashid region, Elbehira Governorate, Egypt at the first day in August. Fish were acclimatized at the new water conditions for three weeks, and fed a diet containing (24%CP).The fingerlings were stocked into18 Concrete ponds at rate of 42 fish/pond (7 fish/ m^3 water), representing six experimental treatments in triplicate. Fish were held under natural light (12:12 h, light: dark schedule). The water level was maintained at approximately $6m^{3,}$ and loss of water due to evaporation and leakage was replaced whenever necessary according to water size in BFT ponds. Aeration and agitation were continuously provided using an air blower.

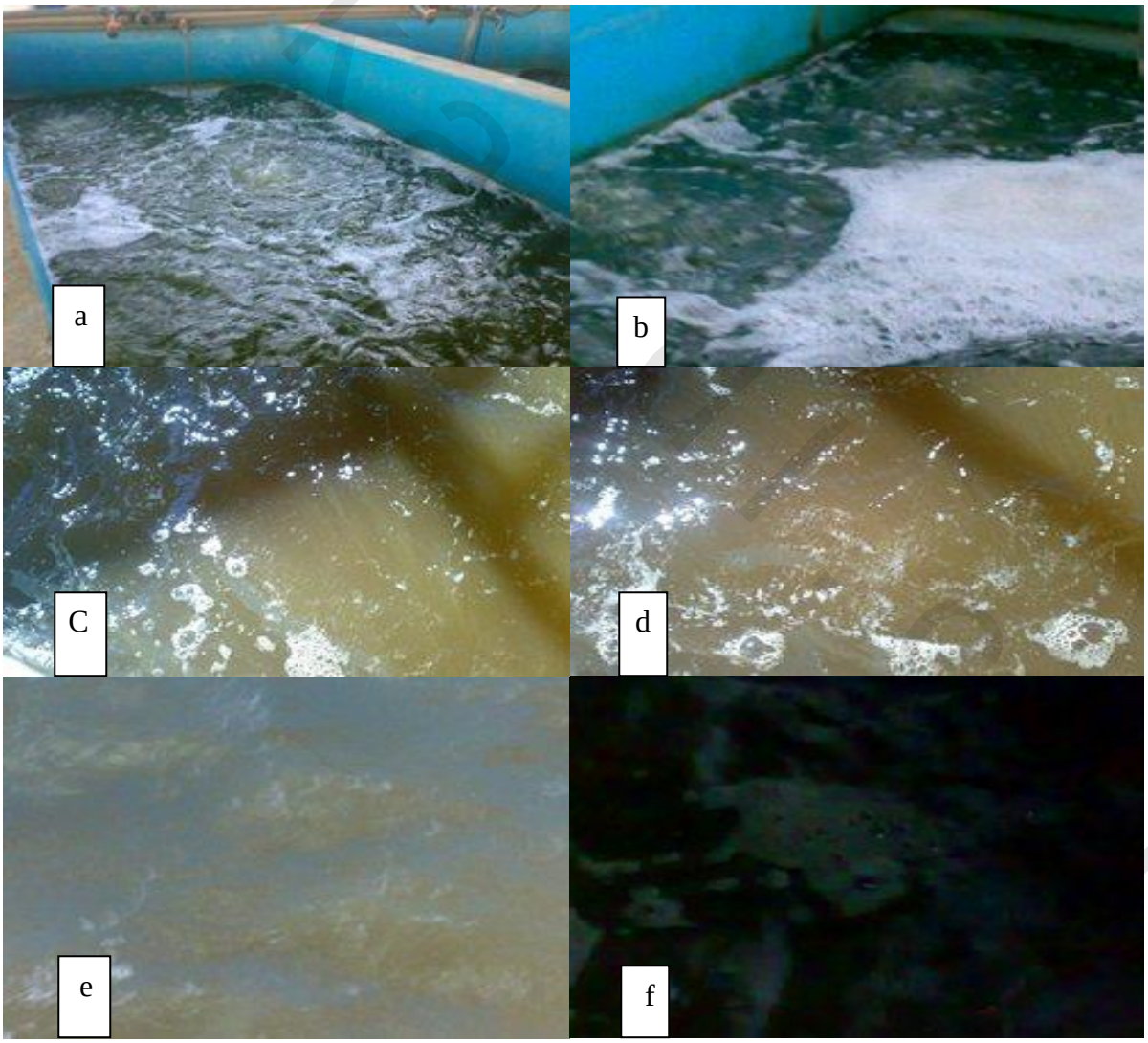

Fig.(11).Condition of biofloc ponds during the second experiment with supplying of Aeration and strong Agitation Technique.

3.1.2.4. Feeding regime:

The experiment established for 70 days, fish were fed the experimental diets (20%, and 24%), under biofloc system using 60% starch of the daily diet, which was noticed for the best fish performance in the first experiment, Table (5). The daily ration was 3% of the total stocked biomass divided into two equal amounts and offered at (9.00 AM and 2.00 PM).

Table (5), design of EXP 2. Two dietary protein levels with three water salinity levels without water exchange using 6o% starch of daily diet.

Crud protein	Water Salinity(ppt)
20%	33 (m) 15 (b) 1>(f)
24%	33 (m) 15 (b) 1> (f)

Where: (m): marine, (b): brackish, (f) : fresh

3.1.2.5. Fish sampling:

Fish in each replicate pond was weighted every 15 days to adjust the feed quantity, and then returned back to ponds after measuring their weights.

3.1.2.6. Water samples:

Water quality parameters were monitored during the study to follow the changes under biofloc system at different salinities. Temperature and pH values of the water samples were measured in the field, using graduating thermometer and portable digital pH meter (Model 201/digital). Water salinity and total dissolved solid (TDS) were measured using Salinometer (Bekman, Model RS-10). Dissolved oxygen was measured using oxygen meter model Hanna oxy check. Organic phosphors were measured by seal AA3 auto analyzer. Ammonia, Nitrite, and Nitrate measured every week calorimetrically by kites according to the Animal Health Research Institute (AHRI).

3.1.2.7. Initiation of biofloc:

All treatments were biofloc at different salinities, starch added at one level, (60%) from amount of feeding ration, which was noticed for the best fish performance in the first experiment, in order to maintain the optimal C/N ratio to activate heterotrophic bacteria growth (>N1:C10). Starch was dissolved in a water at plastic tank, and spread over the pond surfaces at

(10.AM). Also the microbiota used to inoculate the ponds was obtained from a tilapia fish farm (Vitafish, Dottignies, Belgium).

Fig.(12).Initiation of biofloc in the first and the second experiments by adding Starch at El-Max Research Station, National Institute of Oceanography and Fisheries, Alexandria, Egypt.

3. 2. Growth performances, feed utilization parameters, and Survival rate:

3. 2.1. Growth indices:

At the end of the experiment random fish samples were selected and weighted to determine mean final body weight (FBW), weight gain (WG), average daily gain (ADG), specific growth rate (SGR %), feed conversion ratio (FCR), protein efficiency ratio (PER), protein productive values (PPV), and energy retention(ER) which were calculated using the following equations.

- **Total weight gain (g/fish):**

Total weight gain = W_t - W_0

Where:

W_0: initial mean weight of fish by grams

W_t: final mean weight of fish by grams

- **Average daily gain (ADG) (mg/fish/day):**

ADG = W_t - W_0/n

Where:

N: duration period

- **Specific growth rate (SGR) (%/ day):**

SGR = 100 × (ln W_t - ln W_0)/ days

Where:

ln: natural logarithm

Feed Intake (FI): This is the amount of feed given or supplied during the experimental period.

- **Feed conversion ratio (FCR):**

FCR = dry matter intake (g)/body weight gain (g)

Where:

Body weight gain = (W_t - W_0)

- **Protein efficiency ratio (PER):**

PER = weight gain (g)/ protein intake (g)

- **Protein productive value (PPV %)**

PPV=100 × (protein gain (g)/protein intake (g)

- **Energy retention(ER %)**

ER=100× (energy gain (kg)/(energy intake(kg)

3.2.2. Survival rate:

Survival rate was calculated in all experiments according to Ricker (1975) and Newman and Martin (1983) as follows:

Survival rate (%) = (No. of fish at the end / No. of fish at the start) × 100

3.2.3. Condition factor

Condition factor (K) was calculated in all experiments according to Lagler (1956) as follows:

Condition factor (K) = W/L^3*100

Where:

W: weight

L: length

3.3. Chemical analysis:

3.3.1. Fish and diets analysis:

At the beginning and the end of each trial, random pooled samples of fish were collected and sacrificed for determination of initial whole-body proximate composition. Fish sample were oven-dried at 65°C, ground, and stored at -20°C for subsequent analysis. The chemical composition of fish samples were determined according to the procedures of AOAC (1995). Dry matter was determined after drying the samples in an oven (65°C) for 24 h. Ash by incineration at (550°C) for 12 h, crude protein was determined by micro-Kjeldhal method, %N × 6.25 (using Kjeltech aotoanalyzer, Model 1030, Tecator, Hoganas, Sweden), and crude fat by Soxhlet extraction with petroleum ether (60-80°C). Chemical analysis of the experimental diets used in all experiments was done according to AOAC (2000).

3.3.2. Water analysis:

Water quality parameters such as temperature, pH, ammonia, Nitrite, Nitrate dissolved oxygen, and Organic phosphors were monitored weekly to follow the effect of biofloc system in comparison to changing water system.

Water temperature was measured in the field, immediately using graduating thermometer.

Water salinity and total dissolved solid (TDS) were measured using Salinometer (Beckman, Model RS-10).

Dissolved oxygen was measured using oxygen meter model Hanna oxy check.

Organic phosphors were measured by seal AA3 auto analyzer.

.Water samples which were taken for measuring, ammonia, nitrite, and nitrate, were (50ml) from each pond, at (40cm) depth.

Water ammonia, nitrite, and nitrate, were determined weekly. Calorimetrically measurement using (Ammonia $NH^{+}_{4}/NH_{3,}$ Nitrite $NO_{2,}$ and Nitrate) rapid test for water quality testing. All tests were prepared, and quality was controlled and assured by staff members of the Biomedical Chemistry Unite according to the ISO/IEC 17025, 6353, 5664, and 71501. All rights reserved to the Animal Health Research Institute (AHRI), Agricultural Research Center (ARC), and Ministry of agriculture –land Reclamation, Egypt. The ammonia, nitrite, and nitrate, rapid

test is validated to be used for different types of water resources (surface fresh and salt as well as underground waters) and is ideal for aquaculture control (fish ponds).

3.3.2.1. Ammonia rapid test directions:

1-In chosen well (A_{1-12} to H_{1-12}), take one drop of sample by the plastic pasture pipette.

2- Drop one drop using the red labeled plastic pasture pipette from reagent (1) to each sample.

3- Immediately, drop one drop using the red labeled plastic pasture pipette from reagent (2) to each sample.

4- Immediately, drop one drop from reagent (3) to each sample.

5- Shake the plate gently to avoid splash.

6- After seven minutes, put the plate on the color scale card to compare to calculate NH_4/NH_3 where the pH should be measured see the table below.

NH_3			PH		
NH^+_4	7	7.5	8	8.5	9
0.5	0.003	0.009	0.03	0.08	0.18
1	0.006	0.02	0.05	0.15	0.36
2	0.01	0.03	0.11	0.3	0.72
5	0.03	0.09	0.27	0.75	1.8
10	0.06	0.17	0.53	1.51	3.6

7- Rinse many times with tap water the used plate and pasture pipettes after testing to avoid erroneous results.

8- Hit the plate many times while inverted on a paper towel to repel water droplets outside the wells, and then leave it to dry in inverted position.

3.3.2.2. Nitrite:

1-In chosen well ($A1_{-12}$ to H_{1-12}), take one drop of sample by the plastic pasture pipette.

2- Drop one drop using the plastic pasture pipette from reagent (1) to each sample.

3- Immediately, drop one drop from reagent (2) to each sample.

4- Shake the plate gently to avoid splash.

5- After five minutes, put the plate on the color scale card to compare.

6- Rinse many times with tap water the used plate and pasture pipettes after testing to avoid erroneous results.

7- Hit the plate many times while inverted on a paper towel to repel water droplets outside the wells, and then leave it to dry in inverted position.

3.3.2.3. Nitrate:

1-In chosen well (A_{1-12} to H_{1-12}), take one drop of sample by the plastic pasture pipette.

2- Put spoonful solid from reagent (1) to each 1 ml sample in small 2ml bottle provided.

3- Shake vigorously for 15 second.

4- Drop one drop from the supernatant by pasture pipette in micro-titre plate

5- Add one drop from reagent (1) to each sample.

6- Add another drop from reagent (2).

7- Shake the plate gently.

8- After five minutes, put the plate on the color scale card to compare.

9- Rinse many times with tap water the used plate and pasture pipettes after testing to avoid erroneous results.

10- Hit the plate many times while inverted on a paper towel to repel water droplets outside the wells, and then leave it to dry in inverted position.

3.3.2.4. pH :

The pH values of the water samples measured in the field, immediately using portable digital pH meter (Model 201/digital).

3.3.3. Total bacterial counts:

3.3.3.1. Sampling:

Direct manual quantification of total bacteria was carried out to determine the total bacterial counts. A composite water samples (1 L per pond) were collected biweekly, beginning at 8-11 AM, and through 1-4 PM, by combining four 250-mL samples obtained approximately 15 cm below the water surface, and from the middle of each side of the pond. Formalin (40 mL) was added to each 1 liter from the sampling water in a numbering flask, to aggregate algae, zooplankton and all microorganisms. Then, every water sample was filtered through plankton net 55µ mesh size, 25cm diameter and 50cm length, and the volume of aggregated algae and all microorganisms was measured by a numbering tube (0.5-50) ml graduation as alternative to Imhoff cones. Fig (14).

3.3.4.2. Laboratory techniques:

A second sub-sample (50 ml) was removed from the original composite water sample that was collected from each pond for analysis. Formalin (2 ml) was added to each 50-ml sample to preserve algae, zooplankton and all microorganisms until microscopic examination could be performed. Identification and enumeration was performed by transferring a1-ml sample into a microbiology laboratory and using a binocular research microscope with (150×magnification)

Fig.(14). Manually quantification of total bacteria process during the first and the second experiments at El-Max Research Station, National Institute of Oceanography and Fisheries, Alexandria, Egypt.

3.3.4.3. Identification of biofloc

Identification of algae and micro-organisms was made according to the following references: (Paerl and Tucker, 1995); Cocke (1967); Prescott (1962); Foissner and Berger (1996); Wallace and Snell (1991); Pontin (1978).

3.4.5. Statistical analysis:

Data of the first and the second experiment were analyzed using two ways ANOVA as the following model:

$$Yij = M + Pi + Tj + (PT) ij + eij$$

where yij, observation of the ij^{th} parameter measured; m overall mean; pi, effect of i^{th} level of protein; tj, effect of j^{th} starch (for the first experiment) or salinity (for the second experiment); (pt) ij, interaction level of protein by starch or salinity; eij, random error. significant differences ($p \leq 0.05$) among means were tested by the method of duncan (1955). The analyses of variance (ANOVA) were made according to Snedecor and Cochran (1981).

3.5. Economic efficiency and evaluation:

The descriptive analytical and economical style was used during the present experiments in order to study and explore the key economic features of flathead grey mullet culture under BFT conditions according to Helal and Essa (2005). Also, some evaluating performance parameters were used to identify the current operating economics of culture flathead grey mullet according to Abdel Hafez and El – Kariony (1992) as well as Scott *et al*. (1993), such as:

Operating ratio (%) = Total operational costs/Revenue

1. Return on sales (%) = Net income/Revenue
2. Return on costs (%) = Revenue/Total operational costs
3. Capital recovery period (years) = Investments/Annual income
4. Return on equity (%) = Net income/Investments
5. Rate of return as a % of total inputs = Net income/ Total operation costs

Data on all the items of fixed and variable costs and depreciation of capital and the outputs of farmed fish, and price of sale and revenue, were collected during the present study period.