EFFECT OF PROBIOTICS, MINERAL SUPPLEMENTATION AND BIOFERTILIZERS ON GROWTH PERFORMANCE OF SOME EURYHALINE FISH SPECIES IN INLAND SALINE GROUNDWATER

By

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HISAR

2006

CERTIFICATE-I

This is to certify that this thesis entitled, **"Effect of probiotics, mineral supplementation and biofertilizers on growth performance of some euryhaline fish species in inland saline groundwater,"** submitted for the degree of **Master of Science** in the subject of **Fisheries** to the Chaudhary Charan Singh Haryana Agricultural University, Hisar is a bonafide research work carried out by **Mr. Nalle Datta A.** under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of these investigations have been fully acknowledged.

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Dedicated

To

My Revered Parents

&

Loving Brother

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LIST OF ABBREVIATIONS USED

Abbreviation	Description
%	per cent
mt	million tones
ha	hectares
t	tones
lbs	pounds
^{o}C	degree centigrade
min	minutes
kg	kilogram
g	gram
mg	milligram
S.E.	Standard error
BW	Body weight
d	day
l	liter
y	year
Lat.	latitude
Long.	longitude
kJg^{-1}	kilo joules per gram
cm	centimeters
mm	milimeters
h	hour
CFU	Colony forming units
TDS	Total dissolved solids

PREFACE

The underground waters of major part (about 60%) of this state (Haryana) are moderately to highly saline. Because of inefficient utilization of surface water resources, there is a rapid rise in water table and soil salinization in arid and semi-arid canal irrigated areas of central and south-western Haryana. The annual rise in water table (0.3 to 1 m) has been recorded in nearly 75 per cent of its arable land and the water table has almost reached the surface, which by all accounts is alarming. Irrigation water losses on the farm have been reported to be one of the major factors contributing to the rise of water table. To prevent further salinisation and to maintain the productivity of such lands detailed drainage and reclamation studies have been undertaken on pilot scale. The studies include laying of subsurface drainage systems with an inbuilt reuse system in view of the absence of a drainage outlet for the disposal of drainage effluent.

In order to have a sustained agriculture, the brackish water logged areas with poor productivity can be used for raising fish. Traditionally, in India, (especially in this part) fresh waters are utilized for stagnant pond fish culture. As a part of a programme to develop commercial culture of the exotic and Indian carps, fish culture studies utilizing inland brackish waters of low salinities were carried out at Haryana Agricultural University. Studies have shown that the common carp as well as the Indian major carps are able to tolerate the available salinities (upto 8 dSm_{-1}) of the ground water as no mortality was ever observed. Fish culture studies have further revealed that carps perform well at salinities upto 8.0 ppt. Higher salinities (>8.0 ppt) appear to repress fish growth (Garg and Sukkel, 1993; Garg, 1996; Garg and Bhatnagar, 1996). Further, brackish waters are not suitable for the breeding of locally available fish species (Garg, 1993). Since only moderately saline water upto 7.5-8.0 ppt could be utilized for the culture of locally available species, therefore for utilizing waters of high salinity (10 ppt and above) and also for the diversification of aquaculture, high salt tolerant (euryhaline) finfish and shellfish species occurring in the coastal areas were cultured and grown in these waters (Jana *et al.,* 2004, 2005, 2006, Barman *et al.* 2005).

A detailed analysis of underground saline water (Table 1) obtained from deep tubewells have revealed that with increase in salinity (‰), water quality parameters like conductivity, pH, Na^{+}, K^{+}, Ca^{++}, Mg^{++}, total hardness, chlorides, bicarbonates and orthophosphate concentration increased, while no variations were observed in the concentration of sulphates with change in salinity levels. It was further observed that potassium, calcium, magnesium, total hardness and chlorides were very high in comparison with the natural sea water.

Azotobacter a diazotroph, (biofertilizer) which fixes atmospheric nitrogen not only in the terrestrial system but also in the aquatic habitat. Therefore, its utilization in the ponds in conjunction with fish culture will not only reduce the use of inorganic fertilizers but also will help in reducing aquatic pollution (Bioremedian) and at the same time would enhance pond productivity.

More than 25 years ago Parker (1974) introduced the modern concept of probiotics. Aquatic animals are quite different from land animals for which the probiotic concept was developed and therefore, the probiotic usage in aquaculture, especially in culture fisheries has taken a different meaning. The high risk of losing crops due to disease attack has prompted many fish farmers

Table 1. Chemical composition of underground saline water, fresh water and sea-water (From Garg, 2002)

Sr. No.	Parameters	TUBEWELL-1 Brackish water-1	TUBEWELL-2 Brackish water-2	FRESH Water	SEA Water
1.	Salinity %	9.55±0.03	24.15±0.35	0.3±0.0	34.48
2.	Conductivity mScm	16.49±0.03	38.55±0.11	10.54±0.05	44.48
3.	pH	7.22±0.02	7.09±0.02	7.15±0.09	8.86
4.	Sodium mg L^{-1}	278.66±2.21	390.0±1.65	42.00.47	10556.1
5.	Potassium mg L^{-1}	30.4±0.0	40.00±0.94	3.0±0.0`	380.0
6.	Calcium mg L^{-1}	420.51±7.43	1082.83±5.25	102.81±27.36	400.1
7.	Magnessium mg L^{-1}	1360.29±13.72	2183.46±114.72	63.39±2.33	1272.0
8.	Total hardness mg L^{-1}	4625.0±0.64.95	11050.0±134.63	683.33±7.20	1715.57
9.	Chlorides mg L^{-1}	4709.1±20.60	9008.12±20.60	46.38±1.35	18979.9
10.	Sulphate mg L^{-1}	95.68±1.36	93.34±0.55	85.75±0.36	2648.6
11.	Carbonates mg L^{-1}	0.0	0.0	0.0	28.0
12.	Bicarbonates mg L^{-1}	140.5±0.82	158.0±0.70	89.33±0.54	139.7
13.	Total alkalinity mg L^{-1}	140.5±0.82	158.0±0.70	89.33±0.54	167.7
14.	O-PO4 mg L^{-1}	0.034±0.001	0.092±0.001	0.029±0.01	0.001-0.00

All values are mean± SE of mean

all over the world especially in India to use the probiotics during the culture operations.

All forms of aquatic animals requires inorganic elements for their normal life processes. Dietary calcium and phosphorus contents have been reported to increase growth performance in a number of fish species (Sakamoto and Yone 1973). Calcium is an essential constituent of all living cells, its mode of action is not clear but it appears to play an important part in decreasing the permeability of cell membrane. Elements, such as calcium and phosphorus and numerous trace minerals can be absorbed through the gills as well as through the gut. Hence, water concentration may have a major influence on mineral status. Since the use of *Azotobacter* to reploace inorganic fertilizers and use of feed supplemented with the probiotics/minerals not only will check pollution but will also enhance growth and resistance to pathogens, therefore, to fulfill these goals, following objectives have been taken up in this thesis :

1. Effect of Azotobacter strains in primary productivitry and milkfish production in inland saline ground water ponds.
2. Effect of some probiotics and minerals on growth performance and nutrient utilization in *Chanos chanos* and *Oreochromis niloticus*.

***CHANOS CHANOS,* FORSSKAL (SEE PLATE-1)**

Classification

Phytum	:	Chordata
Sub-phytum	:	Vertebrata
Class	:	Teleostomi
Order	:	Chaniformes
Family	:	Chanidae
Genus	:	*Chanos*
Species	:	c*hanos*
Common name	:	Milk fish

Distribution

It is widely distributed in coastal waters of the Indopacific region, entering estuaries, rivers and lakes.

Food and Feeding habits

Fish feeds on unicellular algae and other phytoplankton during early stages. Adult subsists on bottom invertebrates, soft leaves, filamentous algae, dead microorganisms and lab-lab.

Age and growth

Under normal conditions, it grows to a length of 450-500 mm/0.7-0.9 kg in the first year. Its maximum length is recorded as 1800 mm in the nature.

First maturity

Chanos chanos attains sexual maturity when 3-4 years old and 0.7-0.9 m in length.

Fecundity

Generally fecundity varies with age, length and weight. Number of ova ranges between 2.5-5.7 million at their size of 0.9m to 1.5m. It does not breed in confined waters and spawns during summer months (May or September).

Specific characteristics

- Middle dorsal and small and terminal mouth.
- Body elongated and compressed.
- Dorsal profile of head flat and snout broad.
- Pectoral pointed with elongated scale appendage at base.
- Caudal large and deeply forked.
- Body bluish grey above, sides and abdomen silvery and whitish

- Annual growth 200-400 mm and 800g; maximum sizes 1500 mm and 7-15 kg.

OREOCHROMIS NILOTICUS (L) (SEE PLATE 2)

Classification

Phylum	:	Chordata
Sub-phylum	:	Vertebrata
Class	:	Teleostomi
Order	:	Perciformes
Family	:	Cichlidae
Genus	:	*Oreochromis*
Species	:	*niloticus*
Common names	:	Nile tilapia or American koi

Distribution

It is widely distributed in Nile and Niger river basins and in lakes Tanganyika, Albert, Edward, and George, as well as in many smaller drainages and lakes in western and eastern Africa; also in Middle East in Yarkon river, Israel. This species was introduced for aquaculture purposes. It was introduced into open waters, likely through escape or release from fish farms and has established in large reserviors.

Food and Feeding habits

It is a bottom feeder and fish feeds on filamentons algae, dead micro-organisms and lab-lab.

Age and growth

Under normal conditions it grows to a size of 63 cms.

First maturity

Attains maturity during the second 6 months of their existence. They start spawning when the fish measures not more than 15 cm and even less.

Specific characteristics

* Body colour changes during breeding.
* Head straight, eye, mouth terminal.
* The most distinguishing character in the species is the presence of regular vertical stripes throughout the depth of caudal fin.
* Margin of dorsal fin grey or black.
* Vertical bars in caudalfin (7-12).
* Body short and cross section oval.

PART-I

Role of *Azotobacter* and *Gluconacetobacter* in Nitrogen Fixation, Phosphate Solubilization, Nutrient Release and Fish Growth in Inland Saline Groundwater Ponds

Chapter-I

Introduction

Excessive use of manures and inorganic fertilizers in fish culture ponds for enhancing primary productivity has led to the deterioration of water quality which adversely affects fish growth survival and production (Wohlfarth and Schroeder, 1979; van Rijn and Shilo, 1989; Garg and Bhatnagar, 1996). To combat pollution and to reduce the excessive use of organic/inorganic fertilizers for sustaining the production system and also for assuring food security, it has become a necessity to apply newer techniques and technologies that will facilitate the culture of aquatic organisms without adversely affecting the pond ecology. The present day global interest in biological nitrogen fixation is a direct consequence of this necessity to provide some economic assistance to the small and marginal fish farmers and also to reduce pollution. Therefore, attempts have been made to utilize microbial bioinoculants such as *Azotobacter* which is a free living diazotroph and is reported to occur even in aquatic ecosystem (Bhatnagar et al., 2004). Earlier studies of this laboratory (Garg *et al*., 1998; 2001; Garg and Bhatnagar, 1999b) have revealed that inoculation of *Azotobacter chroococcum* (Mac 27-nitrogen fixer and PS-21 Phosphate solubilizer) in freshwater fish culture ponds enhances pond productivity and fish production. Most of these studies have dealt with the fresh water ecosystem. Since the underground water in many of the Indian states such as Haryana, Punjab, Rajasthan and Gujarat is moderately to highly saline, therefore, there is a need to test the efficacy and suitability of these bioinoculants in inland saline groundwaters. Recently, (Narula *et al*., 2005) have reported the development of a high temperature tolerant strain of *A. chroococcum* and high salinity tolerant *Gluconacetobacter diazotrophicus* strain suitable for tropical and subtropical areas where the soil is saline. Therefore, in the present studies, attempts have been made to utilize Mac 27, PS-21, high salinity and high temperature tolerant strains of *A.chroococcum and G. diazotrophicus* in saline water ponds in conjunction with fish culture. Two experiments were conducted and the impact of inoculation of *Azotobacter* strain, Mac 27- (Nitrogen fixer), phosphate solubilizer *Pseudomonas* strain PS-21 (Experiment 1); high salinity tolerant *Gluconacetobacter* 35-47; and high temperature tolerant HT-51 strain of *Azotobacter* (Experiment 2) was studied on physico-chemical characteristics of pond waters, their nutrient status, plankton production and fish biomass.

Chapter-II

Review of literature

Now-a-days a large number of microbial inoculants are available and have been investigated in detail which could be effectively employed as biofertilizers in terrestrial as well as in aquatic systems. These can be broadly classified into following two groups depending upon the nutrients they provide/release in the environment.

(i) Nitrogen fixing biofertilizers.

(ii) Phosphorous solubilizing biofertilizers

(i) Nitrogen fixing biofertilizers

There are many microbes which can fix atmospheric nitrogen in the soil and water, however, *Azotobacter* and *Azospirillum* have been found to be more effective as nitrogen fixers in aquatic environment/medium (Garg *et al.,* 1998, 2001, Garg and Bhatnagar 1999a,b, 2000, 2002b, Garg and Bhatnagar, 2004).

(ii) Phosphorus solubilizing biofertilizers

Next to nitrogen, phosphorous is another key element required for enhancing the primary productivity of fish ponds. Not many phosphorous solubilizing bacteria occur in the aquatic medium, however, certain modified strains of *Azotobacter chroococcum* have the ability to solubilize phosphorous present in the medium through the production of organic acids (Yadava and Dadarwal, 1997; Garg *et al.,* 1998; Garg and Bhatnagar, 2000b). In soil *Pseudomonas striata* is also used as a phosphorus solubilizer which has been found to increase yield of many a crops. *Bacillus polymyxa* is another bacteria occur in soil, which can also solubilize unavailable form of phosphorus to available forms (Bagyaraj and Indira, 1994).

In addition, blue-green algae (*Cyanobacteria*) and a fern Azolla have also been recognised to be useful as biofertilizers for biological nitrogen fixation in aquatic system.

In the present review an attempt has been made to evaluate the usefulness of *Azotobacter* as an effective source of biological nitrogen fixation/phosphate solubilization in aquatic system in relation to primary productivity, nutrient release and fish production.

Azotobacter is a bacterium whose name is derived from the french "azote" which means nitrogen and it denotes their ability to synthesize ammonium from atmospheric nitrogen. The honour of being called "nitrogen bacterium", despite the existence of other nitrogen fixing bacteria stems from the fact that *Azotobacter* was the first nitrogen fixing bacterium isolated by Beijerinck (1901). It is a free living, diazotroph, highly aerobic rhizobacteria which has recently attracted the attention of microbiologists, aquaculturists and environmentalists due to their unique biological properties for nitrogen fixation, heavy metal resistance, biodegradation of agrochemicals, phosphorus solubilization and hormone production etc.

Azotobacter is a heterotrophic bacterium which fixes atmospheric nitrogen independently and does not need the help of the plant. The beneficial effect of *Azotobacter* is not only due to its fixation of atmospheric nitrogen independently but it also secretes some growth promoting substance and siderophores etc. (Narula, 2000).

Members of this genus are strict aerobes. Oxygen is required for metabolism and also to fix nitrogen. It has a very high rate of respiration, and is able to respire sufficiently rapidly to prevent oxygen penetration to the nitrogenase proteins. Evidence for this is that nitrogenase activity is inhibited if the oxygen concentration is raised to a level at which the bacteria can no longer respire quickly enough to prevent the entry of oxygen (Dalton and Postgate, 1969). These microbes have evolved a variety of structural, biochemical and behavioural modifications, including associations with other organisms to deal with the effects of oxygen evolution (Carr and Whitton, 1973).

Following the report of Beijerinck (1901), that *Azotobacter* frequently occurs in water bodies also, the role of *Azotobacter* spp. in nirogen fixation and their wide occurrence in a variety of aquatic habitats have progressed over the years. It has been reported to fix atmospheric nitrogen in a wide variety of aquatic habitats such as canals, rivers, tanks and ponds etc. (See Table 1). The fact that *Azotobacter* is a free living aerobic diazotroph, prompted its use as a biofertilizer in relation to the development of sustainable aquaculture/fish culture system (Garg *et al.,* 1998).

Habit, Habitat and Geographical distribution

Azotobacter occurs in a wide variety of habitats : soils, leaves, roots and in both marine and fresh waters. In general, most of the *Azotobacter* species are restricted to environments which are rich in organic matter.

Investigations on isolation, identification and occurrence in relation to hydrobiological characteristics of fresh and saline water ponds have also been carried out (Bhatnagar *et al.*, 2004) and studies have revealed the occurrence of three species of *Azotobacter*, which showed no relationship with any of the water quality parameters including water salinity (upto 6.0 ‰). Per cent occurrence of different species of *Azotobacter* have revealed that *A. chroococcum* was the dominant species, followed by *A. beijerinckii* and *A. vinelandii*.

Fragmentary information is available on the nitrogen fixing ability of *Azotobacter* in aquatic environments ((Jordan, 1981). Many fresh water habitats have been reported to contain one or more species of *Azotobacter* but little is known about their role in nitrogen fixation. In some African lakes, like

Lake George and L. Tanganika, nitrogen fixation accounts for about half of total nitrogen input (Hecky *et al.,* 1991). The fixation rate of atmospheric nitrogen in Lake George, Uganda (mean depth of 2.5m) was 0.44 to 1.69 mg m^{-2} h^{-1} (Horne and Viner, 1971) and 11 to 58 mg m^{-2} d^{-1} of N (Ganf and Horne, 1975). Nirogen fixation was maximum in Lake George at the surface and at a depth of about 40 cm and minimum at depths of 15 cm and 150 cm (Horne and Viner, 1971). Lin *et al.* (1988) studied the natural biological nitrogen fixation in water of tilapia growout ponds in Thailand and reported that nitrogen fixation ranged from undectable levels to 105 μg^{-1} L^{-1} day^{-1} in water column compared with 200 μg^{-1} L^{-1} day^{-1} input of N from chicken manure loading. These studies, however, do not indicate the source of nitrogen or the organisms responsible for biological nitrogen fixation.

According to Delince (1992) nitrogen fixation in pond sediments is essentially an activity of heterotrophic organisms. Such fixation in anaerobic conditions is less efficient than in aerobic conditions and therefore, more substrate is needed. *A. chroococcum* in microaerobic conditions is able to incorporate 46.5 mg of nitrogen per g of sugar consumed. Nitrogen fixation in Hungarian fish ponds amounted to 4.7 mg m^{-2} d^{-1} or 0.33 mg m^{-2} h^{-1} of N and was temporarily inhibited by the use of nitrogen fertilizers (El-Samra and Olah, 1979). Tripathy and Ayyappan (1998) have reported that nitrogen fixation rate in terms of acetylene reduction activity were 0.09-0.67 nmoles of

C_2H_4 $ml^{-1}d^{-1}$ in case of *Azotobacter* spp. Recent studies (Garg *et al.*, 2001) have revealed that *Azotobacter* can grow and multiply in aquatic ecosystem under *in vitro* conditions *albeit* at slow rates. The rate of nitrogen fixation under *in vitro* condition was found to range between 0.25-0.60 mg L^{-1} d^{-1} in the presence of organic fertilizers such as cowdung.

Recent studies (Bhatnagar *et al.,* 2004) have revealed that the rate of nitrogen fixation in terms of n moles of C_2H_4 h^{-1} ml^{-1} was found to vary between 2.45-36.72 in wild ponds and between 2.45-67.52 in cultivated ponds.

Factors affecting nitrogen fixation

Considerable nitrogen fixation occurs in the dark. Oxygen inhibits the nitrogenase activity as photorespiration and nitrogenation compete for reductants. According to Paerl (1985) calm waters of low mixing depth and high temperatures in the water allow the creation of anoxic microzones around cells, conducive to nitrogen fixation. These microzones are also conducive to the extracellular reduction of microelements, such as molybdenum and iron. Nitrogen fixation is significant when the N:P supply ratio is below the Redfield ratio of 16:1 (Howarth *et al.,* 1988b).

Ammonium and ammonia are strong inhibitors of nitrogen fixation. Nitrogenase synthesis is activated when interstitial ammonium concentrations drops below a minimum level. Ammonium represses the synthesis and inhibits the activity of the aerobic enzyme, while the synthesis of the anaerobic fixation enzyme is repressed, but its activity is not inhibited. Nitrogenase activity is highest at the mud-water interface, where organic matter is in abundant supply and of good nutritional quality (Blauw, 1988). Hence, favourable conditions for nitrogen fixation includes clam waters of low mixing depth and long stratification periods, light intensity and probably, high temperatures (Howarth *et al.,* 1988b). Phosphate and nitrate are the most common pollutants of fresh waters. Nitrates inhibits nitrogen fixation, whereas, nitrogenase activity is enhanced when phosphate is added to phosphorus deficient waters.

(a) Effect of temperature

Thompson and Skerman (1979) have reported the optimum of temperature tolerance range for the three species of *Azotobacter* (*A. chroococcum,* 9°-33°C; *A. vinelandii*, 9°-30°C *A.lacticogenes,* 2.0°-33°C). These authors have further reported that optimum temperature range for high growth of *A. chroococcum* lies between 27°-29°C. High temperature (50°C and above) proves to be fatal.

Garg *et al.* (2001) reported that with increase in water temperature from 24.5°C-32°C an increase in the rate of nitrogen fixation and nutrient release occurs under *in vitro* conditions. Since these are the normal temperatures which normally prevail in this region in fish culture ponds, therefore, strains of *A. chroococcum* have beeen selected as biofertilizers in fish culture ponds for enhancing productivity (Garg *et al.,* 1998; Garg and Bhatnagar, 1999b,2002b).

(b) Effect of pH

In addition to other physico-chemical factors, water pH also regulates the rate of multiplication and nitrogen fixation of *Azotobacter* spp. (Jensen, 1961; Roy *et al.,* 1962). Terzaghi and Terzaghi (1986) reported that *Azotobacter* can be found at a pH range of 3-9. The soil pH between 7.8 and 8.5 supported maximum population of *Azotobacter* species and the count decreases with increase in pH, however, the efficiency of nitrogen fixation is not affected by the increase in soil pH from 7.0 to 8.7 (Raju *et al.,* 1974; Khullar and Chahal, 1975). Most isolations are made from neutral soils and it is generally accepted that the optimum pH for nitrogen fixation is around neutrality, with nitrogen fixation ceasing at pH below 6. Similar conclusions have also been drawn by other workers that the optimum pH required for growth and nitrogen fixation was near or slightly above neutrality (Yamagata and Itano, 1923; Rangaswami and Sadasivan, 1965). However, there are exceptions, most notably the Beijerinckias, which can grow and fix nitrogen at pH as low as 3.5 (Becking, 1978).According to Ninawe and Paulraj (1997) the optimum pH for growth and nitrogen fixation by *Azotobacter* spp. in prawn-cum-paddy fields of Kerala was found to be 7.0 to 8.5. Garg and Bhatnagar (2002b) have reported a decrease in bacterial viable counts with increase in pH and dosage of organic substrate.

(c) Effect of salinity

Free living *Azotobacter* spp. are also responsible for biological nitrogen fixation in brackishwater fields and ponds. Fluctuations in salinity, influence the bacterial activity in biological transformation of the nutrient elements, thus affecting the pond productivity (Ninawe and Paulraj, 1995). Beijerinck (1901) was the first to report the resistance of *Azotobacter* to high salt concentrations. Lipman (1912) on the other hand reported that NaCl concentration of more than 0.5-0.6% was toxic to *Azotobacter*. Iswaran and Sen (1958) found that excess concentrations of salts in soil may also render *Azotobacter* inactive, thereby depressing their nitrogen fixing ability. Babak (1965), Lakshmanaperumalsamy *et al.* (1975) found that *Azotobacter* from saline soils of Central Asia grew in the high concentrations of NaCl.

Studies of Hebert (1975) have revealed that *Azotobacter* are salt sensitive microorganisms and do not show any growth in the presence of salt and thus are absent even in estuaries. According to Dicker and Smith (1981) differences have been reported to occur in the nitrogen-fixing efficiency of *Azotobacter* at different salinities. In general, decreasing nitrogen fixation rates have been reported to occur with increasing salinity; however, nitrogen fixation can still occur at 40 per cent salinity.

Ninawe and Paulraj (1995) studied the effect of salinity on the growth and nitrogen fixation ability of *A. chroococcum* in prawn-cum-paddy culture system. Their studies on 13 strains of *Azotobacter* have revealed that the salinity levels above 45‰ and below 5‰ were found non-conducive for the growth of most of *A. chroococcum* strains. In most of the strains, the maximum growth occurred in the salinity range of 15-35‰. The rate of nitrogen fixation by all the 13 strains also showed the maximum values at optimum salinity levels, i.e., between 20-40‰.

Authors have further reported (Ninawe and Paulraj, 1996) the influence of salinity on the growth and nitrogen fixing capacity of *A.vinelandii* in prawn-cum-paddy culture system. The study on nine strains of *A. vinelandii* revealed that the salinity levels above 40‰ and below 10‰, in general reduced growth. In most of the strains, the maximum growth for this species also occurred in the salinity range of 15-35‰. The rate of nitrogen fixation also showed maximum values at salinity levels of 25-30‰.

Low water salinity (upto 6.0‰) appears to have no effect on the population, occurrence of three *Azotobacter* species in fish ponds or on their ability to fix nitrogen (Garg and Bhatnagar, 2004).

Use of modified strains of *Azotobacter* as biofertilizer in fish culture ponds

To increase the efficiency of *Azotobacter* as biofertilizer in aquaculture, many strains of *Azotobacter* have been developed at Haryana Agricultural University, Hisar (Narula, 2000). Important strains of *Azotobacter* which can be used in aquatic medium are :

1.	High ammonia excretor	E-12
2.	High ammonia excretor	HAE

3.	Ammonia excretor and phytohormone producer	MSX-9
4.	Nitrogen fixer derepressed strain	Mac-27
5.	Phosphate solubilizer	PS-21

Keeping in view the importance of *Azotobacter* and its strains as biofertilizer in enhancing crop productivity, experiments at the Department of Zoology and Aquaculture, CCS HAU, Hisar were carried out on their utilization as biofertilizer under aquatic conditions in relation to fish culture for the development of efficient sustainable aquaculture technology (See Introduction).

Chapter-III

Materials and Methods

Bacterial strains and culture media:

Bacterial cultures/mutants used during these studies are given in Table 2a and 2b.

Table 2a. List of bacterial cultures used

Sr. No.	Culture/mutants used	Characteristics
1.	*Azotobacter chroococcum*- Mac 27	Nitrogen fixer
2.	Azotobacter chroococcum-HT51	Nitrogen fixer and High temperature tolerant
3.	*Pseudomonas* spp.-PS 21	Phosphate solubilizer
4.	*Gluconacetobacter diazotrophicus*-35-47	Indole acetic acid producer and salinity tolerant

Experiments were carried out in 0.375 ha ($15m^2 \times 25\ m^2$ – 1.2 m deep) ponds. Prior to the commencement of treatments, ponds were cleaned and allowed to dry and thereafter filled with inland saline groundwater from bore-well (13 ppt salinity). A minimum of 0.9 m water depth was maintained.

Irrespective of the treatment, all the ponds received cow-dung at 7500 kg ha^{-1} y^{-1}. Cowdung and microbial cultures were applied at fortnightly intervals. Semi-dry cowdung was thoroughly mixed and dissolved in water before application in fish culture ponds.

Cultures were obtained from Department of Microbiology, CCS Haryana Agricultural University, Hisar, India (Table 2a). Cultures were maintained and grown in their respective media at $25^{o}\pm1^{o}C$. Jensens media was used (Jensen, 1951) for *Azotobacter*, LGI (Cavalcante and Dobereiner, 1988) for *Gluconacetobacter* and King's media (King and Ward, 1954) for *Pseudomonas* respectively.

Preparation of inoculum: A loopful of culture was inoculated in 150 ml Jensen N free broth and incubated at $28^{o}\pm2^{o}C$ for 48-72 h. Thereafter, media (500 ml in the flask) was inoculated at 10% inoculum level incubated at $30^{o}C$ for 72 hrs under stationary conditions. Culture used for inoculating the pond water was diluted (10^{9} CFU ml^{-1}) and inoculated in ponds at fortnightly interval. Two experiments were conducted (Table 2b).

Experiment 1 : Effect of inoculation of Nitrogen fixer *A. chroococcum* Mac-27 and Phosphate solubilizer PS-21 strains on pond productivity and fish growth in Inland saline groundwater

In this experiment nitrogen fixer (Mac-27) and phosphate solubilizer (PS-21) strains were used as biofertilizers (See Table 2b for experimental design). Two weeks after the application of the first dose of fertilizer, milkfish, *Chanos chanos* fingerlings (mean body wt. 43.36±0.12g and mean body length 18.51±0.04 cm) were stocked during September. No supplementary feed was given to the fish during the experimental period. Two replicates of each treatment were maintained.

Table 2b. Experimental design

Treatment	Expt.1	Dosage (per 375 m^2)	Expt. 2	Dosage (per 375 m^2)
1	*A.chroococcum* Mac-27	1.5 L	*Glunoacetobacter diazotrophicus 35-47*	1.5L
2	*Pseudomonas* PS-21	1.5 L	*A. chroococcum* HT 51	1.5L
3	*Mixed culture* (Mac 27+ PS-21)	0.75L+0.75L	No biofertilizer	Control
4	No biofertilizer (Control)	-	-	-

* Irrespective of the treatment, ponds were fertilized at biweekly intervals using cowdung at 7500 kg ha^{-1} y^{-1}, Each ml of biofertilizer contains 10^9 cells ml^{-1}

Experiment 2 : Effect of inoculation of high salinity tolerant *G. diazotrophicus* and high temperature tolerant strain of *A. chroococcum* on pond productivity and fish growth

In this experiment high salinity tolerant strain (*G. diazotrophicus* 35-47) and high temperature tolerant strain of *A. chroococcum* (HT-51) were used as biofertilizers (Table 2b for experimental design). Two weeks after the inoculation of first dose of fertilizer, milkfish *Chanos chanos* fry (mean body wt. 0.36 g and mean body length 3.80 cm) were stocked during the month of June 2004.

For experiment 1, water samples were collected in duplicate at weekly intervals for the determination of physico-chemical and biological characteristics. For experimental 2 water samples were collected (in duplicate) twice only i.e. at 30 and 60 days interval.

DO levels were recorded at zero day and the day following inoculation. Plankton samples were collected by filtering 10L of water sample through a 100 μm mesh plankton net. For viable bacterial counts, water samples were collected in sterilized tubes, serially diluted and 100 μl was plated on Jensen's nitrogen free medium and incubated at 28±2^{o}C for 48-72 hrs.

Analysis

Water samples for physico-chemical and biological characteristics were analysed following APHA (1998). Net primary productivity (NPP) was determined by light and dark bottle technique. Phytoplankton and zooplankton were analysed quantitatively according to the standard methods (Wetzel and Likens, 1979). Water temperature (^{o}C) of the ponds was recorded daily. Dissolved oxygen (DO), pH, conductivity were measured with a portable kit (F-set 3, E-Merck Ltd., Germany).

At the end of the experimental period (70 days for experiment 1 and 60 days for experiment 2), ponds were completely drained and fish were harvested and counted. Length (cm) and weight (g) of the individual fish was recorded. Carcass composition of fish (for experiment 1) following AOAC (1995) was also determined.

Statistical analysis

The coefficient of correlation between different parameters and multiple regression between independent and dependent variables was determined by computer. One way ANOVA, followed by Tukey's studentized range (HSD) test was used to compare the group means (Gomez and Gomez, 1984).

Chapter-IV

Results

Experiment 1 : Effect of inoculation of nitrogen fixer *A. chroococcum*-Mac-27 and phosphate solubilizer -PS-21 strains on pond productivity and fish growth in inland saline groundwater.

Viable counts (colony forming units)

High values of total viable count were observed in ponds inoculated with nitrogen fixing strain (Mac-27), followed by ponds inoculated with phosphate solubilizer strain (PS-21). Bacterial counts remained low in ponds where a co-culture (Mac-27 and PS-21) was inoculated. Significantly ($P<0.05$) low bacterial counts were seen in control ponds. Observations have further shown that irrespective of the strain of *Azotobacter* inoculated, a significant ($P<0.05$) increase in viable counts was observed upto day 7 following inoculation and thereafter a significant ($P<0.05$) decline in viable counts on day 14 was observed (Table 3 and Fig.1).

Table 3. Variations in viable bacterial counts (CFU) from fish ponds inoculated with *Azotobacter* strains Mac-27, PS-21 and control ponds

	Control (CFU ml^{-1})	Mac-27 (CFU ml^{-1})	PS-21 (CFU ml^{-1})	Mac-27+PS-21 (CFU ml^{-1})
Day-1				
0 h	$0.53 \times 10^4 \pm 0.07$	$0.74 \times 10^4 \pm 0.05$	$0.74 \times 10^4 \pm 0.09$	$0.575 \times 10^4 \pm 0.52$
1 h after inoculation	$0.54 \times 10^4 \pm 0.04$	$0.93 \times 10^4 \pm 0.02$	$0.80 \times 10^4 \pm 0.05$	$0.58 \times 10^4 \pm 0.52$
48 h	$0.3 \times 10^4 \pm 0.07$	$1.28 \times 10^4 \pm 0.03$	$1.25 \times 10^4 \pm 0.12$	$1.18 \times 10^4 \pm 0.11$
Day-7	$1.2 \times 10^4 \pm 0.20$	$3.66 \times 10^4 \pm 0.17$	$3.06 \times 10^4 \pm 0.06$	$2.98 \times 10^4 \pm 0.10$
DAY-14				
0 h	$1.0 \times 10^4 \pm 0.06$	$1.8 \times 10^4 \pm 0.01$	$1.55 \times 10^4 \pm 0.10$	$2.10 \times 10^4 \pm 0.06$
1 h after inoculation	$0.94 \times 10^4 \pm 0.08$	$2.05 \times 10^4 \pm 0.09$	$1.75 \times 10^4 \pm 0.02$	$2.02 \times 10^4 \pm 0.08$
Day-15	$0.78 \times 10^4 \pm 0.08$	$2.10 \times 10^4 \pm 0.04$	$1.90 \times 10^4 \pm 0.16$	$2.09 \times 10^4 \pm 0.04$
Day-21	$0.86 \times 10^4 \pm 0.12$	$3.30 \times 10^4 \pm 0.08$	$2.90 \times 10^4 \pm 0.00$	$2.66 \times 10^4 \pm 0.00$
DAY-28				
0 H	$0.29 \times 10^4 \pm 0.08$	$1.24 \times 10^4 \pm 0.08$	$1.38 \times 10^4 \pm 0.12$	$0.9 \times 10^4 \pm 0.06$
1 H AFTER INOCULATION	$0.41 \times 10^4 \pm 0.05$	$1.34 \times 10^4 \pm 0.06$	$1.46 \times 10^4 \pm 0.12$	$0.99 \times 10^4 \pm 0.04$
DAY-29	$0.8 \times 10^4 \pm 0.06$	$2.14 \times 10^4 \pm 0.07$	$1.88 \times 10^4 \pm 0.03$	$1.98 \times 10^4 \pm 0.02$
DAY-35	$0.26 \times 10^4 \pm 0.06$	$1.85 \times 10^4 \pm 0.07$	$1.65 \times 10^4 \pm 0.11$	$1.8 \times 10^4 \pm 0.10$
DAY-42				
0 H	0.00	0.00	0.00	0.00
1 H AFTER INOCULATION	0.00	$0.18 \times 10^4 \pm 0.05$	$0.18 \times 10^4 \pm 0.04$	$0.16 \times 10^4 \pm 0.05$
DAY-43	0.0	$0.20 \times 10^4 \pm 0.09$	$0.28 \times 10^4 \pm 0.11$	$0.19 \times 10^4 \pm 0.09$

DAY-49	0.00	0.00	0.00	0.00
DAY-56				
0 H	0.00	0.00	0.00	0.00
1 H AFTER INOCULATIO N	0.00	$0.18\times10^4\pm0.05$	$0.18\times10^4\pm0.04$	$0.16\times10^4\pm0.05$
DAY-57	0.00	$0.20\times10^4\pm0.09$	$0.28\times10^4\pm0.04$	$0.19\times10^4\pm0.09$
DAY-63	0.00	0.00	0.00	0.00

All values are mean±SE of means of eight observations
Mac-27 = Nitrogen fixing strain of *Azotobacter*
PS-21=Phosphate solubilzing strain of *Pseudomonas*
Mixed culture=Mac-27+PS-21
Control=No biofertilizer

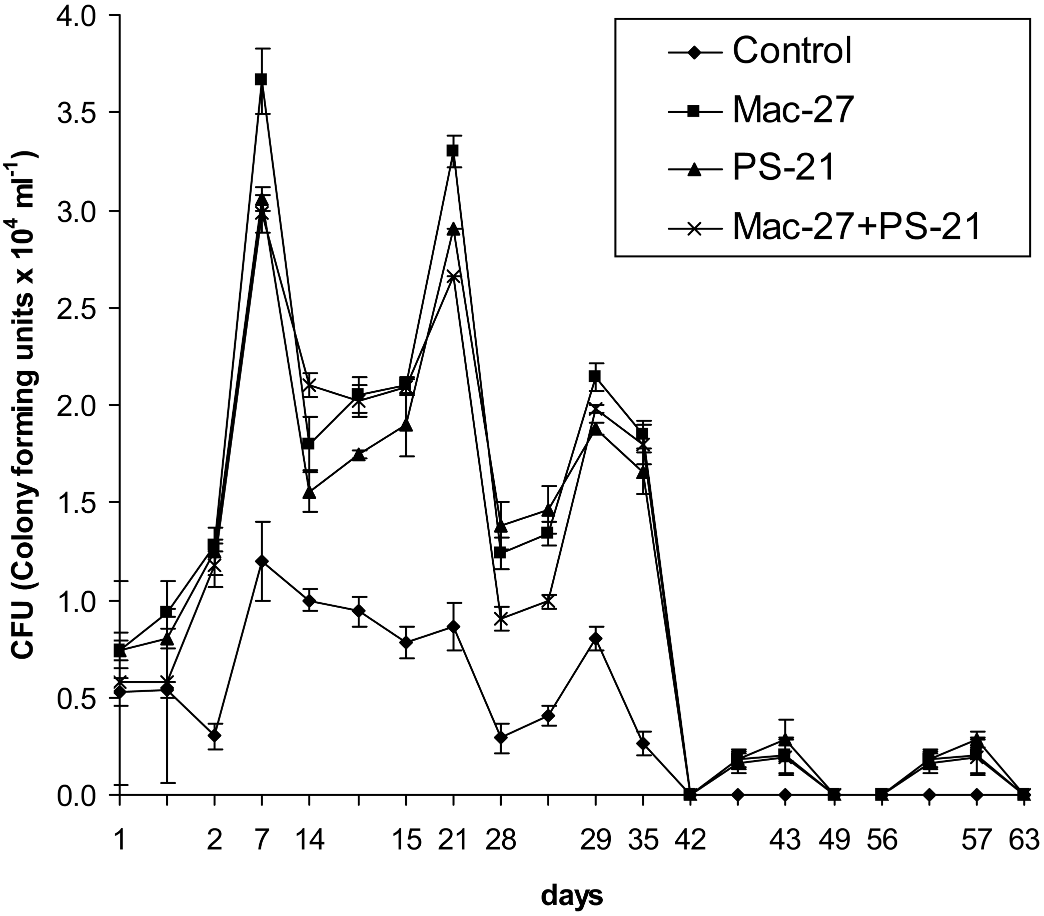
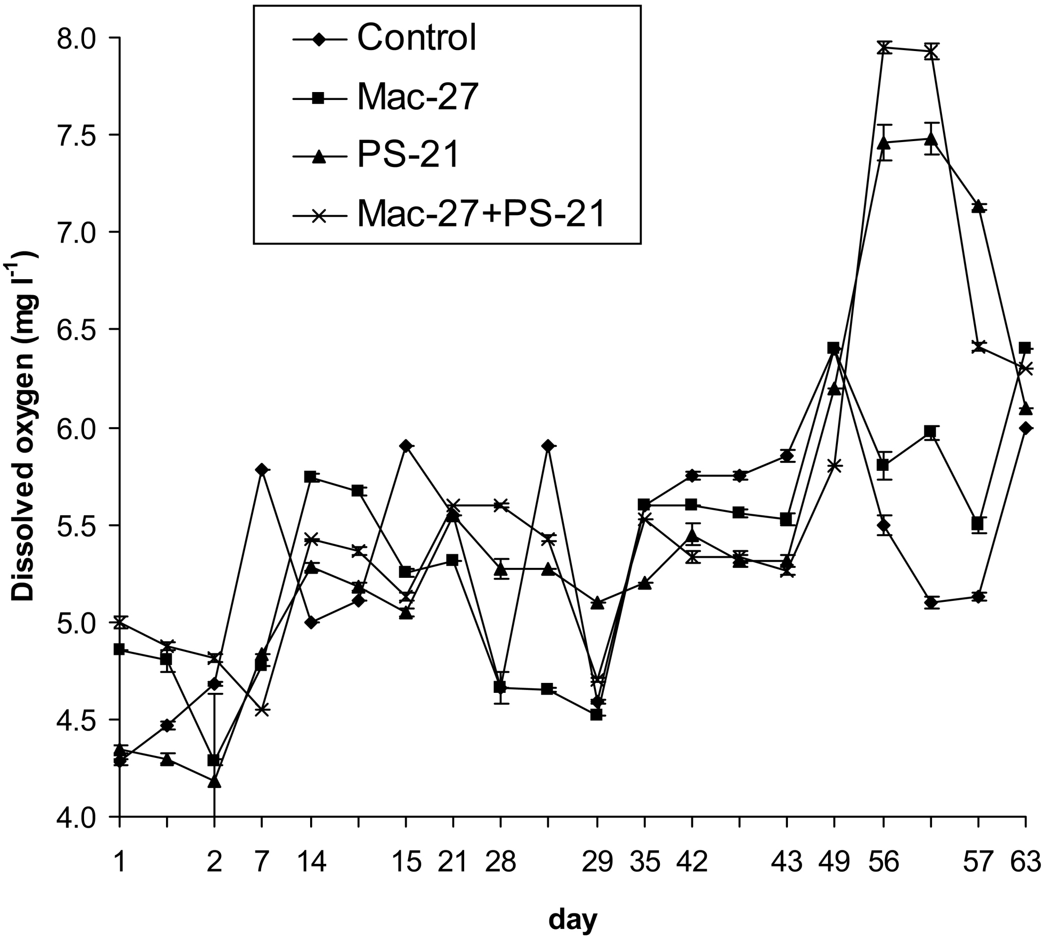


Fig. 1. Variations in viable counts (CFU) from fish ponds inoculated with *Azotobacter chroococcum* strains (Mac-27), *Pseudomonas* (PS-21) and in control ponds. Inoculation day is indicated by arrows (↑)

Effect of microbial inoculation on hydrobiological characteristics of pond waters

Electrical conductivity remained high in microbial inoculated ponds. pH was alkaline in all the treatments and fluctuated between 8.27-8.38. Weekly variations in DO concentration (Fig. 2) showed no significant differences with respect to microbial inoculation, however, the values remained higher in ponds inoculated with mixed culture. Total alkalinity, bicarbonates and turbidity remained high, while carbonates were low in ponds inoculated with mixed culture (Mac-27 + PS-21), followed by PS-21 and Mac-27 alone. No significant effect of microbial inoculation was observed on chlorides, calcium, magnesium, total hardness and sulphates and the values remained high in all the treatments (Table 4).



Control
Mac-27
PS-21
Mac-27+PS-21
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
Dissolved oxygen (mg l-1)
1
2
7
14
15
21
28
29
35
42
43
49
56
57
63
day

Fig. 2. Effect of Mac-27, PS-21 and mixed culture (Mac-27+ PS-21) inoculation on dissolved oxygen (DO) concentration in fish ponds.

In general, release of nutrients viz. total Kjeldahl nitrogen (Fig.3), NO_3-N (Fig.4), and NH_4-N were high in ponds inoculated with mixed culture (Mac-27 + PS-21), followed by ponds inoculated with Mac-27 and PS-21 alone (Table 4). On the other hand, o-PO_4 release was high in ponds inoculated with PS-21 (Fig. 5). No significant variations in NO_2-N levels were observed among different treatments.

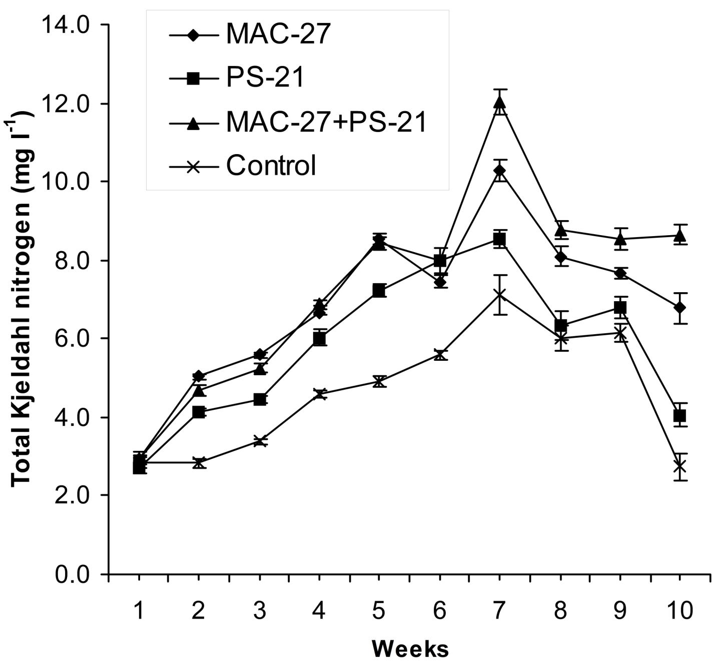


Fig. 3. Weekly variations in total Kjeldahl nitrogen concentration in fish ponds inoculated with Mac-27, PS-21 and mixed culture (Mac-27+PS-21) and control ponds

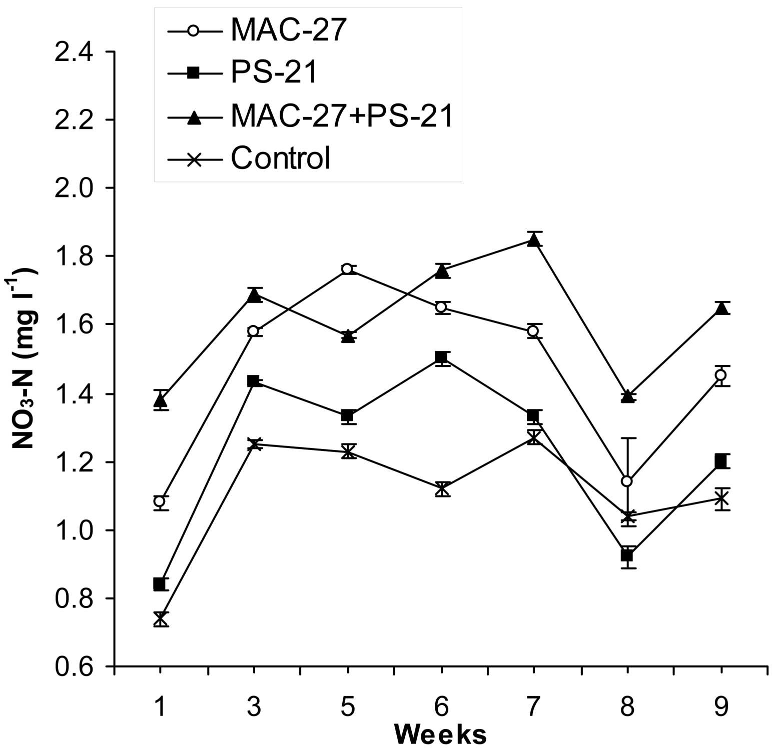


Fig. 4. Weekly variations in NO_3-N concentration in fish ponds inoculated with Mac-27, PS-21, mixed culture (Mac-27+PS-21) and control ponds

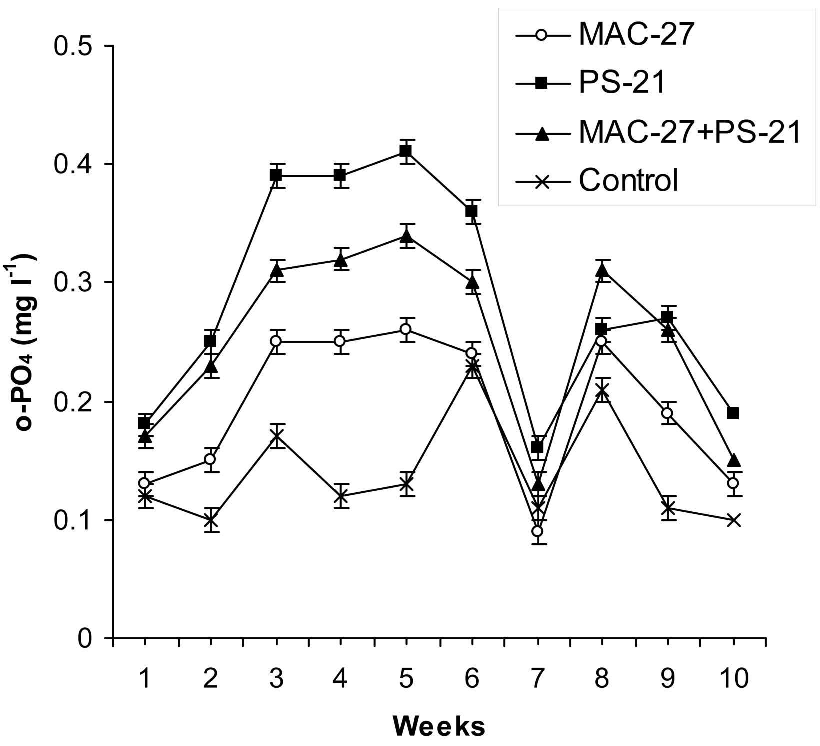


Fig. 5. Weekly variations in o-PO_4 concentration in fish ponds inoculated with Mac-27, PS-21, mixed culture (Mac-27, PS-21) and control ponds.

Primary productivity, pigment concentrations and biotic community

Following inoculation with microbial culture, *a* significant ($P<0.05$) increase in NPP (Fig. 6), GPP (mg C l^{-1} d^{-1}), chlorophyll *a*, pheophytin *a* (µg l^{-1}) and phytoplankton population was observed in all the treatments in comparison to controls (Table 5). High values in most of these parameters, however, were observed in ponds inoculated with mixed culture. No significant ($P<0.05$) variations in plankton (phytoplankton and zooplankton) population (nos.l^{-1}) and species diversity (d) were observed in microbial inoculated ponds (Table 5).

Irrespective of the treatments, plankton communities principally consisted of two groups of phytoplankton (Bacillariophyceae and Chlorophyceae), and one group of zooplankton (copepoda). Phytoplankton were represented by 8 genera, 4 belonging to Bacillariophyceae and 4 to Chlorophyceae. While zooplankton was represented by copepoda (i.e. only *Cyclops*) alone. Among Bacillariophyceae *Synedra* and *Navicula*, while among Chlorophyceae *Closterium* formed the stable community.

Fish growth

No disease was detected during the entire period of investigations. Survival remained high in all the treatments. ANOVA revealed a significant ($P<0.05$) increase in growth performance of milkfish (in terms of mean live weight gain, SGR, growth per day and fish biomass) in all the treatments in comparison with controls (Table 6). High weight gain and biomass was observed in ponds inoculated with mixed culture (Mac-27+PS-21), followed by ponds inoculated with Mac-27 and PS-21 strain alone. A study of length weight relationship (LWR) at the end of the experimental period of 70 days also revealed that the values of exponential 'n' of LWR was high (2.77) for the fish stocked in ponds inoculated with co-culture (Mac-27+PS-21). In other treatments, the value of 'n' fluctuated between 2.00-2.6 (Table 6).

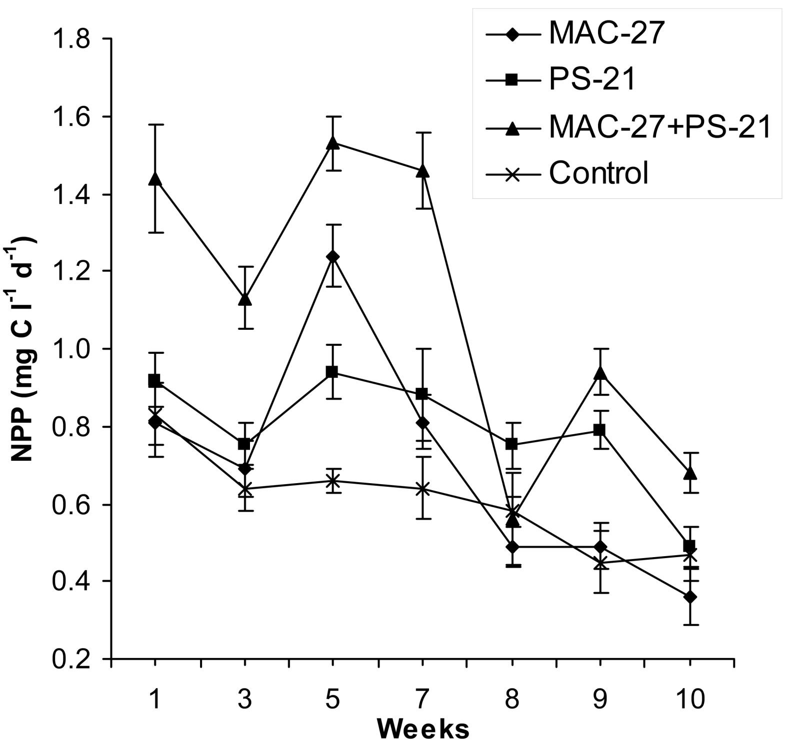


Fig. 6. Weekly variations in NPP (Net primary productivity) levels in fish ponds inoculated with Mac-27, PS-21),

mixed culture (Mac-27 and PS-21) and control ponds

Carcass composition also appears to be significantly affected in different treatments as accumulation of protein, fat and energy were significantly higher in fish ($P<0.05$) grown in ponds inoculated with co-culture (Mac 27+PS-21) in comparison with other treatments (Table 6).

Experiment 2 : Effect of inoculation of high salinity tolerant *G.diazotrophicus* and high temperature tolerant strain of *A. chroococcum* on pond productivity and fish growth.

Viable counts (colony forming units)

Significantly high values of total viable counts were observed in inoculated ponds as compared to controls. Irrespective of the strain inoculated, a significant ($P<0.05$) increase in counts was observed till day 7 and, thereafter a significant ($P<0.05$) decline on day 14 was observed (Table 7).

Hydrobiological characteristics

pH remained alkaline in all the treatments and fluctuated between 8.34-8.36. No significant effect of microbial inoculation was observed on dissolved oxygen, EC, carbonates, chlorides, calcium, magnesium and sulphates. Release of nutrients viz. total Kjeldahl nitrogen, NO_3-N, NO_2-N, NH_4-N and phytoplankton population were high in ponds inoculated with high salinity tolerant strain. On the other hand, total hardness, total dissolved solids, o-PO_4, chlorophyll *a* and zooplankton population were high in temperature tolerant strain (Table 8).

Fish growth

Survival remained high in all the treatments. ANOVA revealed a significant ($P<0.05$) increase in growth performance of milkfish (in terms of man fish weight gain, SGR, growth per day and fish biomass) in inoculated ponds in comparison to controls. High values in growth parameters however, were observed in ponds inoculated with high salinity tolerant strain in comparison to high temperature tolerant strain and controls (Table 9).

The results have further revealed that the rate of nitrogen fixation in terms of per cent increase in total Kjeldahl nitrogen with respect to control ponds were higher in Experiment 1 (21-64%) in comparison to Experiment 2 (5.10-12.0%). On the other hand per cent increase in orthophosphate was high in experiment 2. Bacterial viable counts were higher in experiment 2 where high salinity (*G.diazotrophicus*) or temperature tolerant strain (*A. chroococcum*) were inoculated (Table 10).

Table 4. **Effect of inoculation of Mac-27 and PS-21 on physico-chemical characteristics of pond water stocked with *Chanos chanos* (70-day treatment)**

Parameters	Treatments			
	Mac-27	**PS**	**Mac-27+PS**	**Control**
EC dS m^{-1}	16.48±0.09a	16.49±0.09a	16.73±0.13a	15.98±0.05b
pH	8.27±0.01b	8.32±0.21b	8.38±0.01a	8.29±0.02b
Dissolved oxygen mg^{-1}	5.76±0.09b	5.89±0.13b	6.44±0.12a	5.34±0.08c
Carbonates mg l^{-1}	8.46±0.38a	8.32±0.37a	6.61±0.28b	7.86±0.55a
Bicarbonates mg l^{-1}	203.43±2.24bc	208.79±2.30b	217.4±2.28a	199.14±2.14c
Total alkalinity mg l^{-1}	211.93±2.04c	217.04±2.15b	223.94±2.35a	207.00±1.80c
Chlorides mg l^{-1}	6859.49±66.60a	6918.07±61.11a	6938.49±50.38a	6886.11±76.20a
Total hardness mg l^{-1}	3425.00±40.15a	3414±45.11a	3464.29±37.23a	3430.36±34.28a
Calcium mg l^{-1}	518.90±12.28ab	504.64±13.25b	521.16±11.12ab	542.18±7.78a
Magnesium mg l^{-1}	520.05±9.04a	527.40±10.37a	528.24±9.33a	507.71±7.77a
Total Kjeldahl nitrogen mg l^{-1}	7.13±0.30a	5.73±0.27b	7.80±0.37a	4.74±0.24c
NO_3-N mg l^{-1}	1.38±0.04b	1.13±0.03c	1.52±0.03a	1.05±0.03c
NO_2-N mg l^{-1}	0.75±0.02b	0.80±0.02b	0.68±0.0c	0.88±0.02a
NH_4-N mg l^{-1}	1.26±0.03a	1.08±0.03b	1.25±0.03a	0.94±0.03c
o-PO_4 mg l^{-1}	0.19±0.01b	0.26±0.01a	0.24±0.01a	0.13±0.01c
SO_4 mg l^{-1}	68.68±1.39b	71.16±1.54b	71.44±1.47b	83.01±1.17a
Turbidity NTU	69.42±1.86b	68.61±1.43b	82.73±1.49a	55.61±0.82c

All values are mean±SE of mean

Means with the same letter/s in the same row are not significantly (P<0.05) different

Water temperature during the experimental period dropped from 29.4 to 16.3^{0}C
Mac-27 = Nitrogen fixing strain, PS-21=Phosphate solubilzing strain, Mac-27+PS-21=Mixed culture of both strains
Control=No biofertilizer

Table 5. Effect of inoculation of Mac-27 and PS-21 on biological characteristics of pond water (70 days treatment)

Parameters	Treatments			
	Mac-27	PS	Mac-27+PS	Control
NPP mg C l^{-1} d^{-1}	0.70±0.05bc	0.79±0.03b	1.11±0.06a	0.61±0.02c
GPP mg C l^{-1}	2.20±0.04c	2.33±0.04b	2.86±0.05a	2.00±0.03d
Chlorophyll 'a' μg l^{-1}	1.97±0.04b	1.88±0.04b	2.43±0.05a	1.72±0.03c
Pheophytin 'a' μg l^{-1}	0.75±0.06b	0.73±0.05b	1.26±0.06a	0.64±0.64b
Phytoplankton nos l^{-1}	15975.00±1172.00a	15050.00±944.00a	16150.00±838.00a	11925.00±752.00b
Zooplankton Nos. l^{-1}	750.00±153.00a	425.00±90.00ab	625.00±124.00ab	325.00±65.00b
Phytoplankton (d)	2.22±0.08a	2.43±0.07a	2.37±0.07a	2.30±0.07b

All values are mean±S.E. of mean. Water temperature during the experimental period dropped from 29.4 to 16.3^{o}C
Means with the same letter's in the same row are not significantly (P<0.05) different
Mac-27 = Nitrogen fixing strain of *Azotobacter chroococcum*
PS-21=Phosphate solubilzing strain of *Pseudomonas*
Mac-27+PS-21= Mixed culture

Control=No biofertilizer

Table 6. Effect of inoculation of *Azotobacter* strain Mac-27 and *Pseudomonas* PS-21 on growth performance and carass composition in *Chanos chanos* under field conditions–70 days treatment

Treat ments	INITIAL FISH STOCK			FINAL FISH STOCK (after 70 days)			Increase in mean fish wt. (g) (Mean length cm)	Growth g day^{-1}	CF (k)	LWR
	Stocking density 375m^2	Total Biomass (kg)	Mean fish weight (g) (Length cm)	Survival (%)	Total Biomass (kg)	Mean fish weight (g) (Length cm)				
Mac-27	200	8.62	43.12±0.51a (18.54±0.11)	96.75	17.63b	91.13±2.15b (23.31±0.22)	48.01b (4.77)	0.69b	0.71b	$W=0.000023L^{2.6}$ Log W=-4.63+ 2.6 Log L
PS-21	200	8.75	43.74±0.54a (18.60±0.11)	95.00	13.41c	70.56±0.87c (21.41±0.10)	26.82c (2.81)	0.38c	0.72b	$W=0.00005\times L^{2.33}$ Log W=-4.29+ 2.33 Log L
Mac-27 + PS-21	200	8.64	43.20±0.54a (18.49±0.12)	94.0	20.25a	107.13±4.19a (24.13±0.35)	63.93a (5.64)	0.91a	0.74a	$W=0.000021\times L^{2.77}$ Log W=-4.81+ 2.77 Log L
Control	200	8.67	43.39±0.54a (18.39±0.09)	93.50	10.73d	57.39±0.94d (20.64±0.17)	14.00d (2.25)	020d	0.65c	$W=0.0003\times L^{2.30}$ Log W=-3.88+ 2.30 Log L

Carcass Composion

Treatment	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Phosphorus (%)	Energy (kJ g^{-1})
Initial	71.00±0.17a	17.00±0.09d	3.12±0.12c	3.84±0.04a	0.44±0.02c	6.12±0.07c
Mac-27	68.20±0.18b	18.71±0.10b	3.82±0.11b	3.34±0.14a	0.64±0.01bc	6.95±0.09b
PS-21	68.40±0.13b	18.44±0.12b	3.78±0.11b	3.47±0.16b	0.62±0.01b	6.87±0.06bc
Mac-27+Ps-21	67.65±0.48c	19.10±0.10a	4.15±0.07a	3.01±0.01c	0.67±0.01a	7.20±0.02a
Control	68.48±0.24b	17.94±0.10c	3.46±0.19c	3.51±0.18b	0.55±0.01bc	6.74±0.07bc

Means with the same letter/s in the same column are not significantly (P<0.05) different

Mac-27=Nitrogen fixing strain, PS-21=Phosphate solubilizing strain, Mac-27+PS-21=Mixed culture of both strains
Control= No biofertilize. Condition factor (k) =$Wt \times 10^5/L^3$, where Wt is weight in grams and L=total length in milimeters
Length weight relationship (LWR) : $W=cL^n$ or log W=log c+n log L, where, W=Weight in kg, C=Constant, n=exponential value of length and L=length

Table 7. Variations in viable bacterial counts (CFU) from fish ponds inoculated with salinity tolerant and temperature tolerant strains

	Control (CFU ml^{-1})	Salinity tolerant stain (CFU ml^{-1})	Temperature tolerant strain (CFU ml^{-1})
Day 1			
0 h	$1.55\times10^{4}\pm0.05$	$1.73\times10^{4}\pm0.06$	$1.35\times10^{4}\pm0.21$
1 h after inoculation	$1.42\times10^{4}\pm0.12$	$1.55\times10^{4}\pm0.16$	$1.33\times10^{4}\pm0.10$
Day 7	$2.17\times10^{4}\pm0.10$	$6.35\times10^{4}\pm0.33$	$5.00\times10^{4}\pm0.60$
Day 14			
0 h	$2.19\times10^{4}\pm0.13$	$2.95\times10^{4}\pm0.14$	$2.6\times10^{4}\pm0.09$
1 hr after inoculation	$2.20\times10^{4}\pm0.06$	$3.23\times10^{4}\pm0.020$	$2.78\times10^{4}\pm0.28$
Day 21	$2.73\times10^{4}\pm0.14$	$3.32\times10^{4}\pm0.19$	$2.95\times10^{4}\pm0.36$
Day 28			
0 h	$1.60\times10^{4}\pm0.25$	$1.90\times10^{4}\pm0.05$	$1.20\times10^{4}\pm0.39$
1 h after inoculation	$1.97\times10^{4}\pm0.16$	$1.93\times10^{4}\pm0.08$	$1.98\times10^{4}\pm0.06$
Day 35	$1.97\times10^{4}\pm0.10$	$4.60\times10^{4}\pm0.46$	$4.80\times10^{4}\pm0.58$
Day 42			
0 h	$2.39\times10^{4}\pm0.15$	$2.38\times10^{4}\pm0.33$	$3.73\times10^{4}\pm0.10$
1 h after inoculation	$2.15\times10^{4}\pm0.19$	$2.98\times10^{4}\pm0.22$	$4.05\times10^{4}\pm0.18$
Day 49	$2.09\times10^{4}\pm0.21$	$3.93\times10^{4}\pm0.05$	$4.10\times10^{4}\pm0.18$
Over all mean	**$2.04\times10^{4}\pm0.13$ b**	**$3.07\times10^{4}\pm0.19$ a**	**$2.99\times10^{4}\pm0.26$ a**

All values are mean±S.E.of mean

G. diazotrophicus, Salinity tolerant
A. chroococcum, High temperature tolerant

Table 8. Effect of inoculation of salinity tolerant and temperature tolerant strains on physico-chemical and biological characteristics of pond water stocked with *Chanos chanos*

Parameters	Control	Salinity tolerant strain (*G.diazotrophicus*)	Temperatu... tolerant strain (*A. chroococcum*)
CFU per ml	$2.18 \times 10^4 \pm 0.92$b	$5.0 \times 10^4 \pm 0.4$a	$4.46 \times 10^4 \pm 0.41$a
EC μ mhos cm^{-1}	23.22±0.09a	21.95±0.16b	23.98±0.24a
pH	8.36±0.02a	8.34±0.2a	8.35±0.06a
Dissolved oxygen mg l^{-1}	6.16±0.42a	5.57±0.19ab	5.30±0.23ab
CO_3 mg l^{-1}	5.15±0.47ab	5.33±0.38ab	7.00±0.577a
HCO_3 mg l^{-1}	216.05±1.17a	199.67±0.64b	209.66±1.92b
Alkalinity mg l^{-1}	219.9±1.09a	205.33±0.75b	217.00±1.85a
Chlorides mg l^{-1}	5761.35±54.13a	5736.21±20.71a	5727.92±22.16a
Total hardness mg l^{-1}	3104.00±27.94b	3283.33±27.06b	3525.00±46.26a
Calcium mg l^{-1}	354.77±10.87ab	355.62±7.08b	368.79±23.58a
Magnesium mg l^{-1}	534.07±15.59b	581.23±6.16ab	633.79±19.90a
Total kJeldahl nitrogen mg l^{-1}	4.74±0.06b	5.31±0.08a	4.98±0.09b
NH_4-N mg l^{-1}	1.15±0.01b	1.36±0.09a	1.16±0.03b
NO_2-N mg l^{-1}	0.80±0.01a	0.83±0.05a	0.71±0.01b
NO_3-N mg l^{-1}	1.20±0.01b	1.32±0.02a	1.24±0.02b
o-PO_4 mg l^{-1}	0.09±0.06c	0.32±0.01a	0.19±0.01b
Sulphate mg l^{-1}	73.86±1.35b	78.19±1.40a	78.31±0.68a
TDS mg l^{-1}	7641.30±34.6b	7340.00±18.35c	7962.50±49.28a
Chlorophyll 'a' μg l^{-1}	3.4±0.13b	3.01±0.05c	3.82±0.09a
Phytoplankton nos. l^{-1}	8687.50±3814.28 c	12000.00± 456.43a	11312.00±369.00a b
Zooplankton	7062.00±371.95b	7550.00±857.67b	9042.00±262.00a

nos. l^{-1}

All values are mean± SE of mean

Mean with the same letter's in the same row are not significantly different

Water temperature during the experimental period fluctuated between 26.0– 32.2^{o}C

Table 9. Effect of inoculation of Salinity tolerant (*G. diazotrophicus*) and temperature tolerant (*A. chroococcum*on growth performance of *Chanos chanos* under field conditions–70 days treatment.

Treat ments	INITIAL FISH STOCK			FINAL FISH STOCK (after 60 days)			Increase in mean fish wt. (g) (Mean length cm)	Growth g d^{-1}	SGR
	Stocking density 375m^2	Total Biomass (kg)	Mean fish weight (g) (Length cm)	Survival (%)	Total Biomass (kg)	Mean fish weight (g) (Mean fish length cm)			
Control	375	138.75	0.37±0.03a (4.2±0.07)	91.19	9.43c	27.59±0.64c (16.19±0.23)	27.22c (12.00)	0.45c	7.22±0.16c
Salinity tolerant strain	375	161.25	0.43±0.06a (4.33±0.10)	88.33	16.30a	49.26±1.54a (18.63±0.29)	48.83a (14.30)	0.81a	7.99±0.26a
Temperature tolerant strain	375	127.50	0.34±0.03a (3.53±0.10)	88.26	12.85b	38.96±1.14b (17.70±0.19)	38.62b (14.17)	0.64b	7.90±0.15b

Mean with the same letter in the same row are not significantly (P<0.05) different
Control – No biofertilizer

Table 10. Per cent increase in total Kjeldahl nitrogen and o-PO_4 in comparison to controls

	Treatment	Per cent increase in total nitrogen	Per cent increase in orthophosphate
	Experiment 1		
1	Mac-27	50.42	46.15
2	PS-21	21.00	100.00
3	Mac-27+PS-21	64.00	84.00
	Experiment 2		
1	High salinity tolerant	5.10	111.00
2	high temperature tolerant	12.00	255.00

Chapter-V

Discussion

Since *Azotobacter* is a highly aerobic organism (Lakshminaryana, 1993), therefore inoculation of *Azotobacter* in freshwater fish ponds resulted in decrease in dissolved oxygen (DO) concentration (Garg *et al.,* 1998; Garg and Bhatnagar, 1999a, 2002a). No drastic reduction in DO concentration was observed in the present studies, which may be attributed to the comparatively large surface area (375 m^2) of the ponds used in the present studies. The pH values and alkalinity indicate that the pond waters were well buffered and thus remained suitable for the release of nutrients in optimum concentrations required for growth and survival of biotic communities. Significantly high ($P<0.05$) values of alkalinity, total Kjedahl nitrogen, NO_3-N, NO_2-N, NH_4-N and o-PO_4 in microbial inoculated ponds indicate that ponds were in high trophic status. Statistically significant positive correlation of DO with fish weight gain ($r=0.25$, $P<0.001$; $r=0.52$, $P<0.01$), NPP ($r=0.20$, $P<0.05$; $r=0.25$, $P<0.01$) and GPP ($r=0.33$, $P<0.01$; $r=0.41$, $P<0.001$) was observed both in

Experiment 1 and in Experiment 2, respectively. This may indicate that DO was at optimal levels which favoured high pond productivity/fish growth.

The rate of nitrogen fixation in terms of total Kjeldahl nitrogen, NO_3-N and NH_4-N remained significantly ($P<0.05$) high in ponds inoculated with mixed culture in comparison with other treatments and control ponds in Experiment 1. Total Kjeldahl nitrogen showed a significant ($P<0.05$) and positive correlation with NO_3-N ($r=0.60$, $P<0.001$; $r=0.40$, $P<0.001$), NH_4-N ($r=0.35$, $P<0.001$; $r=0.56$, $P<0.0001$), phytoplankton population ($r=0.82$, $P<0.001$; $r=0.61$, $P<0.0001$), chlorophyll *a* ($r=0.34$, $P<0.001$; $r=0.27$, $P<0.01$), NPP ($r=0.18$, $P<0.05$; $r=0.21$, $P<0.05$) and GPP ($r=0.13$, $P<0.05$; $r=0.18$, $P<0.05$) both in Experiment 1 and in Experiment 2. These results clearly reveal that nitrogen fixation, its conversion and utilization is a continuous process. In experiment 2 rate of nitrogen fixation in terms of total Kjeldahl nitrogen, NH4-N and NO3-N were high in ponds inoculated with salinity tolerant strain indicating this strain can also be used as biofertilizer in inland saline groundwater ponds. DO (Ganf and Horne, 1975) and Ammonia (N-NH_3, N-NH_4) (McFarland and Toetz, 1988) are considered to be the strong inhibitors of nitrogen fixation, however, biological nitrogen fixation process in different treatments was not affected. According to Pearl (1985), calm conditions in the water allow the creation of anoxic microzone around the cells, which may be considered condusive to nitrogen fixation. Even though NH_4-N levels were high in different treatments, the rate of nitrogen fixation was not affected, which can be attributed to the lower effect of ammonia in water than in sediment (Howarth *et al*., 1988a). Nitrogenase activity appears to be closely related to phosphorus availability and phosphorus was not a limiting factor in any of the treatment thus allowing the biological nitrogen fixation process to proceed uninterrupted.

Bacterial viable counts showed an increase upto day 7 following inoculation and thereafter a significant ($P<0.05$) decline in viable counts on day 14 was observed in both the experiments. Viable counts varied with respect to the microbial strain used in the experiments (Fig 1,Table 2 and 6), however, the rate of nitrogen fixation was not affected in relation to multiplication rate of inoculated bacteria. These results suggest that growth rate and nitrogen fixing efficiency of *Azotobacter* need not to have a direct relationship. Ninawe and Paulraj (2003) have also reported that nitrogen fixing efficiency is independent of growth rate of bacterial strain.

Eventhough, viable counts were high, however, the rate of nitrogen fixation by high salinity/temperature tolerant strains remained low in comparison to the nitrogen fixing strains used in experiment 1. Low rate of nitrogen fixation may either be attributed to the high salinity of the medium or to the high water temperature.

A significant and positive correlation of NO_3-N with phytoplankton (r=0.62, P<0.01 and 0.48, P<0.005) and o-PO_4 with chlorophyll *a* (r=0.29, P<0.001), NPP (r=0.23, P<0.001) and GPP (r=0.29, P<0.001) further indicate that nutrients increase natural fertility of the water body on which primary productivity depends (Delince, 1992).

High fish growth observed in the present studies also coincided with high plankton population and nutrients. Statistically also fish weight gain showed a positive correlation with alkalinity (r=0.19, P<0.05), NO_3–N (r=0.46, P<0.001, r=0.92 P<0.0001), total Kjeldahl nitrogen (r=0.34, P<0.001, r=0.72, P<0.001), o-PO_4 (r=0.15, P<0.05), phytoplankton population (r=0.19, P<0.05, r=0.78, P<0.001), zooplankton population (r=0.17, P<0.05), chlorophyll *a* (r=0.38, P<0.001), NPP (r=0.26, P<0.001) and GPP (r=0.37, P<0.001) clearly revealing that fish growth is positively correlated with the trophic status of the ponds also. Many other studies (Garg and Bhatnagar, 1999b; 2005; Garg *et al*., 1998, 2001; Knud-Hansen and Batterson, 1994) have also reported that fish growth is significantly correlated with the trophic status of pond waters.

Although values of constant 'n' (LWR) for *Chanos chanos* were high in inoculated ponds in comparison to controls, the values were <3, indicating that *Chanos chanos* grown in inland saline groundwater (13.0-13.5 ppt) do not follow the cube law. Thus more studies are required to investigate the reasons for the deviation of growth patterns from cube law.

In the present studies a decrease on day 7 in total viable counts was observed after inoculation, while earlier studies have shown a stable population or a decrease in viable counts only at the end of 15 days (Garg and Bhatnagar, 2002b). Since present studies were conducted in brackishwater therefore, a drop in viable counts on 7th day after inoculation may be attributed either to the non-availability of sufficient organic base due to slow degradation owing to salinity of the medium or to the intolerance of *Azotobacter* to high salinity of the medium. Since the values of most of the parameters viz. nutrients, alkalinity, NPP, GPP and pigment concentrations remained high in ponds inoculated with a mixed culture (Experiment 1) and in salinity tolerant strains (Experiment 2), it would be advantageous to use a mixed culture of nitrogen fixing, phosphate solubilizing strains of *Azotobacter* along with salinity tolerant *G. diazotrophicus* strain in inland saline groundwater ponds for reducing the use of inorganic fertilizers and thereby preventing the deterioration of water quality. Use of microbial bioinoculants in inland saline groundwater ponds for the culture of euryhaline fishes such as milkfish, *Chanos chanos* and mullet. *Mugil cephalus* will help in the development of an eco-friendly and economically viable technology required for sustainable aquaculture. However, many more studies are required to investigate the optimum frequency (weekly/fortnightly) and optimum dose of different strains of *Azotobacter* and *Gluconacetobacter* required to fertilize inland saline groundwater ponds for obtaining high fish production.

Chapter-VI

Summary and Conclusion

Fish culture ponds stocked with milkfish, *Chanos chanos* at 10,000 ha^{-1} and fertilized with cowdung at 7500 kg ha^{-1} yr^{-1} were inoculated with nitrogen fixing *Azotobacter chroococcum* (Mac 27), phosphate solubilizing *Pseudomonas* (PS-21) and also in mixed culture in experiment 1. High temperature tolerant (HT-51) strain of *Azotobacter chroococcum* and high salinity tolerant, (*Gluconacetobacter diazotrophicus* 35-47) strain were used in experiment 2. Hydrobiological parameters of pond waters, net primary productivity (NPP) and fish growth were studied. Significantly ($P<0.05$) high values for alkalinity, kjeldahl's nitrogen, NO_3-N, turbidity, pigment concentration and fish growth were observed in ponds inoculated with co-culture, followed by Mac-27 and PS-21. o-PO_4 concentration was significantly ($P<0.05$) high both in PS-21 and in mixed culture inoculated ponds. In experiment 2, alkalinity, hardness, TDS and chlorophyll *a* concentration were significantly ($P<0.05$) higher in ponds inoculated with high temperature tolerant mutant of *A. chroococcum*, while total kjeldahl nitrogen, NH_4-N, NO_2-N, o-PO_4 concentration and fish growth were high in ponds inoculated with salinity tolerant strain of *G. diazotrophicus.* . Not much variations in DO concentration were observed among different treatments. Per cent increase in biological nitrogen fixation indicated high values in experiment 1 as compared to experiment 2. Irrespective of bioinoculants, a significant ($P<0.05$) increase in viable counts were observed upto day 7 after inoculation, and thereafter a significant decline in microbial population was observed.

PART-II

Effect of probiotics supplement on digestibility and excretion of metabolites in Nile Tilapia, *Oreochromis niloticus* (Linn.)

Chapter-I

Introduction

Vaccines are being developed and marketed and they generally cannot be used as a universal disease control measure in aquaculture. Juvenile fish are fully immunocompetent and do not always respond to vaccination. Vaccination by injection, sometimes are the only effective route of administration, is impractical when supplied to small fish or large number of fish. This situation is avoided by an alternative in the production system through the use of beneficial bacteria to fight against pathogenic bacteria i.e., through the use of probioticss which is an acceptable practice in aquaculture. The health of the fish thus can be improved by the elimination of pathogens or at least by minimizing their effects in aquaculture (Patra and Bandyopadhyay, 2003).

Due to outbreak of disease in aquaculture industry in the last 20 years, use of antibiotics have lead to the development of drug-resistant strains resulting in reduction of natural defense mechanism in the aquacultural animals. On the other hand, probiotics can provide better immune response, increase survival and promote growth and nutrient utilization. Its use can thus assure the nutritional security in the next millennium. Probiotics for aquaculture are generally selected by their ability to produce antimicrobial metabolites; however, attachment to intestinal mucus is important in order to remain within the gut of the host (Vine *et al.,* 2004).

The beneficial effects of probiotics have been attributed to their ability to promote the immunological and non-immunological defense barrier in the gut, normalization of increased intestinal permeability and altered gut microflora. They have also been shown to enhance humoral immune response, and consequently to promote the intestine's barrier. Probiotics have also been shown to stimulate non-specific host resistant to microbial pathogens, and thereby aid in immune elimination (Predigon *et al.,* 1995). Probiotics are cultures of special microorganisms, which have been used as feed additives. Probiotics are applied not only as feed supplements or pharmaceuticals but also in dairy products, fruit juices, chocolates, and even meat products. The selection of a suitable strain of a microorganism can be regarded as the primary requirement for their use as a probiotics. These cultures must be able to pass the stomach-duodenum barrier in a viable state and to multiply at the site in the intestine. Additionally, they must be capable of producing antagonistic metabolites against a dominating saprophytic microflora resulting in a competitive growth.

The research on probiotics for aquatic animals is increasing with the demand for environmental-friendly aquaculture. The probiotics were defined as live microbial feed supplements that improve health of terrestrial and aquatic livestock. The gastrointestinal microbiota of fish and shellfish are peculiarly dependent on the external environment, due to the water flow passing through the digestive tract. Most bacterial cells are transient in the gut. Extension of the probiotics concept is pertinent when administrated microbes survive in the gastrointestinal tract. Otherwise, more general terms are suggested, like biocontrol when the treatment is antagonistic to pathogens, or bioremediation when water quality is improved. The first probiotics tested in fish were commercial preparations devised for land animals. Most attempts to identify probiotics have been undertaken by isolating and selecting strains from aquatic environment. These microbes were vibrionaceae, pseudomonads, lactic acid bacteria, *Bacillus* sp. and yeast. Three main characteristics have been searched in microbes as candidates to improve the health of their host. (1) The antagonism to pathogens was shown *in vitro* in most cases. (2) The colonization potential of some candidate probionts was also studied. (3) Challenge test confirmed that some strains could increase the resistance to disease of their host. Many other beneficial effects may be expected from probiotics, e.g., stimulation for immune system. The most promising prospects are sketched out, but further research is needed towards standardization of this beneficial practice. The first question unanswered in many cases, is the fate of the probiotics in the rearing medium and in gastrointestinal tract. It is essential to invstigate the best way of introduction and optimal dose of the probiotics.

Some information on the effect of probiotics for Indian major carps and other indigenous fishes of major economic importance is available in our country and keeping above points in consideration; the present study was therefore undertaken to evaluate the effects of probiotics supplement for the improvement of growth of *Oreochromis nilotics* (L.). The study includes assessment of growth and associated physiological and biochemical changes in fish due to the incorporation of different doses of probiotics supplement in fish feed.

Chapter-II

Review of Literature

'Pro' means favor and 'bios' means 'life' literally means "for life" and an antonym to antibiotics, which involves multiplying few useful microbes to compete with the harmful ones, thus suppressing their growth. Antibiotics have been used profusely as therapeutic agents and as growth promoter since 1950 onwards, however, their excessive use in the production system has led to the development of resistant bacterial pathogens. In 1969, Swan committee restricted a list of antibiotics used in treatment of diseases. The therapeutic use of antibiotics result in intestinal disorders. This is believed to be due to the different effects of antibiotics, which tends to remove indigenous gut flora along with disease causing agents. This situation is avoided by an alternative situation in the production system through the use of beneficial bacteria to fight against pathogenic bacteria i.e., probiotics that is an acceptable practice in aquaculture. The health of the animal is improved by the elimination of pathogens or at least minimizing the effects, which benefits aquaculture.

The first application of probiotics in aquaculture seems relatively recent (Kozasa, 1986), but the interest in such environment friendly treatment is increasing rapidly. Scientific evaluation corroborated seldom the first empirical trials, and the information was mainly spread by "gray literature" (Gatesoupe, 1999). However, a growing number of scientific papers have dealt except with probiotics, and it is now possible to survey the state of the art, from the empirical use to the scientific approach.

Definition Of Probiotics

Biological control has been described as the utilization of natural enemies to reduce the damage caused by noxious organisms to tolerance limits (Debach and Rosen, 1991) or more precisely, the control or regulation of pest populations by natural enemies (Smith, 1919). Strictly speaking, a probiotics ought not to be classified as a biological agent, since a probiotics microorganism doesn't necessarily attack the noxious agent (pathogen).

Elie Metchnikoff's work in the beginning of this century is regarded as the first research conducted on probiotics (Fuller, 1992). He described them as "microbes ingested with the aim of promoting good health." This definition was modified to "organisms and substances which contribute to intestinal microbial balance" (Parker, 1974) and latter by Fuller (1989), to "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Gibson and Roberfroid (1995) introduced the concept of 'probiotics'. It is defined as non-digestible food ingredients that beneficially effect the host by selectively stimulating the growth and the activity of one or a limited number of bacteria in the colon, and thus improves host health. Ruiz-Ponte *et al*., (1998) defined it as a beneficial microorganisms, which can protect organisms against pathogens or enhance their growth.

Probiotics are now also being used in aquaculture and therefore, the definition may have to be modified. In aquatic animals, not only the digestive tract is important but also the surrounding water. Jory (1988) defined probiotics from aquaculture point of view as culture (single or mixed) of selected strains of bacteria that are used in culture and production systems (tanks, ponds and others) to modify or manipulate the microbial communities in water and sediment to reduce or eliminate selected pathogenic species of microorganisms and generally improve growth and survival of the targeted species. Gatesoupe (1999) defined probiotics as "microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health". Gram *et al.* (1999) broadened the definition by removing the restriction to the improvement of the intestine: "a live microbial supplement which beneficially affects the host animals by improving its microbial balance". Neither should probiotics be classified as growth promoters, since their action is not confined to improved growth but is associated with a general improvement in health.

Evaluation of Probiotics in Aquaculture

The range of probiotics examined for use in aquaculture has encompassed gram-negative and gram-positive bacteria, bacteriophases, yeasts and unicellular algae. In particular probiotics have been reported to be successful with a wide range of invertebrates (Gomez-Gill *et al.,* 2000; Riquelme *et al., 2000)* and vertebrates (Makridis *et al.,* 2000; Verschuere *et al.,* 2000). There is some evidence of host specificity but the significance of these observations still awaits further study (Fuller, 1992; Salminen *et al*., 1997*)*. To date, probiotics have been used in artificial feeds (Robertson *et al.,* 2000), live feed, i.e. artemia and rotifers (Gatesoupe, 1991; Harzevili *et al.,* 1998) and in water (Austin *et al.,* 1995; Moriarty, 1999; Ringo and Birkbeck, 1999).

Aerobic gram-positive endospore-forming bacteria i.e. *Bacillus* sp. have been evaluated as probiotics, with use for improvement of water quality by influencing the composition of water born microbial populations and also by reducing the number of pathogens in the vicinity of the farmed species(Wang *et al.,* 1999). Thus, the bacilli are thought to antagonize potential pathogens in the aquatic environments. This is curious because it is generally accepted that laboratory cultures do not survive well when re-introduced into the natural environments; the cells being often out competed/ antagonized by the natural micro flora(Austin,1988). Nevertheless, a direct benefit to the use of the bacilli was the reduction in the use of chemicals in the aquatic environment and in enhanced growth of framed species (Wang *et al.,* 1999).

The use of probiotics has been accompanied by a concomitant reduction in the use of antimicrobial compounds (particularly antibiotics) used in aquaculture and in improved appetite and / or growth performance of the farmed species. Apart from, laboratory preparation of bacteria, some workers have used commercially available products. For example, Queiroz and Boyd (1998) and Moriarty (1998) used commercial preparations containing *Bacillus sp.* in catfish and shrimp ponds, respectively. Furthermore, Kennedy *et al*. (1998) used *Bacillus* 48 to enhance the quality and viability of common snook, *Centropomus undecimalis* (Bloch). These workers found that *Bacillus* improved the survival of larvae, increased food absorption by enhancing protease level and gave better growth. Also the probiotics decreased the number of suspected pathogenic bacteria in the gut. It is noteworthy that Chang and Liu (2002) used *Bacillus toyoi* and *Enterococcus fascism* SF 68, from commercial products to reduce Edwardsiellosis in European eel, *Anguilla anguilla* (L.). The study indicated that *Enterococcus faecium* SF 68, but not *Bacillus toyoi*, reduced mortalities in eels, and suppressed the growth of *Enterococcus tada in vitro*. It is relevant to note that *Enterococcus faecium* has long been known as probiotics for humans, where as *Bacillus toyoi* has been used with terrestrial animals. *Enterococcus faecium* has also been useful in improving growth when fed to sheat fish, *Silurus glanis* L. Thus, after feeding for 58 days with a dose equivalent to 2×10^{8} bacteria g^{-1} of food, the experimental fish achieved better growth (~11%) compared with the controls (Bogut *et al.,* 2000). Also, the enterococci influences the microflora of the intestine, reducing the incidence of *Escherichia coli, Staphylococcus aureus* and *Clostridium* sp.

DS-12, which was assigned to *Weissella hellenica* by DNA; DNA hybridization (Cai *et al.,* 1998) was one of 199 cultures recovered from the intestinal contents of farmed flounder, *Pralichthys olivaceus*, in South Korea, and was antagonistic to some bacterial fish pathogens and is regarded to have potential as a probiotic (Byun *et al.,* 1997). An isolate of *Micrococcus luteus* was found to have potential in combating *Aeromonas salmonicida* infection in rainbow trout, *Oncorhyanchus mykiss* (Walbaum) (Irianto and Austin, 2002).

The lactic acid producing bacteria, i.e. putative lactobacilli (Gildberg and Mikkelsen, 1998) have been the focus of much interest. As a tropical example, Gatesoupe (1991) reported the benefit of using *Lactobacillus plantarum* and *Lactobacillus halveticus* in turbot, *Scophthalmus maximus* (L.), leading to enhanced growth. The human probiotic, *Lactibacillus rhamnosus*ATCC (American Type Culture Collection, Rockville, MD, USA) 53101, was administered at a dose of 10^9 and 10^{12} cells g^{-1} of feed to rainbow trout for 51 days, and reduced mortalities from 52.6 to 18.9% (10^9 cells g^{-1} of feed) and to 46.3% (10^{12} cells g^{-1} of feed) following challenge with *Aeromonas salmonicida* (Nikoskelainen *et al.,* 2001). It is apparent that increased dosage is not necessarily reflective of superior protection. In another example, Gildberg *et al.* (1997) reported that the administration of

Carnobacterium divergens to Atlantic cod, *Gadus morhua* L., fry resulted in resistance to *Vibrio anguillarum*. Moreover, Harzevili *et al.* (1998) used *Lactococcus lactis* AR 21, which stimulated the growth of rotifers and inhibited *Vibrio anguillarum*. Similarly, encouraging data were obtained by Byun *et al.* (1997) and Suyanandana *et al.* (1998) using *Lactobacillus* as feed additives for flounder and tilapia, respectively Conversely, Gildberg *et al.*(1995) did not find any improvement in using lactic acid bacteria, isolated from salmon intestine, with Atlantic salmon, *Salmo salar* L., fry challenged with *Aeromonas salmonicida*.

The *in vivo* results of Joborn *et al.* (1997) on *Carnobacterium inhibens* demonstrated that the bacteria were metabolically active in both intestinal mucus and faeces of salmonids. Other bacteria have been shown to improve the culture of larval crab, Pacific oyster and turbot (Nogami and Maeda, 1992; Douillet and Langdon, 1994; Gatesoupe, 1994). Thus, *Vibrio proteolyticus* improved protein digestion in juvenile turbot when administered by oral administration (DeSchrijver and Ollevier, 2000). Also, Douillet and Langdon (1994) showed that stain CA 2, which was probably an *Alteromonas*, increased the survival of Pacific oyster, *Crassostria gigas*, when administered in water.

Although there have been no published reports on the use of probiotics in Chinese aquaculture, some researchers have been studying the potential benefit of using phototrophic bacteria of the genus *Photorhadobacterium* in grow out ponds culturing *Penaeus chinensis* (Irianto and Austin, 2002).

PROBIOTICS FOR SUSTAINABLE AQUACULTURE

Microbes are very important and have critical roles in aquaculture systems, including shrimp farming at both the hatchery and grow out level, because water quality and disease control are directly related and closely affected by microbial activity. Probiotics protection can be due to different mechanisms, such as nutritional competition or production of antibacterial substances. Improved hygiene and bio-security, development of probiotics and immune-stimulants, and improvement of artificial feeds promise better post larval fitness while reducing cost of reliability of production (Borwdy, 1998).

During growth of fish spawn and prawn larvae in aquaculture, the first food supplied is usually the rotifer; *Brachionus plicatilis* and algae are commonly included in the system as food for the rotifers, thereby maintaining their nutrient quality. The addition of lactic acid bacteria to rotifers fed to turbot larvae, *Scophthalmus maximus*, was found to improve growth performance (Gatesoupe, 1991) and increase resistance against pathogenic *Vibrios,* (Gatesoupe, 1994).

Aquatic animals are quite different from the land animals for which the probiotics concept was developed, and a preliminary questions is the pertinence of probitics application to aquaculture. As fish are poikilothermic, their gut flora is temperature dependent, i.e. there is an increase in total counts and in the proportion of anaerobic forms with temperature. The state-of-the-art concerning probiotics is not as advanced in fish as it is in homeothermic vertegrates, and further studies are thus required in the field to promote the environmental friendly development of fish culture (Mishra *et al.,* 2001).

The digestive tract of fishes contains a much higher number of microorganisms than the surrounding water, as many as 10^8 cells g^{-1} (Ringo *et al.,* 1995). Reports on the presence of lactic acid bacteria in the intestinal microbiota of fish (Schroder *et al.,* 1980), suggest that there exists lactic acid bacteria that constitute non-pathogenic members of the indigenous intestinal microflora of healthy fish. After hatching, the gastrointestinal tract of Atlantic cod (*Gadus morhua*) larvae are colonised by almost the same bacterial genera as found in the eggs (Hansan and Olafsen, 1989). Dry feed containing lactic acid bacteria (*Carnobacterium divergens*) isolated from Atlantic cod (*Gadus morhua*) intestines improved disease resistance of cod fry exposed to a virulent strain of *Vibrio anguillarum* (Gildberg *et al.,* 1997). Some of the bottleneck in ensuring long-term benefits of probiotic-feeding are, competition for attachment size, i.e., colonization, and antagonism shown by these organisms in terms of reduction in pH, production of secondary metabolites, and production of bacteriocins etc.

Some lactic acid bacteria are pathogenic to fish (Cone, 1982). Pathogenic lactic acid bacteria such as Streptococcus, Enterococcus, Leuconostoc, Lactobacillus etc. belong to be normal microbiota of the gastrointestinal tract in healthy fish and have been detected from kidney, liver, spleen and heart (Ringo and Gatesoupe, 1998) and are reported to enhance growth in a number of fish species (Bandyopadhyay, 2004).

Present study examines the effect of probiotics (*Lactobacillus sporogenes*) on growth performance of *Oreochromis niloticus* under laboratory conditions.

Chapter-III

Materials and Methods

Diet preparation

Five experimental diets with containing varying concentrations of *Lactobacillus sprogenes* (Probiotics) (0.25, 0.5, 0.75 and 1.0%) were formulated using processed full fat soybean as the protein source. The dietary ingredients and proximate composition of the formulated diets are given in Table 11.

Table 11. Ingredient content (%) and proximate analysis (% dry weight basis) of five experimental diets with different levels of probiotics (g 100 g^{-1} of diet)

Ingredient	Diets				
	D0 (control)	D1	D2	D3	D4
Groundnut oil cake	65.00	65.00	65.00	65.00	65.00
Rice bran	3.20	2.95	2.70	2.45	2.20
Wheat flour	3.20	3.20	3.20	3.20	3.20
Processed soybean[a]	26.60	26.60	26.60	26.60	26.60
Chromic oxide (Cr_2O_3)	1.00	1.00	1.00	1.00	1.00
Calcium	0.50	0.50	0.50	0.50	0.50
Phosphorus	0.50	0.50	0.50	0.50	0.50
Probiotics[b]	-	0.25	0.50	0.75	1.00
Proximate analysis % (analysed values)					
Parameters					
Dry matter	83.18±0.01	93.17±0.01	93.17±0.01	93.18±0.02	93.17±0.02
Crude protein	39.70±0.28	39.70±0.16	39.81±0.17	39.59±0.22	39.87±0.32
Crude fat	8.13±0.26	8.38±0.35	8.06±0.33	7.94±0.44	7.88±0.35
Crude fibre	5.81±0.21	6.00±0.19	5.75±0.13	5.75±0.19	5.75±0.19
Ash	6.38±0.13	6.44±0.15	6.56±0.18	6.69±0.19	6.56±0.24
Nitrogen free extract (NFE)	33.17±0.37	32.65±0.40	32.99±0.37	33.22±0.38	33.12±0.55
Phosphorus	0.60±0.02	0.59±0.02	0.64±0.02	0.67±0.02	0.63±0.02
Gross energy (kJ g^{-1})	18.29±0.10	18.30±0.09	18.26±0.10	18.19±0.13	18.22±0.11

[a] Soybean was hydrothermically processed in an antoclave at 121^{o}C (15lbs for 15 minute) to eliminate antinutritional factors (ANFs) (Garg *et al.,* 2002)

[b] Probiotics (SPILAC) : Each kg contains-Lactobacillus sporogenes-33,200 cfu. Liver extract-5320mg. Yeast estract (*Saccharomyces cervisiae*) 1250mg. Alpha amylase-5000mg. SPIRULINA-5320mg. Ascorbic acid coated-500 mg. Xanthophyll-9000 μg. Chlorophyll-9500 μg. GLA-38000 μg. Protein-100 gm.

Experimental Design

Nile tilapia, *Oreochromis niloticus* fry were collected from the fish farm attached to the Department of Zoology and Aquaculture. Experiment was conducted in glass aquaria (60×30×30 cm) with aeration facilities in the laboratory where the temperature was kept as 25±1^{o}C and a lighting schedule of LD 12:12. The salinity of the water in the aquaria was kept at 10.0 ppt.

After an initial 10-day acclimation period, fry (mean body weight : 1.22±0.04g) were randomly distributed among the aquaria, with 20 fish per aquarium. Each diet treatment was tested in replicate of four (four aquaria per diet). All fish were fed twice daily, at 08:00h and at 14:00 h. The feeding rate was at 5% body weight day^{-1} for the whole rearing period of 70 days, and the amount of feed was adjusted every tenth day following a bulk weighing of each group of fish. The fish were exposed to their respective diet for 4h during each ration, thereafter, the uneaten feed was siphoned out, stored and dried separately for calculating the feed conversion ratio (FCR). The faecal matter voided by the fish in each aquarium was also collected by siphoning, dried in a hot air oven (60^{o}C) and subsequently analysed for digestibility estimations. The water in the aquaria was renewed daily with water that had been stored and adjusted to the laboratory temperature (25^{o}C). At the termination of the experiment, fish from all the treatments were weighed (length was also recorded) individually to the nearest gram and processed for subsequent analyses. Eight fish were obtained from each aquarium and kept on an ice tray. The viscera of the fish was extirpated for the calculation of the viscero-somatic index (VSI). Liver was removed for culculating HSI and also for the estimation of liver glycogen (Dubois *et al.,* 1956).

Muscle was extirpated and used for the estimation of muscle protein (Lowry *et al.,* 1951).

Intestine was processed for the determination of protease (Walter, 1984) and amylase (Sawhney and Singh, 2000) lipase and cellulase enzyme activity (Thimmaiah, 1999).

Analytical Techniques

The feed ingredients, experimental diets, faecal matter samples and fish carcass (initial and final) were analysed following AOAC (1995). Cr_2O_3 levels in both the diets and the faecal samples were estimated spectrophotometrically following the method of Furukawa and Tuskahara (1966). pH and dissolved oxygen were monitored using an automatic analyser (model F-set-3; Merck, Germany). AT the end of the feeding schedule, water samples from each aquarium were collected at 2-h intervals over a 24-h period and used for estimating (APHA, 1998) the excretory patterns of total ammonia (NH_4-N) and reactive phosphate (o-PO_4) production; calculations were made following Sumagaysay-Chavoso (2003).

Ammonia/orthophosphate excretion rates (N-NH_4/o-PO_4 mg kg^{-1} fish per 2-h interval) were measured at 2-h intervals from the aquaria water and calculated as follows :

$$\text{Total N-NH}_4\text{/o-PO}_4 \text{ excretion (mg kg}^{-1}\text{ BW 2h}^{-1}) = \frac{[(\text{N-NH}_4/\text{o-PO}_4)_{120} - (\text{N-NH}_4/\text{o-PO}_4)_0] \times 30\text{L}}{\text{Fish biomass (kg)}}$$

$(N\text{-}NH_4/o\text{-}PO_4)_0$ and $(N\text{-}NH_4/o\text{-}PO_4)_{120}$ = concentrations at times 0 and 120 min. (2h) post-feeding.

Daily ammonia/orthophosphate production (DP) rates (in milligrams per day) were estimated by summing up the concentrations obtained at 2-h intervals over a 24-h period.

Live weight gain (in grams), growth percentage gain, specific growth rate [% body weight (BW) per day], feed conversion ratio (FCR), gross protein retention (GPR) and gross energy retention (GER) were calculated using standard methods (Steffens, 1989). Apparent protein digestibility (APD) of the diets was calculated according to Cho *et al.* (1982) as follows :

$$APD = 100 - 100 \times \frac{\%\ Cr_2O_3 \text{ in diet}}{\%\ Cr_2O_3 \text{ in faeces}} \times \frac{\%\text{ nutrient in faeces}}{\%\text{ nutrient in diet}}$$

Gross energy content of the diets and fish were calculated using the average caloric conversion factors of 0.3954, 0.1715 and 0.2364 kJ g^{-1} for lipid, carbohydrate and protein, respectively (Henken *et al.,* 1986).

Statistics Analysis

ANOVA followed by Duncan's multiple range test (Dunan, 1955) and student 't' test (Snedecor and Cochran, 1982) were applied to find out the significant differences between different treatments. Data were further subjected to orthogonal polynomials for trend analysis.

Chapter-IV

Results

Fish growth, digestibility and nutrient retention

Survival was not affected by the inclusion levels of probiotics. Growth performance [(in terms of live weight gain (Fig.7), growth percent gain in BW and final length), SGR (Fig.8) and nutrient retention (PER, GPR, GER and APD)] increased when dietary probiotics level were increased from 0.25g to 0.75g 100 g^{-1} of diet; further increase in dietary probiotics level (>0.75g 100g^{-1}) resulted in a significant ($P<0.05$) growth depression and nutrient depletion. Apparent protein digestibility (Fig. 9) was significantly ($P<0.05$) higher in fish which were fed diets containing probiotics at 0.75g 100 g^{-1} than in fish fed probiotics free diet (Control) or diets containing low or high levels of lactobacillus. FCR values were also significantly ($P<0.05$) lower in fish fed diet containing *Lactobacillus* at 0.75g 100g^{-1} than fish fed other dietary preparations including control diet (Table 12).

Fig. 7. Relationship between dietary probiotics supplement level (0.0, 0.25, 0.50, 0.75 and 1.0 g 100g^{-1} of diet) and live weight gain in *Oreochromis niloticus* fry. Where, Y= Live weight gain (g), x = Probiotics supplement levels (g 100g^{-1} of diet), R^2 = Coefficient of determination, n = Nnumber of observations (16).

Fig. 8. Relationship between dietary probiotics supplement level (0.0, 0.25, 0.50, 0.75 and 1.0 g 100g^{-1} of diet) and specific growth rate in *Oreochromis niloticus* fry. Where, Y=Specific growth rate (SGR), x = Probiotics supplement levels (g 100g^{-1} of diet), R^2 = Coefficient of determination, n = Number of observation (16).

Fig. 9. Relationship between dietary probiotics supplement level (0.0, 0.25, 0.50, 0.75 and 1.0 g 100g^{-1} of diet) and apparent protein digestibility in *Oreochromis niloticus* fry. Where, Y=Apparent protein digestibility (APD %), x = Probiotics supplement levels (g 100g^{-1} of diet), R^2 = Coefficient of determination, n = Number of observations (16).

Postprandial excretory levels of total ammonia (N-NH_4) and reactive phosphate (o-PO_4)

Total ammonia excretion and reactive phosphate production were significantly lower ($P<0.05$) in fish fed probiotics at 0.75g 100 g^{-1} of diet than fish fed other dietary preparations or control diet (Table 12). Irrespective of the probiotics level N-NH_4 excretion showed a peak at 6 h post-feeding, while o-PO_4 production showed an initial high level at 2 h post-feeding and a peak between 14 and 16h post-feeding (Table 12 and Figs. 10A and 10B).

Fish carcass composition

The body composition of the fish was also affected by the probiotics concentrations in diets. The accumulation of carcass protein, fat, phosphorus and gross energy were significantly ($P<0.05$) high in groups fed diets containing probiotics at 0.5 to 0.75 g 100g^{-1} of diet. Carcass ash contents remained significantly ($P<0.05$) low at 0.75 g of probiotics level (Table 13).

Digestive enzyme activity, muscle and liver glycogen, muscle protein, VSI and HSI

In general, activities (total and specific) of digestive enzymes (Protease, amylase, lipase and cellulase) remained significantly ($P<0.01$) high in fish fed diet containing *Lactobacillus* at 0.75g 100 g^{-1} of diet in comparison with other treatments and control. VSI and HSI values were also significantly ($P<0.05$) high in this treatment. Muscle protein was significantly high, while, muscle and liver glycogen levels remained significantly low in fish fed at 0.75g of probiotics at $100g^{-1}$ of diet (Table 14).

Fig. 10. Effect of different levels of probiotics supplement on postprandial patterns of total ammonia (A) and orthophosphate (B) excretion (mg kg^{-1} BW) in *Oreochromis niloticus* fry in holding water. All values are mean±SE of mean of eight observations.

Table 12. Effect of different levels of probiotics supplement on growth performance, digestibility, nutrient retention and excretion of metabolites in *Oreochromis niloticus* fry under laboratory conditions (LD 12:12 at 25±1^{o}C) –70 days treatment

Parameters	Diets				
	D0 (control)	D1	D2	D3	D4
Initial weight (g)	1.22±0.02a	1.29±0.04a	1.23±0.01a	1.15±0.03a	1.20±0.02a
Initial length (cm)	4.00±0.05a	4.25±0.06a	4.14±0.07a	4.09±0.06a	4.20±0.06a
Final weight (g)	3.02±0.03e	3.53±0.02d	5.44±0.06b	6.79±0.24a	4.13±0.05c
Final length (cm)	5.77±0.07d	6.12±0.10cd	6.87±0.12b	7.72±0.12a	6.19±0.09c
Live weight gain (g)	1.80±0.02e	2.23±0.03d	4.21±0.06b	5.64±0.24a	2.93±0.06c
Growth (% gain in BW)	147.19±2.16d	174.20±7.26d	341.63±7.16b	491.21±25.59a	243.61±7.31c
Specific growth rate (SGR)	1.29±0.01e	1.44±0.04d	2.12±0.02a	2.53±0.06b	1.76±0.03c
Feed conversion ratio (FCR)	2.07±0.08a	1.99±0.05a	1.95±0.05a	1.74±0.08b	2.04±0.08a
Gross energy retention (GER)	19.97±0.96b	20.76±0.49b	21.47±0.70b	24.65±0.92a	20.49±1.07b
Gross protein retention (GPR)	24.86±1.09b	25.93±0.79b	27.33±0.85b	31.81±1.32a	25.46±1.11b
Protein efficiency ratio (PER)	1.23±0.05b	1.27±0.03b	1.30±0.03b	1.47±0.06a	1.24±0.05b
Apparent protein digestibility (APD%)	80.07±0.19d	81.28±0.26c	83.58±0.20b	85.37±0.36a	81.12±0.20c
Total ammonia excretion (mg kg^{-1} BW day^{-1})	1349.83±6.31a	1008.75±8.95b	621.46±2.11d	534.55±5.98e	799.45±7.54c
Total phosphate production	244.42±0.24a	176.99±11.26c	144.84±4.44d	143.28±3.12d	199.77±4.84b

(mg kg^{-1} BW day^{-1})	

All values are mean±SE of mean.
Means bearing different letters in the same row differ significantly (P<0.05)

Table 13. Effect of different levels of probiotics supplement on proximate composition (% wet weight basis) in *Oreochromis niloticus* fry under laboratory conditions (LD 12:12 at 25±1°C) –70 days experiment

Carcass composition (%)	**Initial value**	**Diets**				
		D0 (control)	**D1**	**D2**	**D3**	**D4**
Moisture	71.26±0.17	68.21±0.03a	67.85±0.12b	67.23±0.03d	66.80±0.07e	67.62±0.03c
Crude protein	15.31±0.35	18.26±0.26c	18.56±0.27c	19.79±0.37ab	20.56±0.28a	19.03±0.36bc
Crude fat	2.74±0.04	3.78±0.03d	3.82±0.03cd	3.97±0.03b	4.08±0.03a	3.89±0.03bc
Ash	2.85±0.06	3.42±0.02a	3.34±0.02a	3.21±0.02b	3.01±0.05c	3.25±0.03b
Phosphorus	0.59±0.02	0.60±0.02b	0.59±0.02b	0.64±0.02ab	0.67±0.02a	0.63±0.02ab
Gross energy (kJ g^{-1})	5.97±0.03	6.90±0.01e	7.00±0.02d	7.24±0.03b	7.42±0.04a	7.10±0.03c

All values are mean±SE of mean
Means bearing different letters in the same row differ significantly (P<0.05)

Table 14. Effect of different levels of probiotics supplement on muscle protein, muscle glycogen, liver glycogen, enzymatic activities (protease, amylolytic, cellulase and lipase) and viscero-somatic index (VSI) and hepato-somatic index (HSI) in *Oreochromis niloticus* fry under laboratory conditions (LD 12:12 at 25±1^{O}C) – 70 days treatment

Parameters	Diets				
	D0 (control)	D1	D2	D3	D4
Muscle glycogen (mg g^{-1})	1.33±0.02c	1.75±0.03a	1.73±0.03a	1.32±0.03c	1.45±0.02b
Liver glycogen (mg g^{-1})	2.58±0.05a	2.38±0.04b	2.23±0.03c	1.87±0.03d	2.56±0.05a
Muscle protein (mg g^{-1})	110.85±1.89c	116.99±1.29b	121.03±1.40b	133.88±2.59a	118.00±1.75b
Total protease enzyme activity (mg g^{-1} h^{-1})	3.51±0.07d	4.34±0.05c	5.44±0.06b	6.32±0.09a	5.27±0.06b
Specific protease enzyme activity[1]	1.17±0.04d	1.52±0.07c	2.35±0.06a	2.42±0.14a	1.98±0.08b
Total amylase activity (mg g^{-1} h^{-1})	0.22±0.04e	0.24±0.004d	0.30±0.005b	0.38±0.004a	0.28±0.006c
Specific amylase activity[2]	0.12±0.02b	0.13±0.21a	0.15±0.003b	0.17±0.004b	0.12±0.003b
Total lipase activity (mg g^{-1} h^{-1})	0.09±0.01d	0.17±0.01c	0.37±0.03b	0.06±0.03a	0.25±0.03c
Specific lipase activity[3]	0.05±0.009b	0.08±0.008b	0.13±0.01b	0.56±0.03a	0.11±0.01b
Total cellulase activity (mg g^{-1} h^{-1})	0.93±0.03c	1.13±0.02c	1.36±0.02b	1.60±0.05a	1.32±0.11b
Specific cellulase activity[4]	0.43±0.03d	0.51±0.04d	0.80±0.04b	0.95±0.02a	0.70±0.04c
Viscero-somatic index (VSI)	8.63±0.29d	9.00±0.20cd	10.75±0.42b	13.36±0.26a	9.92±0.46bc
Hepato-somatic index (HSI)	1.74±0.16c	1.65±0.04c	2.17±0.04b	2.81±0.13a	1.81±0.06c

[1] mg of tyrosin liberated/mg of protein/minute
[2] mg of maltase liberated/mg of protein/minute
[3] micromole fatty acid liberated/mg of protein/hour
[4] mg of glucose liberated/mg of protein/minute
All values are mean±SE of mean
Means bearing different letters in the same row differ significantly ($P<0.05$)

Chapter-V

Discussion

Fish growth, digestibility and nutrient retention

The survival of *O. niloticus* in all different treatments was excellent. The optimum probiotics levels which resulted in high growth in *O. niloticus* in terms of live weight gain (grams), growth percentage gain, SGR and nutrient retention (PER, GPR, GER and APD) was found to be around 0.75g 100 g^{-1} of diet. FCR values decreased with each increase in the dietary probiotics contents of the diet upto 0.75 g 100g^{-1} of diet, thereafter, increases in dietary probiotics levels resulted in an increase in FCR and growth depression.

The high APD values for the diet containing lactobacillus at 0.75g 100 g^{-1} of diet may be attributed to the probiotics concentration, which was used in diet D3 might be helpful for optimum dietary utilization. Similar results were also reported by Ghosh *et al.* (2003) using *Bacillus circulans* as probiotics in *Labeo rohita* fingerlings and Rengpipat *et al.* (1998) using *Bacillus* sp. S11 as probiotics in *Penaeus monodon*. In the present study, although, all the feeds were isonitrogenous but the concentration of probiotics in feed might be helpful for proper nutrient utilization.

Metabolite excretion

Fishes excrete nitrogen mainly in the form of ammonia, and the quality and quantity of the dietary protein is known to influence this excretion. The excretion of wastes into the effluent water remained significantly ($P<0.05$) low when fish were fed on a diet containing 0.75 lactobacillus 100^{-1} g of diet. Even though the production of metabolites was reduced with each increase in dietary probiotics contents (up to 0.75g), diurnal patterns did not seem to be affected by the levels. Peak values in total ammonia excretion occurred at 6h post-feeding, whereas the level of soluble phosphate in the aquaria water was high initially, at 2 h post-feeding, followed at 14-16 h post-feeding by a peak.

Carcass composition

Our results have also revealed that fish fed probiotics (0.75g 100 g^{-1}) containing diets had a significantly high accumulation of carcass protein, fat and energy and a low percentage of moisture in comparison with fish fed diets containing low concentrations of probiotics, with no further improvement at higher probiotics levels. Significantly low ash contents were also observed in fish fed diet D3 containing probiotics at 0.75g $100g^{-1}$ of diet. Protein depletion at high dietary probiotics levels may be due to the deamination of surplus protein.

Activity of the digestive enzymes, muscle and liver glycogen, muscle protein, HSI and VSI

The nutritional value of the diet depends on the digestive capabilities of the fish, which in turn is affected by the activity of the digestive enzymes present in the digestive tract (Phillips, 1989). The activities of the digestive enzymes (protease amylase, lipase and cellulase) were high in the group fed on a diet having a probiotics concentration of 0.75g 100 g^{-1} of diet. Enzyme activity increased with increasing dietary probiotics levels, while at high probiotics concentration (1.0g 100 g^{-1} of diet) a decrease in their activity was observed.

These results indicate that probiotics stimulate the digestion through the supply of digestive enzymes and certain essential nutrients to the animals. Probiotics are known to improve enzymatic activity in the gut by producing several enzymes not produced by the host. Similar observations were also reported by Swain *et al.* 1996. A complex polysaccharides including cellulose are better utilized by the host in the presence of direct-feed microbes like *Aspergillus oryzae*, *S. cerevisiae*. Further, it was reported that yeast in the diet improves feed efficiency, organic phosphorus (phytic acid) utilization and fibre digestion (Swain *et al.,* 1996). Unfortunately, information regarding the mode of action of probiotics used in aquaculture is incomplete. Benefits to the host have been reported to include the improvement in nutrition by detoxification of potentially harmful compounds in feeds, denaturing of potentially indigestible matter in the diet by hydrolytic enzymes including amylases and proteases, the production of vitamins, such as biotin and vitamin B_{12} (Fuller and Turvy, 1971; Parker, 1974; Roach and Tannock, 1980; Sugita *et al.,* 1991; Fuller, 1992; Sugita *et al.* 1992; Sugita *et al.,* 1996), the production of inhibitory compounds (Spanggaard *et al.,* 2001) and the stimulation of host immunity (Gibson *et al.,* 1997). These attributes of probiotics indicate that a given probiotics could elicit more than one protective response to the host.

Ability of the microflora to elaborate various hydrolytic enzymes indicates that majority of them are capable of utilizing various substrates such as starch, gelatin and lipid. Urea splitting forms were relatively less. The beneficial effects of some intestinal microflora, which is popularly known as probiotics in disease resistance, are well documented (Gatesoupe, 1999; Patra and Bandyopadhyay, 2003).

Some investigators have also suggested that microorganisms exert beneficial effects on the digestive process of fish (Riquelme *et al.,* 1996; Gibson *et al.,* 1998; Gram *et al.,* 1999).

Low muscle and liver glycogen levels indicates its utilization, thereby sparing protein and fat for accumulation in the body of fish fed on a diet at 0.75 g of probiotic at 100 g^{-1}. High HSI values also is an indication of high accumulation of glycogen levels. High VSI, high carcass fat and muscle protein values also support high weight gain in *O. niloticus* fed on optimal (0.75g) concentrations of probiotics. High values of nutrient retention (GER and GPR) and digestive enzyme activity and low excretory levels of metabolites (N-NH_4 and o-PO_4) also support high growth, protein digestion/retention in fish fed optimal dietary probiotics levels.

The results obtained in this study thus support the use of probiotic bacteria *Lactobacillus sporogenes* for better growth, proper nutrient utilization and also for the development of eco-friendly aquaculture.

Chapter-VI

Summary and Conclusions

To study the effect of *Lactobacillus sporogenes* (Probiotics) on growth performance of *Oreochromis niloticus*, four diets containing varying concentration (0.25, 0.5, 0.75 and 1.0g 100 g^{-1} of diet) of the probiotics were formulated. A diet without supplementation of probiotics was also formulated which served as control diet. All diets contained about 40% of crude protein. Significantly highest growth performance, carcass protein, apparent protein digestibility, nutrient retention (PER, GPR, GER and APD) digestive enzyme activity were observed in the group fed diet containing probiotics at a concentration of 0.75 g, 100 g^{-1} of diet. Excretion of metabolites remained low, while the values of VSI and HSI remained high at this treatment. Muscle glycogen and liver glycogen were also low, while the values of muscle protein were high in fish fed diet-3 containing probiotics at a concentration of 0.75g, 100g^{-1} of diet. These studies indicate that supplementation of diets with appropriate concentration of probiotics at an appropriate level can be a useful tool in utilization of supplementary diets in aquaculture.

PART-III

Phosphorus and calcium requirements in the diet of milkfish, *Chanos chanos* (Forsskal) and Nile tilapia, *Oreochromis niloticus* (Linn.)

Chapter-I

Introduction

Aquaculture is one of the fastest growing food-producing systems in the world. The continuous increase of aquaculture production is expected to meet an increasing demand for fish products in both developing and developed countries. However, aquaculture is expanding in a period of environmental awareness and is, therefore, subject to regulations designed to limit its effect on the environment. In order for world aquaculture production to continue to increase, it will be necessary to reduce discharge of wastes, especially phosphorus (P), from fish farms to the aquatic environment.

The ultimate source of P in aquaculture effluent is feeds. Any excess P in diet above the minimum requirement for fish will be excreted by the fish. It is, therefore, critical to know precisely the dietary requirement of P in order to minimize excess P in diet without risking P deficiency in cultured fish. The dietary requirement of P has been studied for various fish and other animal species using fry or juveniles and indicators such as weight gain, feed efficiency, P levels in various tissues, bone density, bone-breaking strength and enzyme activities (NRC 1993, 1994, 1995, 1998). Nutrient requirements in most animal species, however, decrease as they bcome older, because as they age, increasing portions of dietary nutrients including P are used for maintenance. In commercial aquaculture, large fish consume most of the feed used in the production cycle and correspondingly, excrete the largest proportion of waste into effluent. Consequently, knowing the dietary requirement of P specific for large fish is necessary to reduce P excretion from commercial aquaculture systems. Unfortunately, the various indicators listed above are not sensitive for large fish because of their slow growth rate. Feeding large fish with research diets for any extended period is also expensive. Because of these difficulties, little is known about dietary requirements of P and other nutrients in fish especially those, which are euryhaline. Hence, further studies are needed on the mineral nutritional requirements in the fish to obtain better and quick growth during culture as the formulated feed should be such that it should lead to maximum utilization and should also contain sufficient amount of energy so that proteins are converted to fish flesh with high efficiency.

It is well known that the inorganic compounds of the body exercise a great diversity of vital functions in the animal economy. Not only these elements must be available in adequate amounts in the animal diet but also in balanced form, as excess of potassium has been found to increase the elimination of sodium; excess of calcium results in decreased absorption or increased excretion of zinc and other trace metals and abnormal ratio of calcium to phosphorus may lead to impairment of body structures etc. However, such imbalances are usually not present under natural conditions with a well balance diet.

It is also known that calcium is an essential component/constituent of all living cells. Krogh (1939) has reported that calcium is involved in osmo-regulation in some aquatic animals which was later confirmed by Podoliak and Holden (1965, 1966) in Brook, Brown and Rainbow trout. It has an interesting effect on the absorption or utilization of other divalent heavy metals by plants, fish and mammals as the toxicity of lead, copper, baryllium, cadmium and Vanadium salts to fish becomes considerably less in the hard (calcium containing) waters than in soft or distilled water (Michibata, 1981; Khangarot, 1982). Though its mode of action is not clear but it appears to play a part in decreasing the permeability of cell membrane and irritability of cells in general. Similarly, since the greater part of the phosphorus of the body is associated with calcium in bone, the metabolism of these two elements is to a considerable extent parallel and follows that of osseous tissue. Phosphorus, which is also present in many of the soft tissues, plays many important roles in life processes such as carbohydrate metabolism of animals through the formation of hexose phosphates, of adenylic acid and of creatine phosphate; in fat metabolism through the intermediary formation of lecithin and in the neutrality regulation of the organism.

Considering the above mentioned facts, the requirements of calcium and phosphorus for the fish should be considered together because the metabolism of the two elements appear to be intimately connected. Though, there are some reports on the effects of mineral (dietary) requirements of calcium and phosphorus on the growth and metabolism in fish (Andrew *et al.,* 1973; Ketola, 1975; Cowey, 1976), however, most of the studies are restricted to marine fishes as test animals. Therefore, the present study has been undertaken to provide a better mineral mixed cheap diet for the cultivation of two aquaculturally important euryhaline fish species, such as *Chanos chanos* and *Oreochromis niloticus.* The results obtained from these studies will serve as the basic information for future studies.

Chapter-II

Review of Literature

Minerals are inorganic substances required in small amounts for normal growth, health and function. They are classified as macro- or trace minerals. Fish can obtain large quantities of some minerals (e.g., calcium) directly from the water, and a dietary source is usually unnecessary. In addition, many practical feedstuffs are rich in minerals, which reduces the need for synthetic dietary supplements. However, many plant feedstuffs contain minerals in unavailable forms. For example, soybean meal and other plant feedstuffs contain 60-70% of their phosphorus as phytate, which cannot be digested by fish. Phosphorus metabolism is closely tied to that of calcium, but phosphorus is not readily absorbed from the water. Therefore, phosphorus supplementation of diets is important for several reasons. First, commercial feeds contain mostly plant feedstuffs. Secondly, cyprinid fishes (including golden shiners, goldfish and fathead minnows) do not have true stomachs that secrete acid to enhance digestion. Mineral availability from common feedstuffs is known to be lower for some fishes that lack acidic digestion. The dietary phosphorus requirements of the main baitfish species are unknown. However, the requirement for common carp (another cyprind) is 0.6-0.7% of the diet. These requirements are established using highly available forms of phosphorus (e.g., sodium phosphate monobasic). The phosphorus that is unavailable from plant feedstuffs in diets are excreted into the pond where it may stimulate undesirably heavy plankton blooms. Therefore, it is important to minimize the amount of unavailable phosphorus in the diet as it reduces both fish production and water quality.

Basic studies to determine mineral requirements for growth, survival, optimal health and reproduction are needed. Applied studies in outdoor systems are necessary also, due to the reliance of fish on natural foods. However, production and composition of natural foods varies between production units (e.g., ponds) and over time, and it is likely that mineral supplementation of commercial feeds will continue.

Phosphorus is a major mineral that must be supplied in the feed. However, much of the phosphorus in commercial fish diets may be released into the environment (Wiesmann *et al.*, 1988) and this is influenced both by the availability of dietary phosphorus and the high levels of phosphorus encountered in feeds because of the high levels found in animal proteins such as fish meal. The effects of phosphorus wastes from intensive cage and pen culture have been reviewed and recent studies of intensive pond systems have also demonstrated the importance of dietary phosphorus in determining algal density and water quality. Consequently, it is necessary to reduce phosphorus load in effluents and one way to achieve this is to reduce the levels in fish and shrimp diets.

Under practical farming conditions, mineral deficiency signs often arise from a dietary imbalance of calcium owing to the antagonistic effect of excess dietary calcium on the absorption of phosphorus (Nakamura, 1982). When there is an excess of calcium over phosphorus, the phosphorus is not absorbed by the intestine because it is combined with the calcium to form calcium phosphates that are not biologically available (Andrews *et al.,* 1973; Cowey and Sargent, 1979).

A phosphorus requirement has been determined for 10 species of fish including *Crysophrys major, Anguilla japonica, Salmo salar* I., 1758. *Cyprinus carpio* L. 1758, *Ictalurus punctatus* Rainesque 1818, *Oreochromis niloticus* L. 1758. *Oreochromis aureus*. Steindachner 1864. *Poecilia reticulata.* Peters 1859 and *Oncorhynchus mykiss* Walbaum 1792 (Sakamoto and Yone, 1973; Arai *et al.,* 1974; Ketola, 1975; Ogino and Takeda, 1976; Lovel, 1978; Watanabe *et al.,* 1980; Wilson *et al.,* 1982; NRC, 1983; Viola *et al.,* 1986; Robinson *et al.,* 1987; Shim and Ho, 1989; Rodehutseord, 1996; Dougall *et al.,*1996). Reported phosphorus requirements vary from about 0.25-1.00 g kg^{-1} of the diet although this rather wide range may be growth related.

There has been little reported investigation of the mineral requirements of fish species, especially the Ca requirement. A dietary Ca supplement may not be necessary for some fish (Ogino and Takeda, 1976, 1978; Shim and Ho,

1989) since fish can easily absorb Ca from the surrounding water (Lovelace and Podoliak, 1952; Ichikawa and Oguri, 1961; Templeton and Brown, 1963; Love, 1980; Ichii and Mugiya, 1983). However, waterborne Ca has not satisfied the requirements of some species reared in low-Ca water (Arai *et al.,* 1975a, 1975b; Robinson *et al.,* 1984, 1986, 1987). It is generally accepted that sea water contains sufficient amounts of ionized Ca that are readily absorbed by marine fish and a Ca supplement to the diet may not be necessary for marine species. Sakamoto and Yone (1976) reported that a Ca supplement was unnecessary in a purified diet for red sea bream *Pagrus major*. In previous studies, however, we found that tiger puffer *Takifugu rubripes* could not absorb sufficient Ca from sea water (Furuichi *et al.,* 1997; Hossain and Furuichi, 1998). Ca from dietary tricalcium phosphate (TCP) was also unavailable to tiger puffer (Hossain and Furuichi, 1998). Recently, El-Zibdeh (1996) reported poor growth in redlip mullet when Ca was excluded from purified diets.

Chavez-Sanchez *et al.* (2000) studied phosphorus and calcium requirements in the diet of the American cichlid *Cichlasoma urophthalmus* (Gunther). These studies have revealed that optimum level of phosphorus in the diet was 1.5g kg^{-1}, the optimum calcium level was 1.8 g kg^{-1} and the optimum Ca-P ratio was 1:3. Carcass lipid levels were found to be inversely related to dietary phosphorus.

Sugiura *et al.* (2000a) studied the primary responses of rainbow trout to dietary phosphorus concentrations. The authors conducted a series of experiments and the fish were fed up to 24 days with semipurified diets that varied in phosphorus content. Concentrations of glucose-6-phosphate, ATP, creatine phosphate, glucose, total lipids and total cholesterol in blood or skeletal muscle were relatively unchanged by the 24 days of dietary phosphorus restriction. Inorganic phosphorus and ATP levels in the blood, however, correlated significantly and positively. Inorganic phosphorus levels in plasma and urine were significantly lower in fish fed phosphorus-deficient diets than phosphorus-supplemented diets. Urinary phosphorus excretion peaked 6-10h after feeding fish with diets containing potassium phosphate. Fish receiving either commercial feeds or experimental diets containing phosphorus as fish bone excreted trace amounts of phosphorus in the urine. Faecal content of phosphorus significantly increased when the diet containing potassium phosphate was supplemented with calcium carbonate. Urinary phosphorus concentration was found to be a rapid and sensitive indicator for dietary intake of phosphorus and probably phosphorus status of the fish and had an advantage over conventional response variables in estimating dietary phosphorus requirement especially with large commercial-size fish.

Hossain and Furuichi (2000) studied essentiality of dietary calcium supplement in redlip mullet *Liza haematocheila*. Studies have revealed that fish fed on a diet containing no calcium supplement grew significantly lower than the groups of fish fed on diet containing clacium supplements. From the studies the authors concluded that a dietary calcium supplement is necessary for the mullet.

Green *et al.* (2002) studied the effects of dietary phosphorus and lipid levels on utilization and excretion of phosphorus and nitrogen by rainbow trout (*Oncorhynchus mykiss*). Nutritional strategies to reduce both phosphorus and nitrogen excretion relative to growth of rainbow trout were tested in a 2×3 factorial experiment. The two factors were dietary P level and dietary lipid level. Reduction in dietary P from 14 to 8 g kg^{-1} dry diet was achieved by partial substitution of dietary fish meal with a combination of full-fat soyabean meal, corn gluten and spray-dried blood meal. A reduction in dietary fish meal from 500 to 200 g kg^{-1} dry diet, corresponding to a reduction in dietary P from 14 to 8 g kg^{-1} dry diet resulted in 50% reductions in both solid and dissolved P waste, but did not affect growth, feed efficiency ratio (FER) or sensory characteristics of rainbow trout.

Mgbenka and Ugwu (2005) studied aspects of mineral composition and growth rate of the hybrid African catfish fry fed inorganic phosphorus-supplemented diets. A 70-day feeding experiment involving sixteen 38% crude protein diet treatments of four inorganic phosphorus sources (monosodium phosphate, monopotassium phosphate, monocalcium phosphate (MCP), dicalcium phosphate (DCP), four levels of P (0.04%, 0.06%, 0.08%, 1.2%) and three replicates of each diet followed. A non-P supplemented diet and a purified diet (controls) were additionally fed. Gross efficiency of food conversion (GEFC), daily rate of growth (DRG), tissue ash, tissue phosphorus (TP), tissue calcium (TCa) and Ca:P ratio of the fish were measured weekly. These parameters varied significantly ($P<0.01$) among the (a) 18 test diets, (b) inorganic P sources and (c) duration. Monocalcium phosphate-supplemented diets resulted in better response to GEFC, DRG, TP and Ca than the P-supplemented diet while the Ca:P ratio was best exhibited by fish fed the DCP diet. The fish fed the control diets had better GEFC, DRG, TP and Ca than the P-supplemented diets probably because of nearer to optimum available P in these diets. In conclusion, supplementation with 0.6% MCP produced comparatively better growth, feed conversion and mineral deposition in the fry than other inorganic P sources (See Table 15 for calcium and phosphorus requirements of other fish species).

In the present study, the requirements of calcium and phosphorus in various combinations and ratio are taken to assess the requirements of milkfish, *Chanos chanos* and Nile tilapia, *Oreochromis niloticus* maintained under laboratory conditions.

Table 15. Calcium and phosphorus requirements (g 100 g^{-1} in the diet) in different fish species (a) Data expressed as percentages in the diet (b) Data recalculated as weight per unit weight of growth, where possible

Fish Species	P (g 100 g^{-1} in the diet)	Ca (g 100 g^{-1} in the	Ca in the water (mg kg^{-1})	Reference

		diet)		
(a)				
Cichlasoma urophthalmus	1.5	1.8	84	Chavez-Sanchez *et al.* (2000)
Ciprinus carpio	0.6-0.7	0.10	5	Nakamura (1982)
C. carpio	0.6-0.7	NR	20	Ogino and Takeda (1976)
Ictalurus punctatus	0.45	0.05	14	Lovel (1978)
I. punctatus	0.8	1.5	56	Andrews *et al.* (1973)
Morone saxatilis	0.55	0.3	28-35	Dougall *et al.* (1996)
M. saxatilis	0.62	0.3	28-35	Dougall *et al.* (1996)
Oncorhynchus keta	0.5-0.6	0.36	20	Watanabe *et al.* (1980)
Oncorhynchus mykiss	0.25	-	40-50	Rodeshutscord (1996)
Oreochromis aureus	0.50	0.70	0	Robinson *et al.* (1987)
O. aureus	0.80	0.50	0	Robinson *et al.* (1987)
Salmo salar	0.6	-	-	Ketola (1975)
Liza haemotecheila	-	0.2	-	Hossain and Furuichi (2000)
Paecilia reticulata	-	0.4	-	Shim and Ho (1989)
Hamarus americanus	1.91	0.37	-	Gallagher *et al.* (1978)
Ictalurus punctatus	0.8	-	-	Deyoe and Tiemiur (1968)
Ictalurus punctatus	0.8-1.0	1.5	-	Andrew *et al.* (1973)
Red Sea bream	-	0.34	-	Sakamotos and Yone (1973)

Anguilla japonicus	29.0	-	-	Arai *et al.* (1974)
Oncorhynchus tshawgtscha	1.8	1.9	-	Richardson *et al.* (1985)
(b)	**P (g kg-1 of growth)**	**Ca (g kg-1 of growth)**	**Ca-P ratio in diet**	
Cichlasoma urophthalmus	21.6	28.8	1.3	Chaverz-Sanchez *et al.* (2000)
Ictalurus punctatus	12.3	18.4	1.88	Andrews *et al.* (1973)

Morone saxatilis	16.3	16.3	2.0	Dougall *et al.* (1996)
M. saxatilis	9.1	5.88	0.4	Dougall *et al.* (1996)
Oncorhynchus keta	8.9	3.56	0.40	Watanabe *et al.* (1980)
Oreochromis aureus	0.80	11.2	0.5	Robinson *et al.* *(1987)*
O. aureus	12.9	2.6	1.6	Robinson *et al.* (1987)

[1]Determination of bone and scale mineralization with different phosphorus contents.

[2]Determination of weight gain, feed efficiency, serum phosphorus and calcium, and scale and bone mineralization

[3]Determination of calcium requirement with fixed dietary phosphorus of 0.50%

[4]Determination of phosphorus requirement with fixed dietary calcium of 0.80%

Chapter-III

Materials and Methods

Diet preparation

Ten diets (D1 to D10) supplemented with calcium and phosphorus with 40% protein levels were formulated using processed soybean as the major protein source. D1 with Ca-P concentration of 0.2 and 0.1 mg $100g^{-1}$ of diet served as control. Diets were prepared by replacing rice bran with different amounts of calcium chloride and dihydrogen orthophosphate. The diets were approximately isoproteic and isoenergetic. Dietary ingredients and proximate composition of formulated diets are given in Table 16. Dietary ingredients were cleaned, milled and mixed in definite proportions. Thereafter thick dough was made using lukewarm water. Wheat flour was added as a binder, while 1% chromic oxide (Cr_2O_3) was added as an external indigestible marker for digestibility estimations. Using a mechanical pelletizer, 0.5 mm. thick pellets were obtained, which were oven (60-62^{o}C) dried, before using in feeding trials.

Table 16. Ingredient content (%) and proximate analysis (% dry weight basis) of experimental diets with different levels of calcium and phosphorus (g 100g^{-1} of diet)

Ingredient	Diets									
	D1 (control)	D2	D3	D4	D5	D6	D7	D8	D9	D10
Groundnut oil cake	65.00	65.00	65.00	65.00	65.00	65.00	65.00	65.00	65.00	65.00
Rice bran	4.20	3.45	3.33	3.20	3.20	3.08	2.95	2.95	2.83	2.70
Wheat flour	3.20	3.20	3.20	3.20	3.20	3.20	3.20	3.20	3.20	3.20
Processed soybean[a]	26.60	26.60	26.60	26.60	26.60	26.60	26.60	26.60	26.60	26.60
Chromic oxide (Cr_2O_3)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Calcium[b]	-	0.50	0.50	0.50	0.75	0.75	0.75	1.0	1.0	1.0
Phosphorus[c]	-	0.25	0.375	0.50	0.25	0.375	0.50	0.25	0.375	0.50
Proximate analysis % (analysed values)										
Parameters										
Dry matter	93.51± 0.34	93.33 ± 0.26	93.22 ± 0.25	93.48 ± 0.22	93.21 ± 0.33	92.95 ± 0.25	93.26 ± 0.26	93.39 ± 0.34	93.23 ± 0.31	93.18 ± 0.30
Crude protein	39.81± 0.37	39.70 ± 0.16	39.81 ± 0.17	39.81 ± 0.17	39.70 ± 0.16	39.81 ± 0.17	40.03 ± 0.32	39.81 ± 0.44	39.48 ± 0.38	39.70 ± 0.16
Crude fat	8.19± 0.27	8.28± 0.29	8.44± 0.26	8.19± 0.27	8.19± 0.27	8.13± 0.23	8.06± 0.32	8.13± 0.30	8.06± 0.29	8.19± 0.33
Crude fibre	6.34± 0.15	6.06± 0.15	6.25± 0.09	5.88± 0.16	5.81± 0.13	6.13± 0.26	5.81± 0.23	5.94± 0.18	5.69± 0.21	5.94± 0.11
Ash	6.44± 0.15	6.38± 0.16	6.43± 0.15	6.18± 0.09	6.56± 0.15	6.25± 0.28	6.50± 0.16	6.38± 0.23	6.31± 0.19	6.25± 0.09
Nitrogen free extract (NFE)	32.63± 0.55	32.92 ± 0.31	32.29 ± 0.37	33.42 ± 0.37	32.95 ± 0.42	32.64 ± 0.60	32.86 ± 0.83	33.15 ± 0.96	33.69 ± 0.70	33.11 ± 0.45
Phosphorus	1.06± 0.03	1.34± 0.03	1.44± 0.02	1.60± 0.02	1.43± 0.02	1.29± 0.02	1.62± 0.03	1.32± 0.02	1.43± 0.03	1.51± 0.03
Gross energy (kJ g^{-1})	18.24± 0.10	18.30 ± 0.10	18.28 ± 0.08	18.38 ± 0.09	18.27 ± 0.11	18.22 ± 0.08	18.29 ± 0.09	18.31 ± 0.10	18.30 ± 0.11	18.30 ± 0.10

[a] Soybean was hydrothermically processed in an antoclave at 121^{o}C (15lbs for 15 minute) to eliminate antinutritional factors (ANFs) (Garg *et al.,* 2002)

[b] Calcium chloride (Ca Cl_2, 2 H_2O)

[c] Potassium dihydrogen orthophosphate (KH_2 PO_4)

The quantities of calcium chloride and potassium dihydrogen orthophosphate substituted for rice bran in the basal diet to formulate the experimental diets

Source and concentrations of calcium and phosphorus

The source for dietary calcium and phosphorus requirements were from dihydrated calcium chloride ($CaCl_2$. $2H_20$) and potassium dihydrogen orthophosphate (KH_2PO_4) respectively [E. Merck (India) Ltd., Bombay].

Altogether ten different diets were prepared by mixing different concentrations of phosphorus and calcium in the following ratios :

Concentrations (%) of calcium (Ca) and phosphorus (P) and their ratios

Diet	Calcium (%) (Ca)	Phosphorus (%) (P)	Ca:P ratio
D1 (Control)	0.20	0.10	2:1
D2	0.50	0.250	2:1
D3	0.50	0.375	4:3
D4	0.50	0.500	1:1
D5	0.75	0.250	3:1
D6	0.75	0.375	2:1
D7	0.75	0.500	3:2
D8	1.00	0.250	4:1
D9	1.00	0.375	8:3
D10	1.00	0.500	2:1

Experimental design

The fry of milkfish (mean body weight 0.24±0.01) was obtained from the Southern coastal areas of India (Wild collection), while Nile tilapia fry (mean body weight 0.49±0.05) were collected from the breeding pond maintained at the fish farm. Fry were maintained in glass aquaria (60×30×30 cms) in laboratory where the temperature was maintained at 25±1^{o}C and lighting schedule (at 12h of light (08^{00}–20^{00}hrs) alternating with 12h of darkness (20^{00}–08^{00} hrs). Fry were acclimated in an inland saline groundwater (10.0 ppt) for a minimum period of 10 days prior to the commencement of experiments. The aquaria water was renewed daily with water adjusted to the laboratory temperature (25^{o}C). During the acclimation period the fry were fed control diet.

Fry of milkfish and Nile tilapia were randomly distributed @ 20 fish per aquarium with four replicates of each dietary treatment. All fish were fed daily twice at 08^{oo} h and at 16^{00} h. The feeding rate being 5% BW d^{-1} for the whole duration of 90 days and the feeding rates were adjusted every fifteen days after bulk weighing. Each group of fish were exposed to their respective diet for four hour during each ration, thereafter, the uneaten feed was siphoned out, stored and dried separately for calculating the feed conversion ratio (FCR). The faecal matters voided by the fish were also collected by siphoning separately from each aquarium. The faecal samples were dried in a hot air oven at 60^{o}C and were subsequently analyzed for digestibility estimations. At the termination of experiment, the fish from all the treatments were weighed (length was also recorded) individually to the nearest gram and processed for subsequent analyses. The pH of aquaria water fluctuated between 7.10-7.95, dissolved oxygen contents ranged between 5.0-6.0 mg l^{-1}.

Analytical Techniques

The feed ingredients, experimental diets, faecal matter samples, fish carcass (Initial and final) were analyzed following the procedure of AOAC (1995). Cr_2O_3

levels in the diets as well as in the faecal samples were estimated specto-photometrically following the method of Furukawa and Tsukahare (1996). pH and dissolved oxygen were monitored using an automatic analyzer (F-set-3 E Merck Germany).

Data Collection

Live weight gain (g), growth percent gain, specific growth rate (% d^{-1}), feed conversation ratio (FCR), protein efficacy ratio (PER), gross protein retention (GPR) and gross energy retention (GER) were calculated using standard methods (Steffens, 1989). Apparent nutrient digestibility of the diets was calculated according to Cho *et al.* (1982). Energy content of the diets and fish calculated using the average caloric conversion factors of 0.3954, 0.1715 and 0.2364 kJ g^{-1} for lipid carbohydrate and protein respectively (Henken *et al.,* 1986).

Statistical Analysis

ANOVA followed by Duncan's multiple range test (Duncan, 1955) and Student 't' test (Snedecor and Cochran, 1982) were applied to find out the significant differences between different treatments. Calcium and phosphorus requirements were determined using broken line analysis according to the method of Zeitoun *et al.* (1976) and by fitting trend lines to the data.

Chapter-IV

Results

Fish growth, digestibility and nutrient retention

Irrespective of the fish species under investigations (*Chanos chanos* and *Oreochromis niloticus*), survival was not affected by the dietary mineral (Calcium/phosphorous) contents. Growth performance [(in terms of live weight gain (Figs.11 and 12) and percentage growth gain in BW], SGR (Figs.13 and 14) and nutrient retention (PER, GPR, GER and APD) gradually (Figs.15 and 16 for APD) increased when dietary P levels were increased from 0.25 g to 0.5 g 100 g^{-1} of diet (calcium concentration kept constant at 0.5g 100g^{-1} of diet) An increase in dietary calcium levels (>5.0 to 1.0g 100 g^{-1}) resulted in a significant ($P<0.05$) growth depression. Apparent protein digestibility was significantly ($P<0.05$) higher in fish fed diets containing Ca and P in equal ratio (0.5g : 0.5g 100 g^{-1}) than in fish fed higher or lower mineral levels. FCR values were also significant lower in fish fed diet containing Ca/P in equal ratio (0.5g : 0.5g 100 g^{-1} of diet) (Table 17 and 18, Figs.17 and 18).

Fig. 11. Live weight gain (g) of *Chanos chanos* fry after 90 days of growth trial (under laboratory conditions LD 12:12 at 25±1^{0}C) on different dietary calcium and phosphorus levels. Trend curves are a polynomial fit.

Fig. 12. Live weight gain (g) of *Oreochromis niloticus* fry after 90 days of growth trial (under laboratory conditions LD 12:12 at 25±1^{0}C) on different dietary calcium and phosphorus levels. Trend curves are a polynomial fit.

Fig. 13. Specific growth rate (SGR) of *Chanos chanos* fry after 90 days of growth trial (under laboratory conditions LD 12:12 at 25±1^{0}C) on different dietary calcium and phosphorus levels. Trend curves are a polynomial fit.

Fig. 14. Specific growth rate (SGR) of *Oreochromis niloticus* fry after 90 days of growth trial (under laboratory conditions LD 12:12 at 25±1^{0}C) on different dietary calcium and phosphorus levels. Trend curves are a polynomial fit.

Fig. 15. **Apparent protein digestibility (APD%) of *Chanos chanos* fry after 90 days of growth trial (under laboratory conditions LD 12:12 at 25±1^{0}C) on different dietary calcium and phosphorus levels. Trend curves are a polynomial fit.**

Fig. 16. Apparent protein digestibility (APD%) of *Oreochromis niloticus* fry after 90 days of growth trial (under laboratory conditions LD 12:12 at 25±1^{0}C) on different dietary calcium and phosphorus levels. Trend curves are a polynomial fit

Fig. 17. **Feed conversion ratio (FCR) of *Chanos chanos* fry after 90 days of growth trial (under laboratory conditions LD 12:12 at 25±1^{0}C) on different dietary calcium and phosphorus levels. Trend curves are a polynomial fit.**

Fig. 18. **Feed conversion ratio (FCR) of *Oreochromis niloticus* fry after 90 days of growth trial (under laboratory conditions LD 12:12 at 25±1^{0}C) on different dietary calcium and phosphorus levels. Trend curves are a polynomial fit.**

Postprandial excretory levels of total ammonia $N-NH_4$) and reactive phosphate ($o-PO_4$)

Irrespective of the fish species total ammonia excretion and reactive phosphate production were also significantly lower ($P<0.05$) in fish fed diet (diet D4) containing Ca/P in equal ratios (1:1) than fish fed other Ca/P ratios. Irrespective of the mineral concentrations and their ratios, $N-NH_4$ excretion showed a peak at 6 h post-feeding (Figs.19A and 20A), while $o-PO_4$ production showed an initial high level at 2 h post-feeding and a peak between 14 and 16 h post-feeding (Tables 17 and 18, Figs.19B and 20B).

Fish carcass composition

The body composition of the fish was also affected by the experimental diets. The accumulation of carcass protein, fat, phosphorus and energy were significantly ($P<0.05$) high when Ca/P ratio was equal (D4) in the diet. Carcass ash contents fluctuated between 3.92–4.60, with highest values in diet (D4) (Tables 19 and 20).

Digestive enzyme activity, muscle and liver glycogen, muscle protein, VSI and HSI

In general (total and specific) activity of digestive enzymes initially increased with increasing dietary phosphorous (from 0.10g to 0.5 g 100 g^{-1} of diet) levels. An increase in calcium levels (from 0.2g to 1.0 g 100 g^{-1}) resulted in reduction of enzyme activities (Table 21 and 22).

VSI, HSI and muscle protein were high in fish fed diets containing Ca/p in equal ratio (1:1) in diet 4. On the other hand, muscle and liver glycogen levels initially decreased in fish fed diets 1, 2, 3 & 4 and thereafter, a gradual increase in muscle and liver glycogen contents was observed in fish fed diets 5-10. Fish fed control diet also had highest values of liver and muscle glycogen (Table 21 and 22).

Fig. 19. Effect of different levels of dietary calcium and phosphorus supplement on postprandial patterns of total ammonia (A) and orthophosphate (B) excretion (mg kg^{-1} BW) by *Chanos chanos* fry in holding water. All values are mean±SE of mean of eight observation.

Fig. 20. Effect of different levels of dietary calcium and phosphorus supplement on postprandial patterns of total ammonia (A) and orthophosphate (B) excretion (mg kg^{-1} BW) by *Oreochromis niloticus* fry in holding water. All values are mean±SE of mean of eight observation.

Table 17. Effect of different levels of dietary calcium and phosphorus supplement on growth performance, nutrient retention, digestibility and excretion of metabolite in *Chanos chanos* fry under laboratory conditions (LD 12:12 at 25±1^{o}C) –90 days treatment

Parameters	Diets									
	D1 (control)	D2	D3	D4	D5	D6	D7	D8	D9	D10
Initial weight (g)	0.23± 0.005a	0.24± 0.004a	0.24± 0.01a	0.25± 0.01a	0.26± 0.005a	0.26± 0.006a	0.24± 0.009a	0.25± 0.008a	0.25± 0.007a	0.25± 0.007a
Initial length (cm)	3.50± 0.04a	3.51± 0.05a	3.51± 0.05a	3.52± 0.04a	3.50± 0.04a	3.52± 0.05a	3.51± 0.06a	3.51± 0.03a	3.51± 0.05a	3.50± 0.04a
Final weight (g)	0.70± 0.03h	1.77± 0.02c	2.27± 0.04b	3.87± 0.11a	1.15± 0.01de	1.27± 0.04d	1.03± 0.02ef	0.88± 0.13fg	0.81± 0.04gh	0.69± 0.03h
Final length (cm)	4.53± 0.06h	5.70 ±0.06c	6.46± 0.05b	7.53± 0.08a	5.44± 0.04de	5.71± 0.03c	5.21± 0.04f	4.85± 0.04g	4.53± 0.07h	4.53± 0.06h
Live weight gain (g)	0.46± 0.02gh	1.52± 0.02c	2.03± 0.04b	3.62± 0.11a	0.89± 0.01de	1.02± 0.04d	0.79± 0.02ef	0.63± 0.13fg	0.56± 0.04gh	0.44± 0.03h
Growth (% gain in BW)	198.48± 10.10f	629.4± 12.48c	854.3± 40.4b	1472.5 ± 61.20a	341.35 ± 9.19de	399.9± 16.9d	333.6± 20.7de	254.73 ± 51.54ef	227.9± 17.6f	182.23 12.1f
Specific growth rate (SGR)	1.21± 0.04ef	2.21± 0.02bc	2.50± 0.05b	3.06± 0.04a	1.65± 0.02de	1.78± 0.04cd	1.62± 0.05de	1.07± 0.47f	1.31± 0.06ef	1.15± 0.05f
Feed conversion ratio (FCR)	2.31± 0.01b`	1.91± 0.01e	1.88± 0.01e	1.70± 0.01f	2.08± 0.02cd	2.02± 0.15cd	2.12± 0.02c	2.03± 0.04cd	2.25± 0.01b	2.43± 0.21a
Gross energy retention (GER)	15.11± 0.13cd	20.45± 0.35ab	20.59± 0.17ab	23.31± 0.20a	18.34± 0.19bc	19.76± 2.47ab	17.36± 0.19bcd	14.02± 2.73d	15.90± 0.13cd	14.91± 0.89c
Gross protein retention (GPR)	17.13± 0.29e	25.65± 0.15bc	27.15± 0.26b	32.30± 0.34a	22.26± 0.29cd	22.74± 2.64cd	19.55± 0.27de	16.10± 3.25e	17.93± 0.35e	16.98± 0.95e
Protein efficiency ratio (PER)	1.09± 0.01bc	1.31± 0.01ab	1.33± 0.01ab	1.48± 0.01a	1.21± 0.01bc	1.33± 0.17ab	1.18± 0.01bc	0.98± 0.19c	1.13± 0.01bc	1.07± 0.06b
Apparent protein digestibility (APD%)	78.13± 0.25g	83.15± 0.09c	84.47± 0.07b	85.65± 0.14a	82.01± 0.13d	80.78± 0.29ef	82.00± 0.20d	80.37± 0.88ef	79.64± 0.14f	79.57± 0.14f
Total ammonia excretion (mg kg^{-1} BW day^{-1})	1481.0± 5.24a	971.87 ± 10.47f	770.3± 65.2g	679.5± 4.12h	1163.5 ± 4.12e	1332.9 ± 5.83c	1233.4 ± 4.96d	1366.6 ± 2.38bc	1421.3 ± 5.42ab	1451.7 ± 3.80
Total phosphate production (mg kg^{-1} BW day^{-1})	222.8± 7.27b	141.88 ± 2.63e	138.81 ± 2.16f	135.38 ± 1.95g	142.7± 2.29e	147.98 ± 2.82e	160.63 ± 3.25d	184.2± 4.28c	273.1± 2.96a	228.9 3.43b

All values are mean±SE of mean.
Means bearing different letters in the same row differ significantly (P<0.05)

Table 18. Effect of different levels of dietary calcium and phosphorus supplement on growth performance, nutrient retention, digestibility and excretion of metabolite in *Oreochromis niloticus* fry under laboratory conditions (LD 12:12 at 25±1°C) –90 days treatment

Parameters	Diets									
	D1 (control)	D2	D3	D4	D5	D6	D7	D8	D9	D10
Initial weight (g)	0.41± 0.03a	0.44± 0.03a	0.41± 0.03a	0.43± 0.02a	0.43± 0.02a	0.43± 0.04a	0.44± 0.04a	0.43± 0.04a	0.42± 0.04a	0.43± 0.03a
Initial length (cm)	2.28± 0.04a	2.83± 0.06a	2.76± 0.05a	2.73± 0.06a	2.78± 0.05a	2.75± 0.05a	2.77± 0.05a	2.80± 0.04a	2.73± 0.05a	2.81± 0.05a
Final weight (g)	0.67± 0.03g	1.59± 0.04c	1.90± 0.04b	2.74± 0.07a	1.29± 0.03d	0.92± 0.03f	1.13± 0.03e	0.92± 0.04f	0.87± 0.03f	0.71± 0.04g
Final length (cm)	3.32± 0.04f	4.85± 0.05c	5.20± 0.05b	5.80± 0.04a	4.40± 0.03cd	3.90± 0.06e	4.15± 0.02d	3.69± 0.04f	2.18± 0.29g	3.31± 0.04f
Live weight gain (g)	0.25± 0.05g	1.15± 0.04c	1.49± 0.06b	2.31± 0.07a	0.85± 0.04d	0.49± 0.05f	0.69± 0.05e	0.49± 0.05f	0.45± 0.05f	0.28± 0.04g
Growth (% gain in BW)	69.98± 17.73e	265.1± 18.34c	395.27 ± 51.46b	554.04 ± 35.3a	202.2± 15.94cd	129.28 ± 25.1de	186.57 ± 44.1cd	127.8± 26.9de	124.5± 26.0de	67.74± 10.21e
Specific growth rate (SGR)	0.55± 0.12f	1.43± 0.06c	1.74± 0.11b	2.08± 0.06a	1.22± 0.06cd	0.88± 0.12e	1.10± 0.14de	0.87± 0.11e	0.85± 0.12e	0.56± 0.07f
Feed conversion ratio (FCR)	2.41± 0.46a	1.85± 0.05b	1.83± 0.04b	1.73± 0.03c	1.96± 0.06b	2.18± 0.22ab	2.04± 0.11ab	2.03± 0.12ab	2.27± 0.20ab	2.29± 0.31ab
Gross energy retention (GER)	18.63± 1.84b	22.07± 0.52ab	22.20± 0.44ab	23.19± 0.37a	21.16± 0.53ab	20.69± 1.55ab	20.49± 1.00ab	21.18± 1.28ab	19.16± 1.34ab	21.24± 2.10ab
Gross protein retention (GPR)	17.40± 2.91e	27.48± 0.73ab	27.69± 0.67ab	30.57± 0.72a	26.36± 0.88abc	23.00± 1.85bcd	22.87± 1.35bcd	23.73± 1.29bcd	20.94± 1.31de	22.00± 2.41cd
Protein efficiency ratio (PER)	1.04± 0.16b	1.36± 0.03a	1.38± 0.03a	1.46± 0.03a	1.29± 0.04ab	1.23± 0.11ab	1.25± 0.08ab	1.28± 0.09ab	1.18± 0.11ab	1.21± 0.12ab
Apparent protein digestibility (APD%)	77.11± 0.16e	83.52± 0.14b	83.93± 0.21b	85.56± 0.20a	82.78± 0.10bc	80.60± 0.26c	81.87± 0.22c	78.80± 0.21d	78.07± 0.20d	79.22± 0.30d
Total ammonia excretion (mg kg^{-1} BW day^{-1})	1298.0± 17.12a	1089.0 ± 7.84d	984.0± 6.17e	883.0± 6.19f	1124.0 ± 10.27c	1079.0 ± 9.31d	1162.0 ± 6.91c	1225.0 ± 7.42b	1228.0 ± 7.12b	1254.0 ± 10.31a
Total phosphate production (mg kg^{-1} BW day^{-1})	470.0± 3.79a	306.0± 4.12de	283.0 ±3.95e	255.0± 2.11f	336.0± 3.47d	384.0± 4.51c	356.0± 3.02d	407.0± 4.12b	433. ± 4.92ab	449.0± 5.17a

All values are mean±SE of mean.
Means bearing different letters in the same row differ significantly (P<0.05)

Table 19. Effect of different levels of dietary calcium and phosphorus supplement on proximate composition (% wet weight) in *Chanos chanos* fry under laboratory conditions (LD 12:12 at 25±1^{O}C) – 90 days treatment

Carcass composition %	Initial value	Diets									
		D1 (control)	D2	D3	D4	D5	D6	D7	D8	D9	D10
Moisture	71.00 ± 0.16	70.19± 0.08a	67.83 ± 0.19ef	67.76 ± 0.07f	66.96 ± 0.07g	68.05 ± 0.12ef	68.14 ± 0.18e	68.68 ± 0.08d	69.08 ± 0.11c	69.41 ± 0.06b	70.00 ± 0.08a
Crude protein	18.20 ± 0.18	16.54± 0.16f	19.31 ± 0.03c	20.12 ± 0.02b	21.55 ± 0.09a	18.31 ± 0.05d	17.40 ± 0.31e	16.95 ± 0.16f	16.85 ± 0.14f	16.67 ± 0.15f	16.67 ± 0.14f
Crude fat	3.86± 0.11	3.62± 0.01b	4.51± 0.63a	4.05± 0.05ab	4.10± 0.09ab	3.89± 0.02b	3.77± 0.02b	3.85± 0.01b	3.69± 0.01b	3.65± 0.005b	3.60± 0.02b
Ash	4.36± 0.12	3.92± 0.01e	4.31± 0.04b	4.32± 0.05b	4.60± 0.03a	4.27± 0.005b	4.31± 0.03b	4.17± 0.02c	4.11± 0.004c	3.97± 0.03de	4.02± 0.03d
Phosphorus	0.60± 0.03	0.62± 0.02cd	0.66± 0.04c	0.67± 0.04b	0.71± 0.02a	0.65± 0.04b	0.64± 0.04c	0.61± 0.03cd	0.62± 0.04cd	0.60± 0.05d	0.59± 0.03d
Gross energy (kJ g^{-1})	6.27± 0.06	6.32± 0.02e	7.04± 0.13b	7.00± 0.02b	7.19± 0.02a	6.80± 0.02c	6.70± 0.02c	6.62± 0.02d	6.52± 0.02d	6.47± 0.02d	6.35± 0.01e

All values are mean±SE of mean
Means bearing different letters in the same row differ significantly (P<0.05)

Table 20 . Effect of different levels of dietary calcium and phosphorus supplement on proximate composition (% wet weight) in *Oreochromis niloticus* fry under laboratory conditions (LD 12:12 at 25±1°C) – 90 days treatment

Carcass composition %	Initial value	Diets									
		D1 (control)	D2	D3	D4	D5	D6	D7	D8	D9	D
Moisture	71.22 ± 0.17	68.21± 0.03a	67.54 ± 0.07cd	67.37 ± 0.04de	67.35 ± 0.12e	67.62 ± 0.03c	67.70± 0.04bc	67.70± 0.03bc	67.87 ± 0.03b	68.16± 0.10a	68 0.0
Crude protein	15.42 ± 0.31	15.97± 0.29e	18.82 ± 0.16b	19.13 ± 0.26b	20.13 ± 0.29a	18.71 ± 0.16b	17.16± 0.15c	17.16± 0.14c	17.07 ± 0.15c	16.63± 0.024cd	16 0.1
Crude fat	2.76± 0.04	3.68± 0.03e	3.83± 0.02bc	3.87± 0.02b	4.00± 0.03a	3.80± 0.02cd	3.77± 0.03cde	3.76± 0.02cde	3.71± 0.04de	3.72± 0.03de	3.6 0.0
Ash	2.87± 0.07	3.12± 0.02c	3.40± 0.02b	3.45± 0.02b	3.77± 0.25a	3.36± 0.02bc	3.32± 0.02bc	3.28± 0.02bc	3.24± 0.02bc	3.24± 0.03bc	3.1 0.0
Phosphorus	0.52± 0.03	0.55± 0.01d	0.65± 0.01bc	0.67± 0.03b	0.74± 0.01a	0.60± 0.02cd	0.55± 0.02d	0.60± 0.04bcd	0.58± 0.03d	0.51± 0.02d	0.5 0.0
Gross energy (kJ g^{-1})	6.06± 0.05	6.78± 0.02e	7.07± 0.02bc	7.12± 0.02ab	7.15± 0.05a	7.04± 0.01c	6.93± 0.01d	6.93± 0.01d	6.89± 0.02d	6.82± 0.02e	6.8 0.0

All values are mean±SE of mean
Means bearing different letters in the same row differ significantly (P<0.05)

Table 21. Effect of different levels of dietary calcium and phosphorus supplement on muscle protein, muscle glycogen, liver glycogen, enzymatic activities (protease, amylase, cellulase and lipase), viscero-somatic index and hepato-somatic index in *Chanos chanos* fry under laboratory conditions (LD 12:12 at 25±1^{o}C) – 90 days treatment

Parameters	**Diets**									
	D1 (control)	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D**
Muscle glycogen (mg g^{-1})	1.85± 0.04a	1.52± 0.04de	1.46± 0.05ef	1.37± 0.03f	1.59± 0.05cd	1.67± 0.04bc	1.70± 0.03bc	1.77± 0.04ab	1.78± 0.04ab	1. 0.
Liver glycogen (mg g^{-1})	2.02± 0.04a	1.46± 0.03ef	1.49± 0.02ef	1.46± 0.03f	1.56± 0.03e	1.77± 0.03cd	1.70± 0.03d	1.84± 0.03bc	1.89± 0.03b	1. 0.
Muscle protein (mg g^{-1})	108.17± 3.24d	116.31 ± 1.94c	127.18 1.99b	142.51 ± 2.40a	109.47 ± 2.64cd	106.45 ± 1.62d	102.24 ± 0.47d	108.07 ± 1.92d	109.65 ± 3.40cd	10 3.0
Total protease enzyme activity (µg g^{-1} h^{-1})	4.21± 0.06h	5.70± 0.05b	5.82± 0.05ab	5.98± 0.04a	5.19± 0.05d	5.41± 0.10c	5.06± 0.05de	4.91± 0.04e	4.69± 0.08f	4. 0.
Specific protease enzyme activity[1]	1.11± 0.02e	2.15± 0.03c	2.32± 0.04b	2.59± 0.04a	1.88± 0.04d	1.57± 0.03f	1.69± 0.03e	1.42± 0.02g	1.32± 0.03h	1. 0
Total amylase activity (mg g^{-1} h^{-1})	2.62± 0.03h	3.60± 0.03c	3.78± 0.03b	3.98± 0.04a	3.43± 0.03d	3.48± 0.02d	3.17± 0.022e	3.01± 0.03f	2.94± 0.04f	2. 0.
Specific amylase activity[2]	0.78± 0.04f	1.54± 0.03b	1.64± 0.04b	1.86± 0.03a	1.20± 0.03d	1.33± 0.04c	1.13± 0.04d	1.15± 0.03d	1.01± 0.04e	0. 0.
Total lipase activity (mg g^{-1} h^{-1})	0.02± 0.002e	0.06± 0.004b	0.07± 0.003b	0.09± 0.003a	0.04± 0.004c	0.05± 0.004c	0.03± 0.003d	0.03± 0.003de	0.02± 0.003e	0. 0.0
Specific lipase activity[3]	0.01± 0.001d	0.02± 0.002bc	0.02± 0.002ab	0.03± 0.002a	0.02± 0.002c	0.02± 0.002cd	0.02± 0.002cd	0.01± 0.002d	0.02± 0.001cd	0. 0.0
Total cellulase activity (mg g^{-1} h^{-1})	0.90± 0.02f	1.20± 0.02c	1.27± 0.02b	1.41± 0.02a	1.15± 0.02cd	1.17± 0.02cd	1.14± 0.01cd	1.12± 0.02d	1.03± 0.04e	1. 0.
Specific cellulase activity[4]	0.36± 0.03f	0.71± 0.02b	0.71± 0.012b	0.87± 0.002a	0.52± 0.02cd	0.57± 0.002c	0.46± 0.03de	0.45± 0.03e	0.43± 0.03e	0. 0.
Viscero-somatic index (VSI)	9.76± 0.30h	13.01± 0.39c	14.13± 0.30b	15.83± 0.25a	12.96± 0.41d	11.92± 0.44ef	12.22± 0.47e	10.85± 0.17g	10.73± 0.39g	10 0.
Hepato-somatic index (HSI)	1.43± 0.07e	1.86± 0.08b	1.89± 0.09b	2.09± 0.06a	1.59± 0.08d	1.76± 0.06c	1.71± 0.12c	1.53± 0.09d	1.39± 0.09f	1. 0.

[1] mg of tyrosin liberated/mg of protein/minute

[2] mg of maltase liberated/mg of protein/minute

[3] micromole fatty acid liberated/mg of protein/hour

[4] mg of glucose liberated/mg of protein/minute

All values are mean±SE of mean. Means bearing different letters in the same row differ significantly (P<0.05)

Table 22 . Effect of different levels of dietary calcium and phosphorus supplement on muscle protein, muscle glycogen, liver glycogen, enzymatic activities (protease, amylase, cellulase and lipase), viscero-somatic index and hepato-somatic index in *Oreochromis niloticus* under laboratory conditions (LD 12:12 at 25±1°C) – 90 days treatment

Parameters	Diets									
	D1 (control)	D2	D3	D4	D5	D6	D7	D8	D9	D1
Muscle glycogen (mg g^{-1})	1.65± 0.02a	1.34± 0.02c	1.22± 0.02d	1.13± 0.02e	1.39± 0.03c	1.52± 0.02b	1.47± 0.03b	1.62± 0.02a	1.62± 0.01a	1.6 0.0
Liver glycogen (mg g^{-1})	1.70± 0.02a	1.48± 0.02c	1.45± 0.02c	1.37± 0.02d	1.57± 0.02b	1.66± 0.01b	1.60± 0.02a	1.67± 0.02a	1.71± 0.02a	1.6 0.0
Muscle protein (mg g^{-1})	96.07± 1.99de	114.16 ± 1.50c	124.94 ± 2.47b	133.13 ± 2.46a	109.94 ± 2.47c	98.04± 2.48de	102.3 ± 1.84d	99.48± 1.52de	97.71± 1.69de	94.8 1.3
Total protease enzyme activity (mg g^{-1} h^{-1})	5.13± 0.02h	5.60± 0.02c	5.68± 0.02b	5.90± 0.03a	5.54± 0.02d	5.45± 0.02e	5.51± 0.02d	5.36± 0.02f	5.25± 0.02g	5.1 0.02
Specific protease enzyme activity[1]	1.29± 0.02h	2.09± 0.02c	2.21± 0.02b	2.37± 0.02a	1.97± 0.03d	1.66± 0.09f	1.84± 0.02e	1.64± 0.02f	1.50± 0.02g	1.4 0.0
Total amylase activity (mg g^{-1} h^{-1})	3.10± 0.03g	4.06± 0.02c	4.25± 0.02b	4.38± 0.02a	3.95± 0.02c	3.62± 0.02e	3.78± 0.01d	3.51± 0.02e	3.51± 0.12e	3.3 0.2
Specific amylase activity[2]	1.40± 0.02h	1.88± 0.02c	1.96± 0.02b	2.15± 0.02a	1.78± 0.02d	1.51± 0.02f	1.66± 0.02e	1.50± 0.02g	1.47± 0.02g	1.4 0.0
Total lipase activity (mg g^{-1} h^{-1})	0.04± 0.001g	0.07± 0.001c	0.08± 0.002b	0.10± 0.003a	0.06± 0.002d	0.05± 0.002e	0.06± 0.001 d	0.04± 0.002e f	0.04± 0.002fg	0.0 0.00
Specific lipase activity[3]	0.01± 0.001f	0.03± 0.001c	0.03± 0.001b	0.04± 0.001a	0.03± 0.001cd	0.02± 0.001e	0.02± 0.001 d	0.01± 0.001f	0.01± 0.001f	0.0 0.00
Total cellulase activity (mg g^{-1} h^{-1})	0.76± 0.02e	1.26± 0.02c	1.35± 0.02b	1.47± 0.02a	1.22± 0.02cd	1.15± 0.025e f	1.19± 0.01d e	1.12± 0.02f	0.96± 0.02g	0.8 0.0
Specific cellulase activity[4]	0.02± 0.001h	0.05± 0.002c	0.06± 0.002b	0.08± 0.002a	0.04± 0.002d	0.02± 0.002f	0.03± 0.002 e	0.02± 0.001f g	0.02± 0.001g h	0.0 0.00 h
Viscero-somatic index (VSI)	8.18± 0.18f	11.08± 0.13b	11.16± 0.12b	12.09± 0.06a	10.92± 0.16b	10.46± 0.14c	10.89 ± 0.08b	10.40± 0.14cd	9.99± 0.17d	9.3 0.2
Hepato-somatic index (HSI)	1.06± 0.05g	1.51± 0.02bc	1.60± 0.03b	1.91± 0.04a	1.42± 0.03cd	1.29± 0.02e	1.39± 0.03d	1.27± 0.03e	1.21± 0.03ef	1.1 0.04

[1] mg of tyrosin liberated/mg of protein/minute

[2] mg of maltase liberated/mg of protein/minute

[3] micromole fatty acid liberated/mg of protein/hour

[4] mg of glucose liberated/mg of protein/minute

All values are mean±SE of mean. Means bearing different letters in the same row differ significantly (P<0.05)

Chapter-V

Discussion

Fish growth, digestibility and nutrient retention

The survival of fish in all the treatments was high. The optimum Ca/P ratio which resulted in high growth in both the fish species in terms of live weight gain, growth percentage gain. SGR and nutrient retention (PER, GPR, GER and APD) was found to be when calcium and phosphorus were given in equal ratio (1:1) i.e. where 0.5g each of the two minerals 100 g^{-1} of diet were given. Further, the growth increased with each increase in the dietary P levels, while growth decreased with each increase in the Ca levels. The deficiency signs observed in this experiment were reduced growth and high food conversion ratio in all diets containing 0.75–0.10 g 100 g^{-1} of diet.

A review on mineral requirements of different fish species shows that phosphorus requirements for various fish species vary from 0.25 to 0.8 g kg^{-1} in the diet, while the calcium requirements vary from zero to 1.5 g kg^{-1} of the diet.

Comparison with other fish species indicates that *Oreochromis niloticus* and *Chanos chanos* have higher requirements for both phosphorus and calcium when expressed as percentage in the diet or as g kg^{-1} of growth, although the Ca-P ratio is within the range required for other fish species. In the present studies mineral contents (Ca and P) of bone and scale were not separately estimated, however, carcass ash content and phosphorus appears to be positively correlated with the growth performance. These results also correlates well with the dietary calcium and phosphorus contents.

Andrews *et al.* (1973) determined that a phosphorus deficiency produced lower bone ash in channel catfish, while Ketola (1975) found that supplement of organic phosphorus in the diet significantly increased bone ash content for Atlantic salmon. Watanbe *et al.* (1980) observed that dietary phosphorus levels greatly affected the ash, calcium and phosphorus contents of the bones of chum salmon. Robinson *et al.* (1987) found that based on calcium levels in bone ash, 0.50g kg^{-1} phosphorus was adequate for normal bone mineralization. In contrast, Rodehutscord (1996) was unable to confirm any necessity for phosphorus for maximum mineralization of rainbow trout.

In experiments with brook trout using labelled calcium, Rodgers (1984) showed that ambient calcium concentration had a pronounced effect on calcium dynamics. Earlier studies of this laboratory indicate that high calcium contents of inland saline water had no adverse effect on growth performance of

C.chanos (Jana *et al.,* 2005) or of *O. niloticus* under field conditions (Kumar, 2004). Present studies also indicate high calcium requirements for high growth of these two fish species under laboratory conditions. A review of literature shows (Table 15) that environmental calcium levels and calcium requirement were also higher for *Cichlasoma urophthalmo* than for other species. One possible explanation given by Chavez-Sanchez *et al.* (2000) for the high demand for phosphorus in this cichlid is that it lives in water with a high calcium content (more than 80 mg kg^{-1}). It is possible that these fish are so adapted to this environment that they are able to utilize high levels of calcium, and this could require a higher than normal level of dietary phosphorus. The increased growth obtained in *C. chanos* and *O. niloticus* when dietary calcium was increased supports this view. This result was also observed for red sea beam *Crysophrys major*, in which a high dietary phosphorus requirement of fingerlings was attributed to an extra calcium supply from the sea water in addition to calcium from the diet (Sakamoto and Yone, 1973). Studies are needed to compare the growth performance of *O. niloticus* and *C. chanos* in different calcium concentrations because they are euryhaline and also their adaptability from fresh water to metahaline environment.

Schwartz (1995) considered that calcium requirements of fish are usually adequately supplied from the water and the calcium content in the food can thus be minimized. Ogino and Takeda (1976) and Watanabe *et al.* (1980) found that the growth of carp and chum salmon, respectively, is affected only by the dietary phosphorus levels and not by the calcium levels. Ogino and Takeda (1976) also found that when diets were used containing very low amounts of calcium and an apparently adequate amount of phosphorus, carp absorbed calcium from environmental water in proportion to the amount of phosphorus absorbed by the digestive tract and used both for growth. Present studies on *O. niloticus* and *C. chanos* indicate that increasing dietary calcium levels (beyond 5 g kg^{-1}) had a negative effect not only on growth but also on all other physiological parameters observed on the present study. Nakamura (1982) also found that increasing dietary calcium had a negative relationship with phosphorus absorbed. On the other hand, Sakamoto and Yone (1973) showed that some calcium was necessary in diets containing high levels of phosphorus because the uptake of the calcium from seawater was not sufficient to meet the requirements of growth in red sea bream *Crysophrys major*. Andrews *et al.* (1973) found that the maximum growth of catfish was obtained in experiments where diets contained 1.5% calcium and considered that the absence of a growth-response relationship at levels below 1.5% calcium indicated a requirement of at least this level in the diet. Robinson *et al.* (1987) showed that weight gain and feed conversion rate was optimum for good growth at 0.70% dietary calcium in *O. aureus*, but that this did not clearly reflect bone or scale mineralization.

A dietary calcium supplement was reported not to be essential in tilapia, *Oreochromis massambica* reared in artificial sea water (Boroughs *et al.,* 1957).

In the present study, it was clear that despite the large amount of Ca in the water (425 mg l^{-1} at 10 ppt), the fish still requires some calcium in the diet. Lall (1979) while reviewing mineral requirements considered that the water chemistry, species differences and the phosphorus level in the diet affect the calcium requirement of finfish.

A definite relationship of dietary Ca-P ratio and the growth performance of fish species and their nutritive physiology was observed in the present studies. While, no definite relationship between the values for the dietary Ca-P ratio and the growth of the experimental fish was found by Chavez-Sanchez (2000) in *Cichlosoma urophthalmus*, Ogino and Takeda (1976) in carp and Watanabe *et al.* (1980) in Chum salmon. Andrews *et al.* (1973) observed that several studies have shown that calcium and phosphorus can be absorbed and excreted through the gills of the fish. Thus, dietary requirements are probably affected by the water levels of these nutrients and the ability to exchange these elements with the environment may explain the absence of a definite relationship between Ca-P ratio and growth rates, i.e. fish may be able to balance the ratio by controlling the absorption or excretion of these elements.

Onishi *et al.* (1981) observed that phosphorus deficiency in carp accompanied accumulation of lipid in muscle and viscera and the activity of the hepatopancreatic enzymes increases. From these results they assumed that the synthesis of fatty acids via citrate from amino acids may be accelerated by phosphorus deficiency (Rodeshutscord, 1996).

It is notable that the range of requirements for calcium and phosphorus determined by various authors is quite wide. Calcium and phosphorus intake may be expected to be related to growth and if this were the case then recalculation of requirements per unit of growth should give more consistent results. Table 15 (given in review) summarizes such data where it has been possible to recalculate values. It can be seen that the range of values is still wide and that *O. niloticus* and *C. chanos* like *C. urophthalmus* have higher requirements for both nutrients than other species. In general, calcium and phosphorus requirements per unit of growth and calcium and Ca-P ratio are positively correlated and in many cases strongly so.

It is clear from this that the methodology used to carry out trials in this field is variable and this certainly widens the range of results. However, there is the underlying influence of environmental levels of these elements affecting the requirements determined and the variable ability of different species to use dietary supplements of these elements undoubtedly adds to the spread of data.

Present feeding trials on *O. niloticus* and *C. chanos* have confirmed the ability of these fish species to benefit from additional calcium in the diet. This ability probably also explains the apparent elevated phosphorus requirements for these species. The variations in the natural habitats of these fish from fresh water to metahaline environment may also be important in that a strong plasticity of physiology has developed to enable the animal to thrive in these widely different environments.

Metabolite excretion

Fishes excrete nitrogen mainly in the form of ammonia, and the quality and quantity of the dietary protein is known to influence this excretion. In the present studies, excretion of wastes appears to be affected by the inclusion levels of Ca and P in the diets. The excretion of wastes into the effluent water remained significantly ($P<0.05$) low when fish were fed on diet supplemented with Ca-P in 1:1 ratio. Even though the production of metabolites was reduced with each increase in dietary P level (upto 0.5g), diurnal patterns did not seem to be affected by the levels of Ca-P added to the diets. Peak values in total ammonia excretion occurred at 6h post-feeding, whereas the level of soluble phosphate in the aquaria water was high initially, at 2 h post-feeding, followed at 14-16 h post-feeding by a peak. These results are in broad agreement to those of Kaushik (1980), Kaushik and Gomes (1988), Ballestrazzi *et al.* (1998), Kalla and Garg (2003) and Jana *et al.* (2006). The excretion patterns observed in the present trial, however, differ from those obtained by Sumagaysay-Chavoso (2003) in milkfish fed formulated and natural food-based diets in that the excretory levels in his studies were very low and Sumagaysay-Chavoso obtained two peaks (6h and 18h) post-feeding for total ammonia nitrogen (TAN) and only one peak, at 21 h post-feeding, for PO_4-P. Since the excretory rates of metabolites depend not only on the fish species but also on the size dietary mineral contents, temperature, salinity (fresh/marine water) of the medium and also on the experimental conditions (Porter *et al.,* 1987);

Ballestrazzi *et al.,* 1994), absolute values can not be compared between different species. However, trends in excretion/production of metabolites and relative magnitude can be compared.

Phosphorus metabolism is closely tied to that of calcium. Any excess minerals in the diet above the minimum requirement for fish will be excreted by the fish in the water. It is, therefore, critical to know precisely the dietary requirement of P and Ca in order to minimize excess P in diet without risking P deficiency in cultured fish. Rodehutscord (1996) found that increasing dietary phosphorus above 4 g kg^{-1} dry diet led to decreasing P utilization (% dietary P retained for growth) of rainbow trout, and no increase in P concentration in gain (g kg^{-1} gain).

According to results of Rodehutscord *et al.* (2000) and Sugiura *et al.* (2000b), below a threshold level of dietary P (considered to be the P requirement), dissolved water P is fairly constant, but above this threshold, dissolved waste P increases with dietary P level. Based on these studies (Bureau and Cho, 1999; Rodehutscord *et al.,* 2000; Sugiura *et al.,* 2000b), it can be concluded that regulation of renal phosphate excretion contributes to the maintenance of P homeostasis in fishes, as has been demonstrated in eels (Fenwick and Vermette, 1989) and salmonids (Watanabe *et al.,* 1980).

Carcass composition

Our results have also revealed that fish fed diets containing Ca-P in the ratio 1:1 (0.5g $100g^{-1}$ of diet) had significantly high carcass protein, fat, ash and energy and a low percentage of moisture in comparison with fish fed other diets.

Activity of the digestive enzymes, muscle and liver glycogen, muscle protein, VSI and HSI

The nutritional value of the diet depends on the digestive capabilities of the fish, which in turn is affected by the activity of the digestive enzymes present in the digestive tract (Phillips, 1969). The activities of the digestive enzymes (protease, amylase, lipase and cellulase) were high in the group fed on diet 3. High amylase and protease activity in the digestive tract of milkfish has also been reported by Chiu and Benitez (1981) and Benitez and Tiro (1982), respectively.

Low muscle and liver glycogen levels indicates its utilization, thereby sparing protein and fat for accumulation in the body of fish fed diets containing appropriate levels of Ca-P. High HSI, VSI and high carcass fat values also support high weight gain in *C. chanos* and *O. niloticus*. High values of nutrient retention and digestive enzyme activity and low excretory levels of metabolites ($N\text{-}NH_4$ and $o\text{-}PO_4$), further support high growth, protein digestion/ retention in fish fed optimal levels of minerals. Increased excretion of metabolites and decreased digestive efficiency have also been shown in fish fed high dietary minerals contents than required.

Chapter-VI

Summary and Conclusion

An experiment was carried out with *Chanos chanos* and *Oreochromis niloticus* to determine their phosphorus requirements and its interaction with dietary calcium. Ten isoenergetic and isonitrogenous diets were prepared using basal ingredients (groundnut oilcake, processed soybean, rice bran and wheat flour etc.) containing various concentrations of phosphorus as potassium dihydrogen orthophosphate (0.25g, 0.375g and 0.5g $100g^{-1}$ of diet) and calcium as calcium chloride (0.5g, 0.75g and 1.0 g 100 g^{-1} of diet). These concentrations resulted in varying Ca-P ratios (1:1, 2:1, 3:1, 3:2, 4:1, 4:3, 8:3). Calcium and phosphorus concentrations in the water at 10 ppt were 425 mg and 0.03 mg l^{-1} of water, respectively. A diet without supplementation with Ca and P served as control. Irrespective of the fish species, significantly ($P<0.05$) high growth performance (growth per cent gain in body weight, SGR and final length, APD, GPR, GER and PER were observed in the groups which were fed on a diet containing calcium and phosphorus at a concentration of 0.5g $100g^{-1}$ of diet in the ratio of 1:1. Low FCR and excretion of metabolites ($N\text{-}NH_4$, $o\text{-}PO_4$) was also observed at these concentrations. Significantly ($P<0.05$) highest digestive enzyme activity was also observed in fish fed on these concentrations. Low muscle and liver glycogen levels and high muscle protein content coincided with the highest growth performance observed in these studies. VSI and HSI values were also high in fish, which had the highest growth performance. Carcass composition also indicated high accumulation of protein. These studies indicated that diets must be supplemented with minerals especially calcium and phosphorus in optimum concentrations and ratios for obtaining the full benefit of supplementary diets.

ANNEXURE-I

TECHNIQUES FOR ANALYSIS

A. PHYSICO-CHEMICAL CHARACTERISTICS OF WATER

Following physico-chemical parameters of the water samples were recorded (APHA, 1998).

1. Temperature

Water temperature was recorded every day in the morning hours using electronic digital thermometer (centigrade) (Model N. 4052, M/s S. Define Chem. Ltd., Bolsar).

2. Turbidity

Turbidity was measured in the laboratory with the help of Nephlo turbidity meter (Systronics) in N.T.U.

3. TDS

Total dissolved solids was recorded by a digital water proof microprocessor TDS meter (EUTEOH instruments, Singapore).

4. pH

The pH of water was recorded by a digital pH meter (Multiline F/set-3 Germany).

5. Electrical conductivity

The specific or electrical conductivity was determined with the help of conductivity meter (Multiline F/set-3 Germany). The results are expressed as micro (μ) mhos cm^{-1}or dSm^{-1}.

6. Salinity

The salinity of water was recorded in ppt (parts per thomand) using Multiline (f/set-3 (Germany) and also by a salinity refractometer (Artago-Japan).

7. Dissolved oxygen (DO)

Dissolved oxygen was determined at the collection site by modified Winkler's method. To the sample collected (without bubbling) in 250 ml stoppered bottles, 2 ml of manganous sulphate (Winkler's A) and 2 ml of alkaline Iodide-azide solution (Winkler's B) were dispensed at the bottom of the bottle to fix dissolved oxyen. It was thoroughly mixed and the flocculent precipitate was allowed to settle. 2 ml of conc. H_2 SO_4 was added through the side of the bottle and was shaken well to dissolve the precipitates. 50 ml of the above solution was taken in a conical flask and titrated with 0.025 N sodium thiosulphate till pale straw colour appeared. 2-3 drops of starch indicator solution (1%) was added and the sample was further titrated to colourless end point.

$$\text{DO in mg } l^{-1} = \frac{8 \times 1000 \times N\,(0.025) \times V'}{V}$$

Where

V = Volume of the water sample taken (ml)

V' = Volume of the titrant (sodium thiosulphate used ml)

N = Normality of the titrant

8. Biochemical oxygen demand (BOD_5)

Biochemical oxygen demand was determined by dilution and incubation method. It is the decrease in oxygen concentration after incubation in the dark at a constant temperature (20°C) for a fixed period of 5 days.

Dilution water was prepared in a glass container by bubbling compressed air in distilled water for about 30 minutes. Two sets of suitable dilutions were prepared according to the range given in APHA (1998). One set of bottles were kept in the BOD incubator at 20°C for 5 days and from the other set of bottles, DO was determined immediately. After the completion of 5 days of incubation period, dissolved oxygen was determined from the other set of bottles.

BOD_5 in mg l^{-1} = $(D_i - D_5) \times$ dilution factor

D_i = Initial DO in the sample (mg l^{-1})

D_5 = DO after five days of incubation (mg l^{-1})

9. Free carbon dioxide

Free carbon-dioxide was estimated using sodium hydroxide titrant and phenolphthalein indicator. 50 ml of the water sample was taken in a conical flask and two drops of phenophthalein was added. If the colour turned pink, free CO_2 was taken as absent. If it remained colourless it was titrated against standard NaOH (0.02 N) titrant until a slight pink colour appeared.

$$\text{Free } CO_2 \text{ mg } l^{-1} = \frac{\text{ml of titrant used}}{\rule{0pt}{0pt}} \times 100$$

ml of the sample taken

10. Total alkalinity

Carbonate and bicarbonate alkalinity was determined titrimetrially using phenolphthalein and methyl orange indicators respectively. To 50 ml of the sample in erlenmeyer flask, two drops of phenolphthalein indicator was added. If the colour changed to pink, titration was done with 0.02 N H_2SO_4 to a colourless end point (A). If the sample remained colourless on addition of indicator, it revealed that carbonates are absent. In the same sample, 2-3 drops of methyl orange indicator was added and it was further titrated against 0.02N H_2SO_4 till the colour changed from yellow to orange (B).

$$\text{Phenolphthalein alkalinity (carbonate alkalinity) mg l}^{-1} = \frac{\text{ml of titraint 'A'}}{\text{ml of the sample}} \times 1000$$

$$\text{Total alkalinity mg l}^{-1} = \frac{\text{ml of titrant B}}{\text{ml of the sample}} \times 1000$$

11. Total hardness

50 ml of the water sample was taken in a conical flask, one ml of ammonia buffer and a pinch of Eriochrome Black-T indicator were added and titrated against 0.01 N EDTA (Ethylene diamine tetra acetic acid) titrant till colour changes from purple to blue.

$$\text{Total hardness mg l}^{-1} = \frac{\text{ml of EDTA used}}{\text{Volume of the sample (50 ml)}} \times 1000$$

12. Calcium

Calcium was determined by titrimetric method using EDTA as titrant. 2.0 ml of 8% NaOH solution and 0.2g of murexide indicator was added to a suitable volume (50ml) of the water sample taken in a conical flask. Pink colour appeared on shaking. It was titrated against 0.01N EDTA. Appearance of purple colour was noted as end point.

Calculations

$$\text{Ca mg l}^{-1} = \frac{\text{ml of titrant used} \times 400.5 \times 1.05}{\text{Volume of the sample taken}}$$

13. Magnesium

Magnesium was determind as the difference between total hardness (A) and calcium hardness (B) multiplied by a factor (0.244).

Calculations

$$\text{Magnesium mg l}^{-1} = (\text{A-B}) \times 0.244$$

Where

A = total hardness in mg l^{-1} (as described earlier)

$$\text{B} = \frac{\text{ml of titrant used for Ca} \times 1000 \times 1.05}{\text{ml of sample taken for Ca}} = \text{Calcium hardness as } CaCO_3$$

14. Sulphate

Sulphate was determined spectrophotometrically. 10 ml each of NaCl-HCl solution and glycerol-ethanol solution one after the other was added to 50 ml of filtered (through Whatman filter paper No. 1) water sample. 0.15g of barium chloride was added and mixed for 30 minutes on magnetic stirrer. Absorbance at 420 nm was noted against a distilled water blank and the sulphate in mg l^{-1} was deduced from the standard curve prepared by dissolving 0.1479 of anhydrous sodium sulphate in distilled water.

15. Chlorides

50 ml of the sample was taken in a conical flask, 2-3 drops of potassium chromate solution (5%) was added (yellow colour) and titrated against 0.014 N Ag NO_3 until a brick red colour appeared.

$$\text{Chloride in mg } l^{-1} = \frac{\text{ml of titrant used} \times N \times 35.5}{\text{ml of sample}} \times 1000$$

N = Normality of the titrant

16. Orthophosphate (Soluble reactive phosphate)

25ml of the water sample was taken in a conical flask. 1 ml of ammonium molybdate solution, followed by 3 drops of freshly prepared stannous chloride solution was added. After 10 minutes absorbance was read on spectrophotometer at 690 nm. Value of O-PO_4 was deduced from the standard curve prepared by dissolving 0.175 g of potassium dihydrogen phosphate in distilled water (0.2-1.0 mg l^{-1}) O-PO_4.

17. Total Kjeldahl nitrogen

Total kjeldahl nitrogen was determined on micro kjeldahl apparatus. To 40 ml of the water sample, 6g of potassium sulphate, 4 ml of conc. H_2SO_4, 0.1

ml of copper sulphate and 1 ml of 10% NaCl solution was added and allowed to be heated gently in a kjeldhal flask. Heating was stopped when 2-5 ml of residue was left in the flask. Sample was brought to room temperature and distilled water was added to make the volume 100 ml. 25 ml of this solution and 10 ml of 10 N NaOH were heated in the micro kjeldhal assembly and 40 ml of the distillate was collected. Mixed indicator (methyl red and bromocresol green) was used and titrated against 0.01N HCl till the colour changed from blue to brown. Distilled water blank was also run simultaneously.

$$\text{Total K. nitrogen mg l}^{-1} = \frac{(a-b) \times 0.01 \times 1000 \times 14 \times D}{\text{ml of sample distilled}}$$

a = ml of HCl used with sample

b = ml of HCl used with blank

D = dilution factor

18. Ammonical-nitrogen (NH_4-N)

50 ml of the filtered water sample was taken in Erlenmeyer flask. 2 drops of Rochelle salt solution was added, followed by 2 drops of Nesseler's reagent. After 10-20 minutes (colour development) absorbance was read at 425 nm against a distilled water blank. NH_4-N concentrations were calculated from the standard curve prepared by dissolving NH_4Cl (E.merck) in distilled water (dilutions from 0.2-1.0 mg l^{-1}).

19. Nitrite (NO_2-N)

50 ml of the filtered water sample was taken to which 1 ml of the sulphamilamide reagent was added. After 3 minutes, 1 ml of aromatic amine reagent was added and shaken for 1 minute. Absorbance was read at 543 nm against a distilled water blank. Value of NO_2-N was deduced from the standard curve prepared by dissolving sodium nitrite (E. merck) in distilled water (dilutions from 0.2-1.0 mg l^{-1}).

20. Nitrate (NO_3-N)

25 ml of the water sample was evaporated to dryness, 0.5 ml phenol disulphonic acid was added and the residue was dissolved using a glass rod. Thereafter, 5 ml of the distilled water was added followed by 2 ml of 12 N KOH. Sample was shaken for 5-10 minutes and a yellow colour developed, which was measured spectrophotometrically at 410 nm against a distilled water blank. NO_3-N (mg l^{-1}) was determined with the help of a standard curve prepared by dissolving potassium nitrite (E. Merck) in distilled water (dilutions from 0.2 to 1.0 mg l^{-1}).

B. BIOLOGICAL CHARACTERISTICS OF WATER (APHA, 1998)

Following biological characteristics of the water samples were recorded (APHA, 1998) :

1. Phytoplankton and Zooplankton density

Samples were collected by filtering 10 litres of pond water through a plankton net of 125 µm mesh and concentrated to 40 ml in small plastic bottles. Plankton filtrate was then immediately preserved in 4% formalin.

Plankton abundance was expressed as organisms per litre. The organisms were counted in a sedgwick rafter cell. 1 ml of the concentrated plankton sample was transferred to the cell cavity. Planktons were allowed to settle and 10 randomly selected fields of chamber were counted under the microscope and calculated l^{-1} as follows :

$$\text{No. of planktons } l^{-1} = \frac{(P \times C \times 100)}{L}$$

Where,

P = The number of plankton counted in ten fields;

C = Volume of final concentrate of sample (ml)

L = The volume of water sample (l) filtered

Identification of planktons to genus level was carried out using the keys from Ward and Whipple (1959), Prescott (1962) and Bellinger (1992).

2. Species diversity (d)

Species diversity of phytoplankton and zooplankton was determined using Shannon and Weaver's Diversity index :

$$d = -\Sigma\ (ni/N)\ \log_2 ni/N$$

Where

d = Species diversity

ni = Number of individuals of i^{th} species

N = Total number of individuals in the sample

3. Net primary productivity (NPP) and Gross Primary productivity (GPP)

NPP and GPP was determined by light and dark bottle technique (APHA, 1998). One set of light bottles (LB) and one set of dark bottles (DB) were taken and filled with the pond water sample. After tightly stoppering the bottles were hung in the ponds for a period of 24h in the same water body at a fixed depth. After 24 h bottles were removed and DO was fixed and determined according to method described earlier. Initial DO of the water samples was also determined before hanging the bottles in the ponds. Net primary productivity was calculated as follows :

Net oxygen productivity (NOP) (mg l^{-1}) = LB-LIB

Gross oxygen productivity (GOP) (mg l^{-1}) = LB-DB

where

LB = DO concentration of the sample in light bottle after 24 h of incubation mg l^{-1}

DB = DO concentration of the sample in dark bottle after 24 h of incubation mg l^{-1}

LIB = DO concentration of the water samples in the beginning of the experiment mg l^{-1}.

Net oxygen productivity (NOP) and Gross oxygen productivity (GOP) was converted and expressed as NPP and GPP g C m^{-3} d^{-1} = mg C l^{-1} d^{-1} by multiplying NOP and GOP with a factor 0.375 and dividing the products by the period of incubation in days.

$$\text{NPP mg C } l^{-1} d^{-1} = \frac{\text{NOP} \times 0.375}{t}$$

$$\text{GPP mg C } l^{-1} d^{-1} = \frac{\text{GOP} \times 0.375}{t}$$

t = Period of incubation in days

4. Chlorophyll 'a' and Pheophytin 'a'

The chlorophyll 'a' pigments were extracted using aqueous cold acetone from the concentrated plankton sample spectrophotometrically.

Concentrated water sample was filtered through Whatman filter paper No. 40. Filter paper was rolled and placed in plastic tubes. 5-10 ml of 90% acetone (ice-cold) was added so as to cover the filter paper. 0.2 ml $MgCO_3$ was added to prevent degradation of pigments. Then the filter paper was ground using tissue grinder. 5 ml of 90% acetone was again added and tubes were kept in a refrigerator for 24h. Thereafter, the tubes were centrifuged at 3000 rpm for 10 minutes. Supernatant was decanted into glass cuvettes. Absorbance was noted at 630 nm, 647 nm and 664 nm wave lengths. Samples were then acidified by the addition of three drops of 0.1 mol L^{-1} HCl and absorbance was measured again at 750 and 665 nm after 90 seconds of acidification. Chlorophyll 'a' and pheophytin 'a' concentrations were calculated according to the equations given in APHA (1998) using the corrected values of optical density. Zero was adjusted with distilled water.

$$\text{Chlorophyll 'a' mg l}^{-1} = 11.85(664) - 1.54\,(647) - 0.08\,(630).$$

$$\text{Pheophytin 'a' (mg/m}^3) = \frac{[26.7 \times \{1.7\,(665a) - 665b\} \times \text{Vol. of extract}]}{\text{Volume of sample, m}^3}$$

Note : Substract the absorbance values recorded at 750 nm (correction factor for turbidity) from all other absorbance values recorded at 630, 647, 664 and 665 nm before calculations.

C. GROWTH PARAMETERS

1. Live weight gain

Weight gain was measured in terms of differences between final weight and initial weight.

$$\text{Weight gain} = W_2 - W_1$$

Where,

W_1 = Initial weight

W_2 = Final weight

2. Growth per cent gain in body weight

Growth per cent gain in body weight was measured by the following formula :

$$\text{Growth \% gain in body weight} = \frac{W_2 - W_1}{W_1} \times 100$$

Where,

W_1 = Initial weight

W_2 = Final weight

3. Growth per day in percentage body weight

Growth per day in percentage body weight was calculated as follows:

$$= \frac{2(W_2 - W_1)}{t\,(W_2 + W_1)} \times 100$$

Where,

W_1 = Initial weight (g)

W_2 = Final weight (g)

t = Duration of experiment (No. of days)

t = Duration of experiment (No. of days)

4. Specific growth rate (SGR)

SGR was calculated using the following formula :

$$SGR = \frac{\text{In } W_2 - \text{In} W_1}{} \times 100$$

t

Where,

W_1 = Initial weight

W_2 = Final weight

t = Duration of experiment (days)

5. Length specific growth rate

Length specific growth rate was calculated using following formula:

$$SGRL = \frac{\text{In } L_2 - \text{In } L_1}{t} \times 100$$

Where,

L_1 = Initial length (cm)

L_2 = Final length (cm)

t = Duration of experiment (days)

6. Condition factor (k)

At the completion of each experiment, fish from each treatment were individually weighed to the nearest 0.01g and total length was measured to the nearest mm to determine the condition factor as follows :

$$k = \frac{W_t \times 10^5}{L^3}$$

Where, Wt is weight in grams, and L is total length in millimetres.

7. Length weight relationship (LWR)

In order to find out the growth pattern of fish at the end of the experiment (field experiment) individual length (cm) and weight (kg) of fish were recorded and length-weight relationship (LWR) was calculated according to the following equation :

$$W = c\,L^n \quad \text{or} \quad \log W = \log C + n \log L$$

Where, W = weight in kg, c = constant, n = exponential value of length and L = length of fish in cm.

Calculations

Σ log L (ΣX) Σ log W (ΣY) Σ log L ×log W (ΣXY)

Σ log L^2 (ΣX^2) Σ log W^2 (ΣY^2)

Mean log L (X) Mean log W (Y)

Calculate sum of squares as follows :

$$xy = \Sigma XY - \frac{(\Sigma X) \times (\Sigma Y)}{N}$$

$$x^2 = \Sigma X^2 - \frac{(\Sigma X)^2}{N}$$

$$y^2 = \Sigma Y^2 - \frac{(\Sigma Y)^2}{N}$$

$$b = \frac{xy}{x^2} \qquad b = n$$

$$a = Y - b X \qquad a = c$$

$W = c L^n$ N = Number of fishes examined

8. Viscero-somatic index and Hepato-somatic index

At the end of experimental treatment fish from different treatments were obtained, after recording weight to the nearest gram the fish was diacted, viscera and liver were removed, weighed to the nearest 0.01 g for calculating VSI and HSI as follows :

$$VSI = \frac{\text{Viscera weight}}{(A)} \times 100$$

$$HSI = \frac{\text{Liver weight (g)}}{(A)} \times 100$$

Where A is total weight of fish in grams.

D. BIOCHEMICAL ANALYSIS OF FISH

Biochemical analysis of fish carcass was carried out following AOAC (1995).

1. Per cent moisture

To determine the moisture contents, weighed amount of the sample was dried at 100±5°C initially for 30 minutes, and thereafter at 60°C until a constant weight was obtained.

$$\text{Moisture content (\%)} = \frac{\text{Weight of fresh sample} - \text{weight of dry sample}}{\text{Weight of fresh sample}} \times 100$$

Dry matter content was determined as the difference between fresh sample and moisture content.

Crude protein, crude lipid, crude fibre, nitrogen-free extract (NFE) and ash contents of the ingredients were calculated as per cent of dry matter.

Dry matter (%) = 100 - % moisture

2. Crude protein by kjeldahl method

The crude protein was estimated by micro-kjeldahl technique. 0.1-0.5g of the dried sample was taken in a long necked 100 ml digestion flask. To this 0.5-2.0 g of the catalyst (K_2SO_4 and $CuSO_4$ in the ratio of 9:1) and 10-30 ml concentrated H_2SO_4 were added. The mixture was gently heated till a clear solution was obtained. The digestion mixture was then transferred to 100 ml capacity volumetric flask, cooled to room temperature and the volume was made to 100 ml by adding distilled water. 10 ml of this solution was taken in a distillation flask to which 15-20 ml of NaOH solution (40% w/v) was added. Thereafter, about 40 ml of the distillate (ammonia liberated) was collected in 10 ml of 0.01N H_2SO_4 with 2-3 drops of methyl red indicator. The distillate so collected was titrated against 0.01 N NaOH till the blue colour (end point) appears. Crude protein (%) was calculated as follows :

$$\% \text{ crude protein} = \frac{V \times 0.00014 \times D \times 100 \times 6.25}{W \times A}$$

V = Volume of 0.01 N H_2SO_4 taken - volume of 0.01 N NaOH used
(i.e. 10 ml) (i.e. ml of titrant used)

D = Dilution factor (i.e. volume made in volumetric flask)

W = Weight (g) of the sample (i.e. 0.1 g)

A = Aliquot taken (i.e 10 ml)

3. Crude lipid

Crude lipid content was determined using Soxhlet's apparatus. 1.0 g of dried sample was taken in a pouch made of Whatman filter paper (No. 40) and the same was placed in a thimble connected with Soxhlet apparatus. The initial weight of the Soxhlet flask was recorded and gradually filled up with 200 ml petroleum ether (boiling point 60-80^{o}C). The total apparatus was then placed over a mantle and the petroleum ether was allowed to boil for 6-8 h for circulation through the thimble by siphon process. After boiling, the flask was taken out and the petroleum ether was allowed to evaporate. The crude lipid was determined by difference between the final weight of flask and initial weight.

Formula :

$$\% \text{ crude fat} = \frac{\text{Wt of fat (g)}}{\text{Wt of sample (g)}} \times 100$$

4. Ash

The ash content was determined by igniting the weighed amount of the sample in a Muffle furnace at 550±50°C for 6 hours.

$$\text{Per cent (\%) ash content} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

5. Nitrogen-free extract (NFE)

NFE was calculated by difference as 100-(% of crude protein +% crude lipid+% moisture +% ash +% crude fibre)

6. Caloric value

Energy (E) contents were calculated as follows :

E (KJ g^{-1})=0.2364 × protein (%) + 0.3954 × fat (%) + 0.1715 × carbohydrate (%).

7. Phosphorus from fish and feed

1.0 gram of finely powdered sample (feed/carcass) was heated slowly in 10.00 ml of nitric acid and perchloric acid mixture till it becomes colourless and reduced to 2-2.5 ml. After cooling, the volume of the digest was made to 50.0 ml by adding distilled water (A). Then 1.0 ml of ammonium molybdate and 0.15 ml of stannous chloride solution was added to 25.0 ml of solution 'A'. Blue colour appeared. Absorbance at 690 nm was recorded to calculate the value of phosphate from the standard curve by using the following equation :

$$P(\%) = \frac{\text{Concentration} \times \text{Volume of extract made}}{\text{Aliquot}} \times \frac{100}{\text{Wt of sample taken from extract}}$$

8. Estimation of muscle and liver glycogen

The muscle/liver was taken out from the fish and the excess of blood was removed by blotting in between the folds of filter paper immediately. It was put into a weighed stoppered test tube, containing 30% KOH and was again weighed. The amount of alkali was adjusted at the rate of 2 ml per gram of muscle/liver. The tissue was digested in a boiling water bath for one and half hours. It was cooled in ice cold water and two volumes of 95% ethanol were added. The mixture was heated just to boiling avoid spurting. It was left overnight under cold conditions. The contents were centrifuged at 3000 rpm. The precipitates were dissolved in 5-10 ml of warm water. The glycogen were re-precipitated with two volumes of 95% ethanol, centrifuged and washed 2-3 times with 60% ethanol.

The precipitates were further transferred with 2N H_2SO_4 (2.0 ml per gram of muscle/liver). The contents were hydrolyzed in a boiling water bath for 3-4 hours. The solution was neutralized with NaOH using phenol red as indicator. Then made to volume and filtered. Glucose was determined in a aliquot. The factor 0.93 was used to convert glucose to glycogen. The sugar was estimated by Dubois *et al*. (1956). Carbohydrates were estimated by Dubois *et al.* (1956) as follows :

Reagents

1. 5% phenol

2. Concentrated H_2SO_4

To 0.1 ml of the extract, 1.0 ml of 5 per cent phenol and 5.0 ml of concentrated sulphuric acid were added. The contents were shaken rapidly and allowed to stand for half an hour at room temperature. The optical density was read at 490 nm against a reagent blank. Standard curve was plotted using graded concentrations of D-glucose.

9. Estimation of proteolytic enzymes activity (Kunitz, 1947) and muscle protein by Lowry's method

Reagents

1. 1% Bovine Serum Albumin (BSA)
2. 0.1 M phosphate buffer pH 7.6
3. $CaCl_2$ solution
4. 5% TCA

Proteolytic enzyme activity was measured using Bovine Serum Albumin (BSA) as 1% substrate following the methods of Kunitz (1947). The reaction mixture contained 1ml of substrate solution, 1 ml of 0.1 M phosphate buffer (pH 7.6), 1 ml of $CaCI_2$ and 1 ml of crude enzyme extract. The digestion was continued for one hour of incubation at 37°C. It was stopped with 3 ml of 5% TCA solution. After 10 minutes the precipitates were removed by centrifugation. One portion of the supernatant was tested for proteins left digested with 5 ml of Lowry's reagent. The protein was determined by methods of Lowry et al., 1951. Three separate blanks namely (a) containing buffer only, (b) buffer plus substrate and (c) buffer plus enzyme were used. The instrument was set at zero with blanks (a). Enzyme activity in the assay mixture was obtained by measuring absorbance at 660 nm and subtracting the combined absorbance of blank (b) and (c) from it. The equivalent amount of tyrosine released was calculated from the standard curve of tyrosine. One unit of enzyme activity represents the amount of enzyme activity represents the amount of enzyme required to liberate one μg of tyrosine min-I under assay conditions and expressed ml^{-1} of enzyme. Proteins were estimated by Lowry's et al. (1951) given below:

Lowry's Method

Reagents

1. 2 per cent (w/v) Na_2 CO_3 in 0.1 N NaOH.
2. Per cent (w/v) aqueous solution of sodium potassium tartarate.
3. One per cent aqueous solution of $CuSO_4.5H_2O$
4. Reagents 2 and 3 mixed equal volume and 1 ml of the mixture was added to 50 ml of reagent 1.
5. 1 N Folin's reagent.

Procedure

To 1.0 ml of diluted protein solution, 5 ml of reagent 4 was added and mixed thoroughly. It was allowed to stand for 10 min at room temperature. Then 0.5 ml of reagent 5 was added and mixed immediately. After 30 minutes, blue colour was developed and the optical density was measured at 660 nm using spectrophotometer. A blank was also run using 1:0 ml distilled water in place of protein solution. The amount of protein was calculated from the standard Curve prepared for (20-200 µg) of Bovine Serum Albumin as standard protein.

10. Estimation of intestinal amylase enzyme activity

Reagents

1. Starch solution (1%)
2. NaCl solution (1%)
3. 3, 5 Dinitrosalicyclic reagents
4. 0.1 Phosphate buffer (pH 7.0)

Intestinal amylase activity was measured using starch solution as 1% substrate following the methods of Bernfield (1955). The reaction mixture contained 1 ml of substrate solution, 1 ml of 0.1M-phosphate buffer (pH 7.0), 1 ml (1%) NaCl and 1 ml of crude enzyme extract. The digestion was continued for one hour of incubation at 37^{O}C.It was stopped with 0.5ml of 3, 5-dinitrosalicyclic acid. Enzyme activity in the assay mixture. Was obtained by measuring absorbance at 540nm and deducing the value from standard curve prepared by using maltose monohydrate. Soluble protein from the enzyme extract was determined by Lowery's methods. One unit of enzyme activity represents the amount of enzyme activity represents the amount of enzyme required to liberate one µg of maltose min under assay conditions and expressed ml of enzyme.

11. Estimation of intestinal cellulase enzyme activity

Reagents

1. Microcrystalline cellulose (1%)
2. 3, 5-dinitrosalicyclic reagent
3. 0.1 M Phosphate buffer (pH 7.0)
4. Standard Glucose solution

Intestinal cellulose enzyme activity was measured using microcrystalline cellulose as 1% substrate. The reaction mixture contained 1ml of substrate solution, 1ml of 0.1M phosphate buffer and 1ml of crude enzyme extract. The digestion was continued for one hour of incubation at 37^{o}C. It was stopped with 0.5 ml of 3, 5-dinitrosalicyclic acid. Enzyme activity in assay mixture was obtained by measuring absorbance at 540 nm and deducing the value from standard curve prepared by using glucose soluble protein from the enzyme extract was determined by Lowery's method.

One unit of enzyme activity represents the amount of enzyme activity represents the amount of enzyme required to liberate one µg of glucose min^{-1} under assay conditions and expressed ml^{-1} of enzyme.

12. Intestinal lipase activity

Principle

The amount of free fatty acid released per unit time is estimated by the amount of sodium hydroxide required to maintain pH constant. The lipase activity is measured as milli equivalents of alkali consumed.

Reagents

1. Substrate - 2 ml of any clear vegetable oil was neutralized to pH 7.0. It was stirred with 25 ml of distilled water in the presence of 100 mg sodium taurocholate (bile salt) till an emulsion is obtained. 2g of gum arabic was added to increase the emulsification.
2. 0.1 NaOH

   (0.4g of NaOH was dissolved in distilled water and volume was made to 100 ml)
3. Acetone.
4. Acetone-ether mixture (1:1, v/v)
5. Diethyl ether
6. 50 m M phosphate buffer, pH 7.0.

Enzyme assay

- 5 ml of phosphate buffer was added to 20 ml of substrate in a beaker.
- The contents were stirred on the magnetic stirrer cum hot plate maintaining the temperature at 35°C.
- pH of the reaction mixture was adjusted to 7.0.
- 0.5 ml of enzyme extract was added and pH was record immediately and at regular intervals (Say 5 minutes).
- As the pH drops by about 0.2 unit, 0.1 N NaOH was added to bring the pH back to 7.0. Titration was repeated for 30 minutes period and volume of NaOH consumed was noted.
- The protein content in the enzyme extract was estimated according to Lowry's method.
- Enzyme activity was calculated as the amount of enzyme required to release one milliequivalent of free fatty acid/minute/g sample and specific activity as milliequivalents/minute/mg of protein.

$$\text{Lipase activity (meq/minute/g sample)} = \frac{\text{Volume of NaOH consumed} \times 0.1}{\text{Wt. of intestinal tissue} \times \text{time (minutes)}}$$

$$\text{Specific lipase activity (mg/mg of protein/h)} = \frac{\text{Lipase activity (meq/min/g of sample)}}{\text{Protein (mg/g)}}$$

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ABSTRACT

Title of thesis	:	**EFFECT OF PROBIOTICS, MINERAL SUPPLEMENTATION AND BIOFERTILIZERS ON GROWTH PERFORMANCE OF SOME EURYHALINE FISH SPECIES IN INLAND SALINE GROUNDWATER**
Full name of degree holder	:	**Nalle Datta A**
Admission No.	:	2003BS166M
Title of degree	:	Master of Science
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(*An abstract of the thesis submitted to the CCS Haryana Agricultural University, Hisar for partial fulfillment of the requirement for the degree of Master of Science in Fisheries*).

Key words : Biofertilizer, probiotics, calcium, phosphorus, *Chanos chanos*, *Oreochromis niloticus*, fish growth

In this thesis an attempt has been made to study the role of microbial fertilizers in pond productivity, role of probiotics, *Lactobacillus sporogenes* and minerals (Ca-P) supplementation on growth performance and some aspects of nutritive physiology of two euryhaline fish species. The thesis is divided into three parts. Part I describes the role of *Azotobacter* c*hroococcum* and *Gluconacetobacter diazotrophicus* 35-47 in nitrogen fixation and phosphate solubilization in inland saline ground-water ponds stocked with milkfish, *Chanos chanos*. Studies have shown significantly ($P<0.05$) high values for alkalinity, kjeldahl's nitrogen, NO_3-N, turbidity, pigment concentration and fish growth in ponds inoculated with co-culture (Mac-27+ PS-21), followed by Mac-27 and PS-21, o-PO_4 concentration was significantly ($P<0.05$) high both in PS-21 and in mixed culture inoculated ponds. Alkanity, hardness, TDS and chlorophyll *a* concentration were significantly ($P<0.05$) higher in ponds inoculated

with high temperature tolerant mutant of *A. chroococcum*, while total kjeldahl nitrogen, NH_4-N, NO_2-N, o-PO_4 concentration and fish growth were high in ponds inoculated with salinity tolerant strain of *G. diazotrophicus*. Not much variations in DO concentration were observed among different treatments. Irrespective of bioinoculants, a significant (P<0.05) increase in viable counts were observed upto day 7 after inoculation, and thereafter a significant decline in microbial population was observed.

To study the effect of *Lactobacillus sporogenes* (Probiotics) on growth performance of *Oreochromis niloticus*, four diets containing varying concentration (0.25, 0.5, 0.75 and 1.0g 100 g^{-1} of diet) of probiotics were formulated. A diet without supplementation of probiotics was also formulated which served as control diet. All diets contained about 40% of crude protein. Significantly highest growth performance, carcass protein, apparent protein digestibility, nutrient retention (PER, GPR, GER and APD), digestive enzyme activity were observed in the group fed diet containing probiotics at a concentration of 0.75 g, 100 g^{-1} of diet. Excretion of metabolites remained low, while the values of VSI and HSI remained high at this treatment. Muscle glycogen and liver glycogen were also low, while the values of muscle protein were high in fish fed diet-3 containing probiotics at a concentration of 0.75g, 100g^{-1} of diet. These studies indicate that supplementation of diets with appropriate concentration of probiotics can be a useful tool in the utilization of supplementery diets in aquaculture.

An experiment was carried out with *Chanos chanos* and *Oreochromis niloticus* to determine their phosphorus requirements and its interaction with dietary calcium. Ten isoenergetic and isonitrogenous diets were prepared using basal ingredients (groundnut oilcake, processed soybean, rice bran and wheat flour etc.) containing various concentration of phosphorus as potassium dihydrogen orthophosphate (0.25g, 0.375g and 0.5g 100g^{-1} of diet) and calcium as calcium chloride (0.5g, 0.75g and 1.0 g 100 g^{-1} of diet). These concentrations resulted in varying Ca-P ratios (1:1, 2:1, 3:1, 3:2, 4:1, 4:3, 8:3). Calcium and phosphorus concentrations in the water at 10 ppt were 425 mg and 0.03 mg l^{-1} of water, respectively. Irrespective of the fish species, significantly (P<0.05) high growth performance (growth per cent gain in body weight, SGR and final length, APD, GPR, GER and PER were observed in the groups which were fed on a diet containing calcium and phosphorus at a concentration of 0.5g 100g^{-1} of diet in the ratio of 1:1. Low FCR and excretion of metabolites (N-NH_4, o-PO_4) and significantly (P<0.05) highest digestive enzyme activity were also observed in fish fed on this concentration. Low muscle and liver glycogen levels and high muscle protein content coincided with the highest growth performance observed in these studies. VSI and HSI values were also high in fish which had the highest growth performance. Carcass composition also indicated high accumulation of protein. These studies indicated that diets must be supplemented with minerals especially calcium and phosphorus in optimum concentrations and ratios for obtaining the full benefit of supplementary diets.

MAJOR ADVISOR **SIGNATURE OF STUDENT**

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