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Effect of Extramin on growth enhancement of white leg shrimp *Litopenaeus vannamei* (Boone, 1931) in low saline semi-intensive pond culture system

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Abstract
The present work emphasizes the impact of the commercial mineral product, Extramin on achieving higher productivity in low saline (0 to 10 psu) *Litopenaeus vannamei* culture. Water quality parameters like salinity, pH, temperature, dissolved oxygen, total hardness, nitrate, transparency, alkalinity, and phytoplankton population dynamics were monitored in control and test ponds treated with Extramin. In addition, product response parameters like the effect of Extramin on molting, white muscles, muscular cramps, arising due to mineral deficiency and impact on survival and food conversion ratio (FCR) were also assessed. Affected shrimp ponds achieved 100% recovery upon administration of Extramin. Test ponds treated with Extramin has developed stable and proper bloom with good population of planktons ranging from 254-359×10^4 cells ml^{-1}, whereas, in control ponds 214-279×10^4 cells ml^{-1} with frequent crash were observed. Overall, the ionic composition and stoichiometry were well balanced in Extramin treated ponds for shrimp growth favouring higher productivity.

Keywords: *Litopenaeus vannamei,* low saline waters, Extramin, muscle cramps, white muscles, and phytoplankton population

1. Introduction
Culturing shrimps on low saline waters has become a common practice in the current scenario throughout the world including Brazil, China, Ecuador, India, Thailand, Mexico, United States and Vietnam [1]. While *L. vannamei* is capable of tolerating a wide range of salinities [1], proper acclimation to low saline waters is the first step in the production process for successfully rearing this species at low salinity. Due to its remarkable ability to thrive in low saline milieus, the Pacific white shrimp, *L. vannamei*, is the principal candidate of choice for shrimp farmers farming in low saline waters [2]. Over the past decade, substantial advances have been made in understanding the low salinity tolerance of this species. Improved understanding of the physiology has resulted in the development of successful culture practices and strategies utilizing low saline waters.

In addition to basic water quality parameters in *P. vannamei* culture, the mineral profile of water plays a vital role in profitable farming. Literally, the quantification of mineral requirement was found to be problematic due to the variability in the ionic profiles of pond waters. In order to develop a successful culture system, levels of minerals in pond water has to be more or less similar to the levels in seawater diluted to the same salinity. However, pond water characteristics of even the very closely located ponds will not be exactly the same.

Ionic ratios were observed to be quite different between seawater and waters from different sources. The ratio of Na (Sodium) to K (Potassium) and Mg (Magnesium) to Ca (Calcium) in water appears to be more significant than pond water salinity. Improper ratios of these minerals in water lead to osmotic stress which may influence growth and survival of shrimp negatively, in terms of mortality [3]. In order to maintain optimum concentration of minerals and ionic balance, modifications in mineral supplementation through water and diets is possible. Ionic levels in ponds with low saline waters have to be raised to match their required concentration.

White muscle disease (WMD) and Muscle cramp syndrome (MCS), which is caused due to mineral deficiency and poor feeding remains as a major threat and sources of mortality in shrimp culture [4].

The current work emphasizes on rectifying the ionic ratio imbalances in pond water using the commercial mineral product, Extramin and thereby influencing the growth and survival of shrimps with lesser Food Conversion Ratio (FCR). The work was conducted with special emphasis on the efficacy of Extramin in recovering animals from muscular cramps, white muscles and stress free molting in order to achieve desired productivity.

2. Materials and methods

2.1 Study site

Six shrimp ponds of equal size (0.5 ha each) located in Kanchivayil, Ponneri (13.39˚ N, 80.22˚ E), Tamil Nadu (Fig. 1) were selected for the study during the period of March to June 2016.

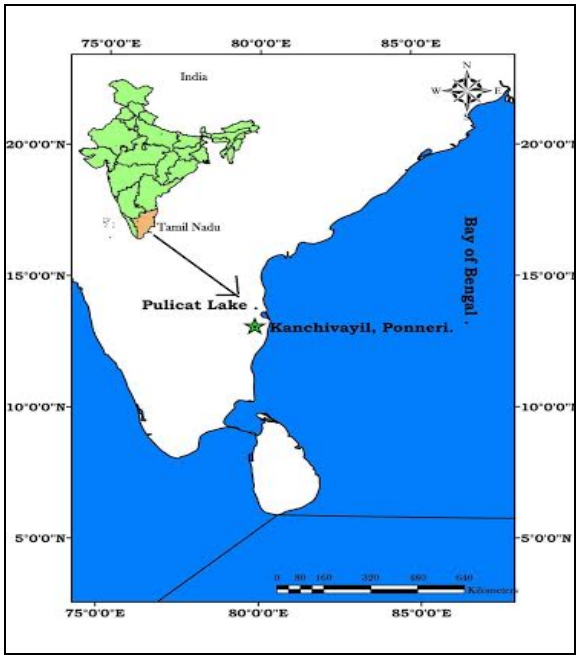

Fig. 1: Figure showing the study area, Kanchivayil Village, Ponneri Taluk, Tamilnadu, India

2.2 Product composition

Extramin is a commercial mineral product from the house of Tablets (India) Limited, Chennai, India, certified by Coastal Aquaculture Authority of India (CAA), Government of India. The composition of Extramin (CAA Reg. No. CAA/F16/FA/00013) per 1000gms is presented in table 1.

Table 1: Composition of EXTRAMIN

Composition of EXTRAMIN per 1000 gms		
Calcium	210	gms
Phosphorus	105	gms
Magnesium	13	gms
Manganese	596	gms
Potassium	9.6	gms
Ferrous	1.5	gms
Copper	1.25	gms
Cobalt	4	mg
Sulphur	10	gms
Zinc	9	gms
Selenium	10	mg
Chromium	20	mg
Iodine	4	mg
Lysine Hydrochloride	19	gms

2.3 Experimental design

Proper pond preparation was carried out in all the selected ponds. Bore water with salinity ranging from 7 to 9 psu was pumped onto the shrimp ponds. Post larvae 12 were stocked at a density of 30/m^2 with the water depth of 1.5m. Three test ponds were treated with Extramin and three ponds were considered as control. 3 kgs/ha of Extramin for each 0.5 hectare pond was mixed thoroughly with same pond water and was broadcasted across the pond between 4 to 7 pm. No water exchange was carried out in any ponds. Physico-chemical parameters of the water and plankton analysis were carried out once in week and was analysed. Product response parameters including recovery from white muscles, muscular cramps and improper molting was calculated on control and test ponds. Survival rate and FCR were calculated at the end of the culture.

2.4 Sampling analysis

2.4.1 Physico-chemical analysis

The physicochemical parameters of water were analysed in the control and test ponds pre and post usage of Extramin once in a week at three different points/spots. Water samples were collected between 07.00 and 08.00 hrs for *in situ* examination and laboratory analysis. Samples were shuttled in 250 ml polyethylene bottles to laboratories. The collected samples were transported in ice-container to the laboratory and were analysed immediately for alkalinity, hardness and nitrate. Water samples were filtered using 0.45 µm membrane filter prior to analyses in order to remove any suspended particles and materials. The salinity in the ponds was recorded *in situ* by means of a portable hand-held optical refractometer (Atago, Japan) and cross-checked in laboratory using Mohr-Knudsen method as salinity is a key factor in this research work. pH was measured using electronic pH pen (Erma, Japan), and temperature was measured using standard Celsius Thermometer. The dissolved oxygen was estimated by modified Winkler's method as described by Strickland and Parsons [5]. The total hardness of the water was estimated by complexometric titration using EDTA [6] and alkalinity was measured as per APHA [7]. Transparency was measured based on the penetration of light using a Secchi disc.

2.4.2 Plankton analysis

Plankton samples were collected using standard plankton net of 1 m length, 25 cm mouth diameter, and mesh size of 20 µm. The plankton net was towed horizontally and vertically to sample the plankton. For horizontal towing, plankton sample was collected by lowering the net horizontally into the water then pulled until the net extended and began to tow. The net was scooped through the shrimp pond water while walking slowly along the pond's bank. For vertical towing, the net was lowered into the water to approximately 1 m depth and was kept vertical and off the bottom. The net was pulled straight up through the water column. The samples were then rinsed into collection vessels.

The samples were preserved in 4% neutral formaldehyde (final concentration) in polyethylene bottles for plankton abundance and identification. Samples were observed and morphologically identified with ZEISS research microscope coupled with an image analysing system using keys and illustrations by Prescott [8], Patrick and Reimer [9], Round [10], Tomas [11], and other taxonomic literature.

Total number of phytoplankton (standing drop) present in a litre of water sample was calculated using the formula:

$N = nv/V$
Where,
N = Total number of phytoplankton cells per litre of water filtered
n = average number of plankton cells in 1mL of plankton sample
v = volume of plankton concentrate (mL)
V = volume of total water filtered (l)
The units of standing drop are N/l or $N\times10^3$ /m^3
Phytoplankton abundance and diversity in the shrimp ponds were thus determined.

2.4.3 Product response parameters
The number of animals affected with white muscles, muscular cramps and improper molting was calculated by conducting sampling with cast net of known diameter (3.2 m) at four corners of the pond and the recovery percentage was calculated post administration of Extramin on water application and dietary administration. Prescribed dosage of 3kgs/ha was applied on water, followed by 10gms/kg through dietary administration.

2.5 Statistical analysis
The data were presented as mean ± SE. Student's t-test was applied to determine the significant difference ($P<0.001$) between the control and test ponds. All statistical calculations were performed using SPSS for Windows version 11.5 (SPSS Inc, Chicago, IL, USA). All column charts were plotted using Origin 6.1(OriginLab Corporation, Massachussets, USA).

3. Results
Maximum temperature in the control group throughout the study period was reported to be 34˚C and the minimum was observed to be 25˚C. Maximum and minimum temperature was reported to be the same in both the experimental groups and hence, no significant difference ($P < 0.001$) was observed between the control and test group (Fig. 2).
pH in control and test ponds ranged from 7.8 to 8.5 and hence no significant difference ($P < 0.001$) was observed between the control and test ponds (Fig. 3). Salinity ranged from 7 to 9 psu in the control and test ponds. However, no significant difference ($P < 0.001$) was observed between the two experimental groups (Fig. 4). The dissolved oxygen level was observed to be in the range of 4 to 4.4 ppm in the control and test groups with no significant difference ($P < 0.001$) between the two groups (Fig. 5).
Transparency was reported to be high on both test and control ponds at the initial days of the culture. Test ponds administered with Extramin soon developed optimal and stable levels, thus favouring the growth of the post larvae leading to improved survival rate. In addition, no bloom crash was reported in the test treated ponds. In contrast, control ponds encountered bloom crash twice during the culture period, first on the 77th day and second on 112th day. The transparency level varied across week's symbolizing the unstable bloom development (Fig. 6). The means of the two experimental groups were notable and the variation was observed to be statistically significant ($P < 0.001$) by Student's *t*-test between the control and test groups with the *P* value of 1.1185E-12.
Total hardness estimated using complexometric titration method showed statistically significant difference ($P < 0.001$) between the means of two experimental groups with *P* value of 3.92364E-13 (Fig. 7). Total hardness ranged from 2300 to 3000 mg/L in the control ponds and test ponds. Total alkalinity was reported to be on the range of 130 to 170 ppm in both test and control ponds (Fig. 8). Nitrate level in the control pond ranged from 0.12 to 0.17 ppm and 0.12 to 0.25 ppm in Extramin treated test ponds (Fig. 9). Phytoplankton diversity and abundance exhibited fourteen species of phytoplankton in Extramin treated ponds and twelve species in control ponds. Test ponds demonstrated density of 254-359×10^4 cells/ml and control ponds with 214-279×10^4 cells/ml (Fig. 10).
100% recovery was observed in test ponds with muscular cramps (Fig. 11) and white muscles (Fig. 12) upon administration of Extramin on water and dietary administration, whereas, control ponds were found to be unrecovered. Application of Extramin in water, followed by feed has recovered the animals from white muscles and muscle cramps very effectively. Number of animals in molting crossed the margin of 60% in test ponds with margin of 40% in control ponds (Fig. 13). Percentage survival of shrimps was calculated at the end of the culture and was 90% in test ponds and 71% in the control ponds (Fig. 14). FCR was calculated post-harvest and was found to be 1.2 in test ponds and 1.8 in control ponds (Fig. 15). Total biomass produced in the test ponds was higher with lesser feed conversion ratio (FCR).

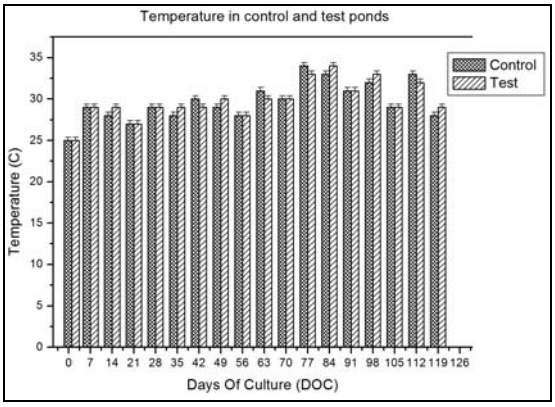

Fig 2: Values of temperature recorded on control and test ponds for a period of 119 days at regular intervals of 7 days. *Results are presented as means with standard errors of three replicates for each experimental group (mean ± SE; n = 3)

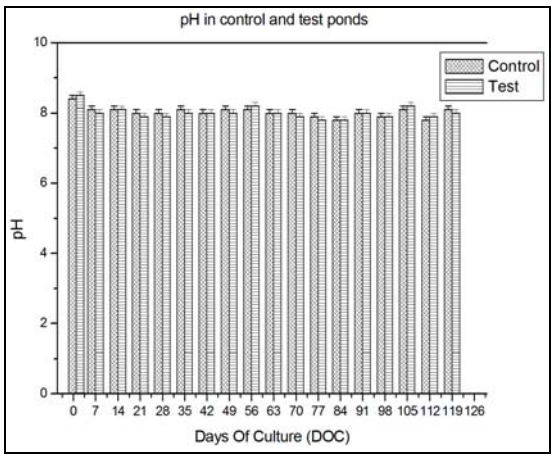

Fig 3: Values of pH recorded on control and test ponds for a period of 119 days at regular intervals of 7 days. *Results are presented as means with standard errors of three replicates for each experimental group (mean ± SE; n = 3)

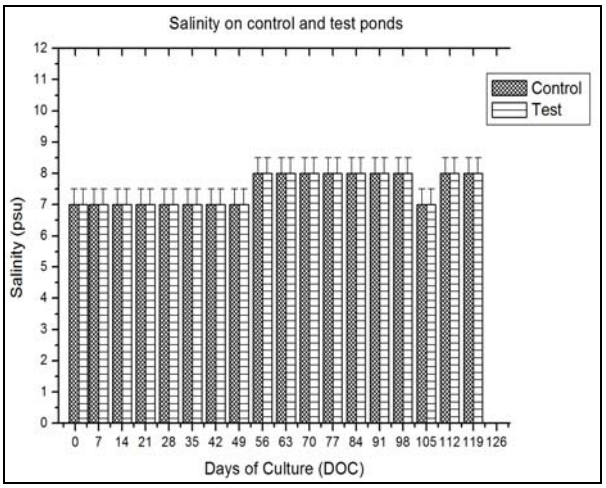

Fig 4: Values of salinity recorded on control and test ponds for a period of 119 days at regular intervals of 7 days. *Results are presented as means with standard errors of three replicates for each experimental group (mean ± SE; n = 3)

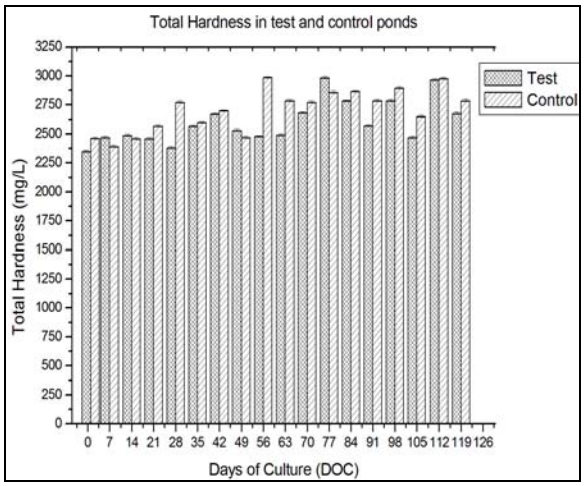

Fig 7: Values of hardness recorded on control and test ponds for a period of 119 days at regular intervals of 7 days. *Results are presented as means with standard errors of three replicates for each experimental group (mean ± SE; n = 3)

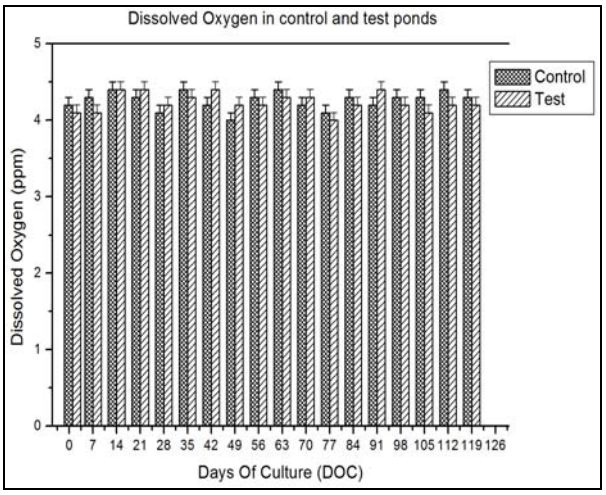

Fig 5: Values of dissolved oxygen recorded on control and test ponds for a period of 119 days at regular intervals of 7 days. *Results are presented as means with standard errors of three replicates for each experimental group (mean ± SE; n = 3)

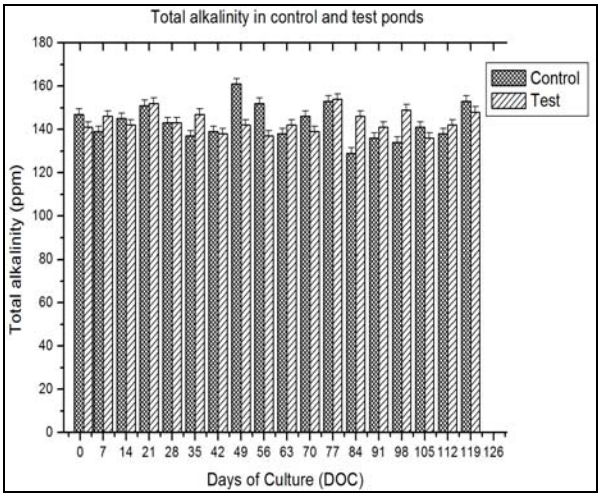

Fig 8: Values of total alkalinity recorded on control and test ponds for a period of 119 days at regular intervals of 7 days. *Results are presented as means with standard errors of three replicates for each experimental group (mean ± SE; n = 3)

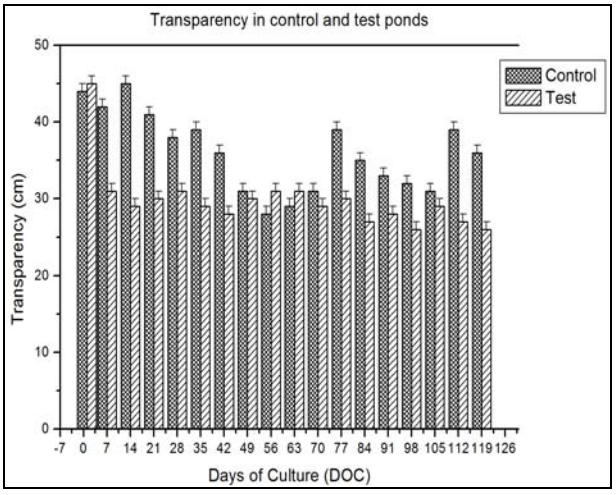

Fig 6: Values of transparency recorded on control and test ponds for a period of 119 days at regular intervals of 7 days. *Results are presented as means with standard errors of three replicates for each experimental group (mean ± SE; n = 3)

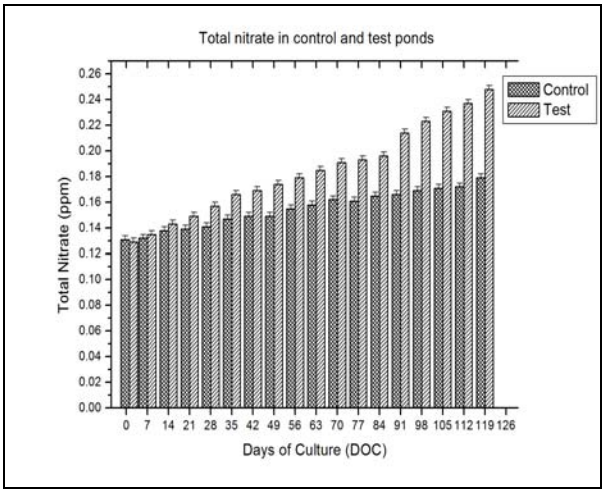

Fig 9: Values of total nitrate recorded on control and test ponds for a period of 119 days at regular intervals of 7 days. *Results are presented as means with standard errors of three replicates for each experimental group (mean ± SE; n = 3)

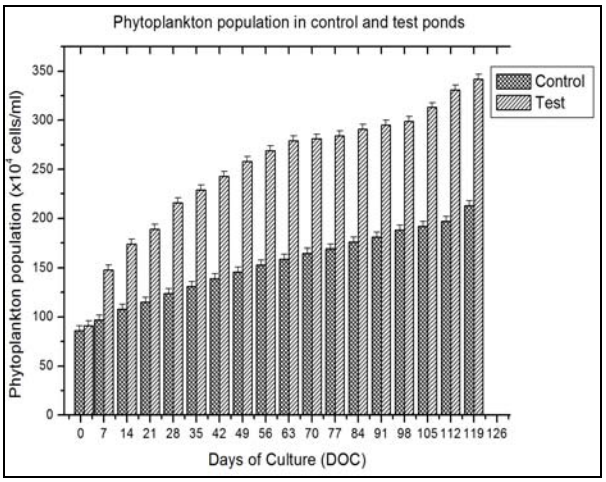

Fig 10: Values of phytoplankton population recorded on control and test ponds for a period of 119 days at regular intervals of 7 days. *Results are presented as means with standard errors of three replicates for each experimental group (mean ± SE; n = 3)

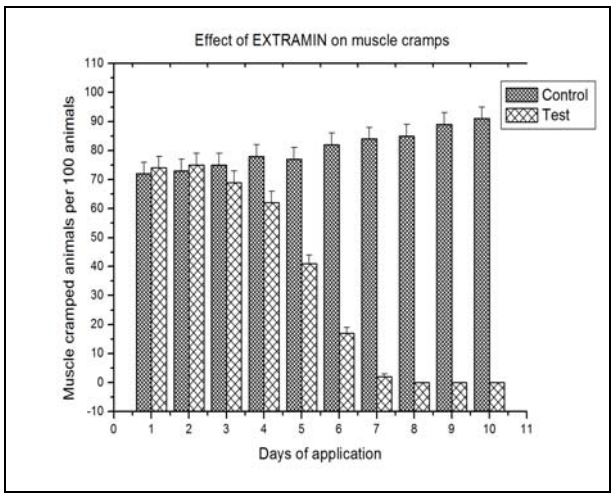

Fig 11: Figure showing the effect of EXTRAMIN on controlling muscular cramps in shrimps. *Results are presented as means with standard errors of three replicates for each experimental group (mean ± SE; n = 3)

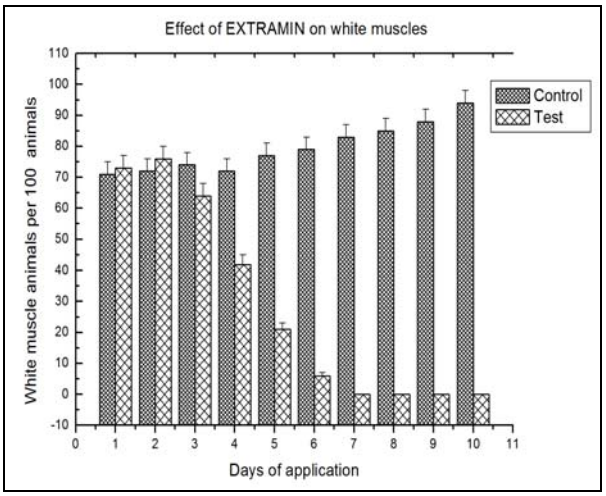

Fig 12: Figure showing the effect of EXTRAMIN on controlling white muscles in shrimps. *Results are presented as means with standard errors of three replicates for each experimental group (mean ± SE; n = 3)

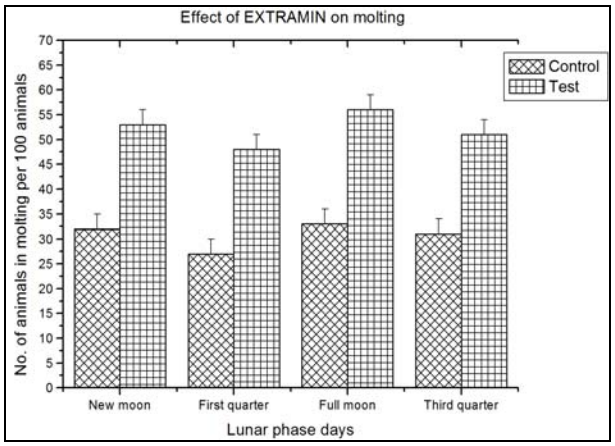

Fig 13: Figure showing the effect of EXTRAMIN on controlling improper molting in shrimps. *Results are presented as means with standard errors of three replicates for each experimental group (mean ± SE; n = 3)

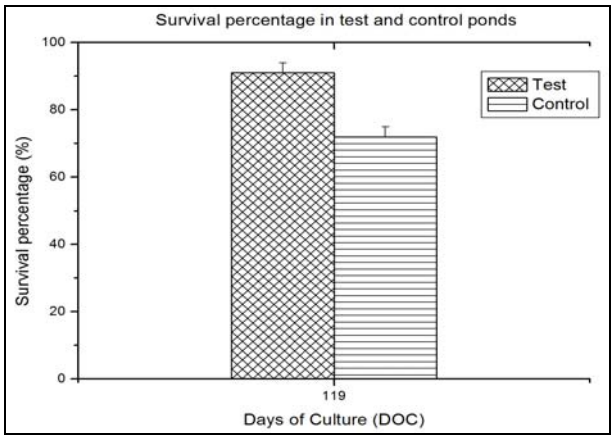

Fig 14: Percentage survival of shrimps in control and test ponds. *Results are presented as means with standard errors of three replicates for each experimental group (mean ± SE; n= 3)

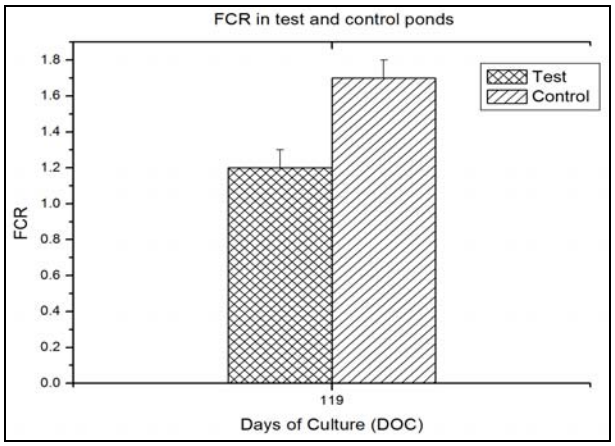

Fig 15: FCR in control and test ponds. *Results are presented as means with standard errors of three replicates for each experimental group (mean ± SE; n = 3)

A dietary source of 23 minerals has been demonstrated as essential in one or more animal species [12]. These elements are divided into two groups: the 7 macro minerals and 16 trace minerals (Table 2). The noticeable biological functions of the macro and micro minerals and deficiency signs in shrimps are explained in table 3.

Table 2. The essential mineral elements [Underwood (1971)]

Macroelements		Trace or microelements	
Principal cations	Principal anions		
Calcium (Ca)	Phosphorus (P)	Iron (Fe)	Fluorine (F)
Magnesium (Mg)	Chlorine (Cl)	Zinc (Zn)	Vanadium (V)
Sodium (Na)	Sulphur (S)	Manganese (Mn)	Chromium (Cr)
Potassium (K)		Copper (Cu)	Molybdenum (Mo)
		Iodine (I)	Selenium (Se)
		Cobalt (Co)	Tin (Sn)
		Nickel (Ni)	Silicon (Si)

Table 3: List of essential macro and micro minerals, their noticeable functions and deficiency signs observed in shrimp

No.	Mineral	Functions	Deficiency signs
	Macro-minerals		
1.	Calcium	Skeletal tissues, membrane permeability	Impaired growth and hard tissue mineralization
2.	Chloride	Osmotic balance	Impaired growth
3.	Magnesium	Enzyme activator	Tetany, muscle flaccidity
4.	Phosphorus	Skeletal tissue, phospholipids	Impaired growth, reduced hard tissue mineralization, skeletal deformities, fat accumulation
3.	Potassium	Osmotic balance, acid-base equilibrium	Convulsions, tetany
4.	Sodium	Osmotic balance, acid-base equilibrium	Impaired growth
	Micro-minerals		
1.	Copper	Metalloenzymes	Impaired growth and reduced activity of copper containing enzymes
2.	Cobalt	Vitamin B_{12}	Anemia
3.	Chromium	Carbohydrate metabolism	Impaired glucose utilization
4.	Iodine	Thyroid hormones	Thyroid hyperplasia
5.	Iron	Haemoglobin	Impaired growth, anemia
6.	Manganese	Organic matrix of bone	Impaired growth, skeletal abnormalities, cataracts
7.	Molybdenum	Xanthine oxidase	Reduced enzyme activity
8.	Selenium	Glutathione peroxidase	Impaired growth, anemia, exudative diathesis, reduced activity of glutathione peroxidase
9.	Zinc	Metalloenzymes	Impaired growth, skeletal abnormalities, cataracts, reduced activity of various zinc metalloenzymes

4. Discussion

Nutrition encompasses the chemical and physiological process which provides nutrients to an animal for normal function, increase in immunity, disease resistance, maintenance and growth. It involves ingestion, digestion, absorption and transport of nutrients and removal of waste. Poor nutrition and feeding affects the shrimp in various ways and may lead to irregular metabolism consequently leading to emergence of diseases opening up the system for pathogens and nutritional imbalance thus deteriorating the dynamics. The indicators of physical health status of the animals as described by Fegan and Clifford [13] including growth, weight, molt stages, gut emptiness, gross signs on body and appendages such as opaqueness of abdominal muscle, deformities, appendage segments, colored gills and exoskeleton examination for necrotic lesions on the external body surface plays a key role in knowing the health status of the animal.

Environmental factors may also have an effect on immunological response [14]. Temperature is one of the most important environmental parameters in shrimp aquaculture as it directly influences the metabolism, oxygen consumption, growth, moulting and survival [15]. Sudden change in temperature may affect the shrimp immune system. Temperature in the current study was optimal favouring the growth of the shrimp.

pH is yet another significant physicochemical parameter effecting the growth of the shrimps to a higher extent by impelling the metabolism and other physiological processes in the biological system. pH and photosynthesis are almost directly linked as higher pH infers higher photosynthesis and higher fertility. Perhaps, this is due to the fact that, water fertility leads to healthier plankton bloom, which subsequently leads to higher photosynthesis. Ramanathan *et al* [16], reported that, the optimum range of pH required for maximum growth and production of penaeid species is 6.8 to 8.7.

Salinity is considered to be the most vital factor in propelling many functional responses of the shrimp biological system as metabolism, growth, migration, osmotic behaviour, and reproduction [15]. Dissolved oxygen is yet another critical factor required for the respiration of shrimps, in maintaining a balance between the biological system and chemical environment and maintaining the hygiene in the culture ponds. It has been shown that low dissolved-oxygen levels increase the toxicity of ammonia to the shrimp [17]. Decreased oxygen level induces the reduction of nitrates to ammonia, which is toxic to the shrimps. It also impedes metabolic performances in shrimp and can reduce growth and moulting which leads to increased mortality [18].

Transparency is a factor exemplifying the plankton bloom. Phytoplankton plays a significant role in the pond ecosystem and minimizes the water quality fluctuations thereby enriches the water quality and suppress the bacterial growth [19]. Higher

the transparency, lower the plankton growth. Hardness is the concentration of divalent cations, mostly calcium and magnesium in water expressed in milligrams per liter (parts per million) of equivalent calcium carbonate. Calcium hardness plus magnesium hardness, of course, is total hardness. The factors for converting between hardness cations and hardness are as follows: calcium × 2.5 = calcium hardness, and magnesium × 4.12 = magnesium hardness.

Nutrients such as nitrogen and phosphorus in the shrimp ponds were originated mainly from prepared feed [20], fertiliser used, water pumped into the pond, juveniles stocks, rainfall [21] and shrimp excretion [20]. Nitrification is a transformation process of ammonia (oxidation by bacteria) to nitrite and then to nitrate. Algal blooms can produce hypoxia or anoxia with resulting shrimp [22]. Nitrates to ammonia reduction process were induced by decreased level of available oxygen level, which is thus toxic to the shrimps. Decreased oxygen level also interferes in the metabolic performances of the shrimp and can reduce growth and moulting, consequently leading to increased mortality [15].

The high nitrate concentrations may be responsible for the dominance of diatoms. Vanni and Findlay [23] and Cremen *et al.* [20] agreed that high phosphate concentrations usually encouraged the growth of Cyanophyta, whereas high nitrate concentration encourages diatoms growth. Cremen *et al.* [20] revealed that high ammonium and nitrite levels result in high N: P ratio that will promote diatom blooms. In addition, Smith [24] reported that some shrimp ponds with high nitrogen loading rates could cause the absence or rare occurrence of Cyanophyta.

The application of fertilizers, containing sources of K and Mg, has dramatically increased growth, survival, and overall production of shrimp in low saline waters where these ions are deficient. Several studies have focused on the importance of Na:K ratios on survival and growth of *L. vannamei* in low saline waters. In full strength seawater, the Na:K ratio is approximately 28:1. In K deficient waters, such as those used by farmers to grow shrimp in west Alabama, lowering the Na:K ratio (by increasing water K concentrations by the addition of fertilizers) dramatically increases growth and survival of shrimp reared in low saline waters. Zhu *et al.* [25] and Zhu, Dong and Wang [26] reported that high Na:K levels can have an effect on shrimp growth even at salinities as high as 30 psu. In the laboratory study conducted with juvenile *L. vannamei* in a recirculating system with artificial low saline waters (4 psu) in Alabama, growth, survival, and weight gain, were affected by Na:K ratio following seven weeks of culture.

A higher growth rate implies an increase in the molting frequency [27] and high molting frequency might not only increase energy expenditure for exuviations, but also alter the animal's entire energy allocation strategy [28]. Extra energy allocated to growth and molting may be derived from other functions involved in reactions and/or adaptation to environmental variations, including response to pathogens.

The cuticle of most crustaceans contain minerals primarily $CaCO_3$ with small amounts of magnesium, phosphorus and sulphur. About 99 % of the total inorganic composition of the exoskeleton widely varies among species, location on the body and stage of the molt cycle [29]. Penaeids may need dietary sources of minerals for growth because of repeated moltings wherein minerals are lost [30]. The availability of minerals to shrimp is dependent on the dietary source and form of the mineral that is ingested, amount stored in the body, interaction of other elements present in the gastrointestinal tract and body tissues and mineral interactions with other dietary ingredients or metabolites. Soluble monobasic, inorganic salts or bioavailable organic salts must be provided in the diet of stomachless shrimps. Phosphorus and calcium availability and absorption is dependent on the presence of an acid secreting stomach [31]. The composition of Extramin balances the mineral requirement of the animal, thus recovering the animal from muscle cramps and white muscles. Paul Raj [32] reported that the average Indian cultured food conversion ratios were varied from 1.5 to 1.75.

A major hazard which may be associated with the use of dietary feed ingredients is the presence of potentially toxic mineral elements such as the accumulative elements copper, lead, cadmium, mercury, arsenic, fluorine, selenium, molybdenum and vanadium. For example, contamination with copper may arise from products fermented within copper lined vessels (ie. brewery by-products). However, Extramin is a purified combination of essential minerals with lysine hydrochloride for improved meat conversion with zero toxic minerals, antibiotics and steroids and was tested and proven to be so by Coastal Aquaculture Authority (CAA) with registration number CAA/F16/FA/00013.

5. Conclusion

Extramin ensures successful crop by providing nutrients to the water for developing optimal bloom to favour the development of phytoplanktons which form the diet of shrimp post larvae. It also induces the alkaline levels required for proper and stable bloom development. By influencing alkalinity, it also improves the colonisation of beneficial probiotic bacteria which in turn aids biofloc development. It supports molting process in shrimp and thus promotes growth. Regular application of Extramin on water and feed administration ensures white muscles and muscular cramps free crops.

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