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Evaluation of the Efficacy of Dietary Inorganic and Chelated Trace Minerals Premixes in Juvenile Korean rockfish, *Sebastes schlegeli* (Hilgendorf) and whiteleg shrimp, *Litopenaeus vannamei* (Boone)

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치어기 조피볼락과 흰다리새우에 있어서 사료 내 무기 및 유기 미네랄 의 영향 평가

Advisor: Sungchul C. Bai

by

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A dissertation

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Table of contents

List of Tables

List of Figures

Evaluation of the Efficacy of Dietary Inorganic and Chelated Trace Minerals (Cu, Zn, Mn, & Fe) Premixes in Marine Fish, Korean rockfish, *Sebastes schlegeli* (Hilgendorf) and Marine Shrimp, whiteleg shrimp, *Litopenaeus vannamei* (Boone)

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Abstract

Four experiments were conducted to evaluate the efficacy of different levels of dietary inorganic and chelated trace minerals (Cu, Zn, Mn & Fe) premixes on the growth performance, tissue mineral saturation whole-body proximate composition, non-specific immune response, disease resistance, histological and hematological response in juvenile Korean rockfish (Initial body weight 9 ± 0.2 g) and juvenile Whiteleg shrimp (Initial body weight 0.6 ± 0.01 g). In experiments I, results suggested that the dietary inorganic trace minerals (Cu, Zn, Mn & Fe) premix supplementation at a minimum level of 0.3% of the diet could be necessary for juvenile Korean rockfish. While, based on the results from experiment II, it could be concluded that the optimum inclusion level for dietary chelated trace minerals premix could be greater than 0.075% but less than 0.3% in juvenile Korean rockfish. While, the results from the experiment III, suggested that the optimum inclusion level for the dietary inorganic trace minerals (Cu, Zn & Mn) premix could be greater than 0.5% but less than 2% of the diet in whiteleg shrimp. While, overall performance from the experiment IV, suggested that the optimum dietary chelated trace minerals (Cu, Zn & Mn) inclusion level could be greater than 0.25% but less than 0.75% for whiteleg shrimp. *L. vannamei*.

요약문

본 4가지의 실험은 섭취할 수 있는 무기물과 킬레이트 화합물의 미량 미네랄 (Cu, Zn, Mn & Fe) 프리믹스의 효능을 알아보기 위하여 수행되었다. 실험어는 최초 어체중 9 ± 0.2 g 인 치어기의 조피볼락과 최초 어체중 0.6 ± 0.01 g 인 흰다리 새우를 대상으로 성장률, 전어체 일반조성 조직미네랄포화도, 비특이적 면역 반응, 질병저항성, 조직학적 반응, 혈액상의 반응을 보았다. 실험 1 에서 최소 수치인 0.3% 에서 섭취할 수 있는 무기물의 미량 영양소 프리믹스 공급은 치어기의 조피볼락에 있어서 필수적인 것으로 나타났다. 반면에, 실험 2 의 결과에서 섭취 할 수 있는 킬레이트 화합물의 미량 미네랄 프리믹스 적정 농도는 조피볼락에 있어서 0.3% 이하이지만 0.075% 이상이 가장 우수한 것으로 나타났다. 이와 달리, 실험 3 의 결과에서 섭취 할 수 있는 무기물의 미량 미네랄 (Cu, Zn & Mn) 프리믹스 적정 농도는 흰다리새우에 있어서 2% 이하이지만 0.5%가 가장 우수한 것으로 나타났다. 반면에, 실험 4 결과에서 섭취 할 수 있는 킬레이트 화합물의 미량 미네랄의 적정 농도는 흰다리 새우에 있어서 0.75%이하이 지만 0.25%가 가장 우수한 것으로 나타났다.

Chapter 1. General Introduction

Minerals are inorganic compounds, smaller than vitamins, needed for the growth and regulation of the physiological body processes. Dietary trace minerals typically required in smaller quantity is called as essential trace element/microminerals *viz*. iodine (I), zinc (Zn), manganese (Mn), selenium (Se) cobalt (Co) iron (Fe), fluorine (F), chromium (Cr), molybdenum (Mo), copper (Cu) (Bai, 2012). Microminerals or trace minerals required by aquaculture species in small quantities participate in a wide variety of biochemical processes. The roles of microminerals or trace elements in basic metabolic functions, with the exception of osmoregulation, are the same for aquatic and terrestrial animals (Nengas, 2012). Although, the importance of trace minerals supplementation in fish feed formulation has been well accepted since some of the trace minerals from ambient water and feed itself cannot supply the optimal level required by the cultured aquatic species. Trace minerals being an essential component of fish nutrition which lags in inquiry when compared to research devoted to other nutrients (Lin et al., 2010). Even though, our knowledge in fish nutrition has advanced significantly, the information on trace minerals requirement is still limited and fragmentary. The reluctance among researchers to determine the trace minerals requirement has been partly due to the related difficulty of conducting research on mineral nutrition. Problems associated with the quantification of mineral requirements include identification of the potential contribution of minerals from the water, leaching of mineral from the diet prior to consumption, availability of suitable test diets that have a low concentration of the targeted mineral and limited bioavailability (NRC, 2011). Whereas, the sustainability issue has put a new dimension in aquafeed formulation with a wide array of new ingredients and additives, on the other hand, the importance of basic nutrient such as trace minerals is still in sideline. Substantial investment and integrated scientific efforts are warranted to bridge the knowledge gap and further improve our understandings on the significance of dietary trace minerals in fish nutrition and health at the least cost to the environment (Bai, 2014).

The trace minerals including copper (Cu), manganese (Mn), iron (Fe), zinc (Zn), selenium (Se), chromium (Cr), iodine (I) and fluorine (F) participate in a variety of metabolic processes. Some of the vital biochemical processes involving minerals are the formation of skeletal structures and other hard tissues (ex. Fin, rays, scales, teeth and exoskeleton), electron transfer, regulation of acid: base equilibrium, the production of membrane potentials and osmoregulation. Among the essential trace minerals, copper (Cu) is an essential element for all organisms including fish (Watanabe et al. 1997; Lorentzen et al. 1998; Shao et al. 2010). It functions in hematopoiesis and in numerous copper dependent enzymes including lysyl oxidase, cytochrome c oxidase, ferroxidase, tyrosinase (O'Dell, 1976). It is also important as a part of antioxidant enzymes (e.g. Cu-Zn SOD) (Lorentzen et al. 1998). Zinc (Zn) is an essential trace element, required by fish for many important biochemical processes (Lall, 1989), including growth, protein metabolism, energy production, gene regulation, and maintaining the health of cell membranes and bones (Watanabe et al., 1997; Yamaguchi, 1998). Although fish can absorb Zn from water, ambient waterborne Zn concentrations in most environments are insufficient to compensate for low dietborne Zn concentrations (Watanabe et al., 1997). Another essential trace mineral, Iron (Fe) has an active part in oxidation/reduction reactions and electron transport associated with cellular respiration. It is found in complexes bound to proteins such as haem, in enzymes such as microsomal cytochromes, catalase, etc., and in non-haem compounds such as transferrin, ferritin and flavin iron enzymes. Hemoglobin occurs in erythrocytes while transferrin is found in plasma;

the latter is the principal carrier of iron in blood (Watanabe, 1997). Iron is one of the primary metals involved in lipid oxidation. Ferrous iron, which is more potent than ferric iron, catalyses the formation of hydroperoxides and free radical peroxides by providing a free radical initiator in the presence of unsaturated fatty acids and oxygen (Chvapil et al., 1974; Lee et al., 1981; Fujimoto et al., 1982). Manganese (Mn) is another important trace mineral for fish and is widely distributed in fish and animal tissue. The mitochondria have a greater concentration of manganese than cytoplasm or other cell organelles. Manganese is necessary for the normal functioning of brain and for proper lipid and carbohydrate metabolism. Manganese activates specific enzymes such as glycosyltransferase and non-specific enzymes such as kinases, transferases, hydrolases and decarboxylases. The activation of leucine amino peptidase by manganese has been demonstrated in sole (Clark et al., 1987). In strict sense, these four trace minerals *viz*. Cu, Zn, Mn and Fe play vital role in fish nutrition and must be supplied in adequate quantity through diet to ensure the optimum growth and health of the cultured fish/shrimp.

Moreover, feed formulations for farmed aquatic animals have historically relied on fishmeal (FM) to provide a major part of their nutrient requirements. With the intensification and expansion of aquaculture worldwide use of FM has also increased dramatically. Not surprisingly, world aquaculture has been reported to consume 73% of the total FM production in 2010 (IFFO, 2012). On the other hand, average global FM production has been reported to decline at 1.7% annually from 1994 to 2009 (FAO, 2012). Lee and Bai (1997) noted that the world supply of FM increased by only about 27% during the past two decades and FM output by the major FM-producing countries actually declined. Consequently, economic and sustainability issues have exerted substantial pressure for the reduction of FM in aquafeeds. Numerous scientific studies in last three decades have investigated the efficacy of different plant protein as an alternative to fishmeal. Consequently, there has been a massive shift towards the use of plant protein in aquafeed formulation. Whereas, plant protein contain a wide array of antinutrients, among them the presence of antagonists factors such as phytic acid has been acknowledges as the major barrier hindering the bioavailability of trace mineral. Phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate) is the major phosphorus (P) storage compound in plant seeds and can account for up to 80 percent of total phosphorus. Phytic acid binds with divalent cationic trace minerals rendering them unavailable to the animal and these are consequently lost to the environment as waste (Cheryan, 1980; Davis and Gatlin, 1996; Davis et al., 1993; Li and Robinson, 1997). Since, fish nutritionist and economic experts predict the trend to be continued and dietary plant protein will have a substantial role in the sustainable development of aquaculture. Thus, the limited bioavailability and potential dietary deficiency of trace minerals are serious concern as adequate trace mineral ensuring the optimum growth and health of cultured species in aquaculture.

Whereas, an ideal approach to improve the bioavailability of trace minerals has been recommended as the inclusion of microbial phytase in fish diet. Phytase is an enzyme chemically known as myo-inositol-hexaphosphate phosphohydrolase (Class 3: Hydrolases), produced either by microorganisms or present in some plant ingredients. Monogastric animals cannot produce this enzyme. Presence of phytase in some animals is of microbial origin. Microbial phytase either as a dry powder or as a liquid is available commercially (Baruah. et. al., 2004). However, microbial phytase has been reported to have limited efficiency due to lower Ph in fish gut apart from its high cost. Deterioration of phytase activity at high temperature of fish feed processing, especially in the case of extrusion processing are additional factors, all together limiting the use of phytase in aquafeed formulation. Another arguable approach to increase the bioavailability of trace minerals as highlighted in NRC (2011), "as the aquatic animal feed industry increase its use of plant feedstuffs, the need for mineral supplementation should increase". Worthy to note that, environmental pollution due to high rate of mineral excretion by mineral antagonisms at higher level of dietary inclusion has been a common encountered problem in livestock husbandry. It remains an important research area for scientific community to clearly understand the ultimate fate of the trace minerals at higher level of dietary inclusion in aquafeed formulation.

Knowledge on bioavailability of supplemental trace minerals sources is critical in selection of a trace minerals source in feed production (Spears et al. 2004; Luo et al. 2005; Shao et al. 2010). The most common form of trace minerals used in fish feeds for growth promotion is the sulfate salt (SO_4). The nitrate salt of copper has been reported to be also effective in feed production (Clearwater et al. 2002). Inorganic form (sulfate/nitrate) of trace mineral has traditionally been used in aquafeed formulation. However, the limited bioavailability of inorganic source of trace minerals due to its higher affinity to antinutrients has hastened the search for alternative form of inorganic trace minerals. As a result, scientific communities have attempted to develop more stable and bioavailable form of trace minerals suitable for aquaculture. In last decade, research is increasing shedding light on the potential benefit of using organic/chelated form of trace mineral in aquaculture. Typically, organic trace minerals are more stable in the digestive tract and less prone to interactions and antagonisms as they are bound to organic molecules and less available to interaction and binding. Some of the commonly available organic trace minerals are metal proteinates, metal amino acid complexes and metal amino acid chelates. Earlier studies have demonstrated improved bioavailability, growth and disease resistance in fish fed metal proteinates (zinc proteinate) and metal amino acid complexes (zinc methionine) compared to fish fed inorganic sources (Hardy and Shearer, 1985; Paripatananont and Lovell, 1995a,b, 1997). The glycine chelates of traceminerals have been shown to improve performance, tissuemineral retention, hematology parameters, immune function and disease resistance in the rainbow trout (Apines et al., 2003; Apines-Amar et al., 2004a,b; Satoh et al., 2001) and in red sea bream (Sarker et al., 2005), despite the presence of dietary antagonists such as phytic acid or tricalcium phosphate (Bharadwaj et. al., 2014). Recently, few studies investigated the efficacy of chelated minerals consisted of a range of divalent cationic minerals chelated to two molecules of HMTBa (2-hydroxy-4-methylthiobutanoic acid or hydroxy analog of methionine; Mintrex™) claimed to have in an extremely stable configuration. The stability of these molecules renders chelated trace minerals less available to binding to phytic acid and to interference from other dietary antagonists. These molecules are thus able to reach the receptors in the gut epithelium where they are absorbed into the circulation of the animal (Eide, 2004; Wang and Zhou, 2010; Yi et al., 2007).

Korean rockfish (*Sebastes schlegelii*) is the second most important marine fish aquaculture species in the Republic of Korea. It has been reported that marine finfish aquaculture dominated by olive flounder (56.35%) and Korean rockfish (23.89%) in 2012. It is not only the encouraging Korean Government support to be appreciated but also the wide area of phenomenal fish research conducted in last three decades for these two fish species. Consequently, major macro and micro nutrients requirement has been well defined for these two species. However, there has been absolute lack of information on the efficacy of trace minerals in Korean rockfish. At present in Korea, crustacean culture is primarily concerned with a single species of shrimp (whiteleg shrimp) and some crabs. Fleshy prawn (*Penaeus chinensis*) and kuruma prawn (*P. japonicus*) used to be the prime species of shrimp being farmed with the former raised mostly in farms along the west of the peninsula and the latter in farms in the southern region. While, whiteleg shrimp (*L. vannamei*) has become the principal one after the introduction of imported seed in 2003 from USA (Bai et. al., 2012). Whiteleg shrimp, also worldwide popular as Pacific white shrimp, white shrimp etc. has been the subject of a vast area of research from fish nutrition as well as allied science in last two decades. However, the importance of micro nutrients in shrimp nutrition has been overlooked, particularly true in the case of dietary trace minerals. Therefore, these present consecutive studies were conducted to evaluate the efficacy of different levels of dietary trace minerals (Cu, Zn, Mn & Fe) premix from inorganic and chelated sources on the growth performance, bioavailability, and health of Korean rockfish (*Sebastes schlegeli*) and whiteleg shrimp, (*L. vannamei*).

Chapter 2:

Evaluation of the Efficacy of Dietary Inorganic and Chelated Trace Minerals (Cu, Zn, Mn & Fe) Premixes in juvenile Korean rockfish, *Sebastes schegelli*

Evaluation of the efficacy of dietary Inorganic Trace Minerals (Cu, Zn, Mn & Fe) Premix in Juvenile Korean rockfish (Experiment I)

Abstract

A 16 weeks of feeding trial was conducted to evaluate the efficacy of dietary inorganic trace minerals (Cu, Zn, Mn & Fe) premix in diet supplemented with natural mineral antagonist, Phytic acid in juvenile Korean rockfish. Four semi-purified diet were formulated comprising a deficient basal control, and diets supplemented with the inorganic trace mineral premixes at three different levels *viz.* 0.3% ($Inr_{0.3}$), 0.15 ($Inr_{0.15}$) and 0.075% ($Inr_{0.075}$). Each experimental diet was supplemented with 2% of dicalcium phosphate and 1% of phytic acid to match up with the minerals antagonism of the commercial practical diets. Twenty four numbers of juvenile Korean rockfish averaging 9 ± 0.2 g (mean ± SD) were randomly distributed in to 16 experimental tanks as quadruplicate groups . Fish were fed at the feeding rate of 2~3% body weight per day for 16 weeks. At the end of the experiment, there were no significant differences in weight gain (WG), specific growth rate (SGR), feed efficiency (FE) and protein efficiency ratio (PER) among the fish fed Basal, $Inr_{0.3,}$ $Inr_{0.15}$ and $Inr_{0.075}$ diets. Trace minerals content in liver tissue showed a significantly highest Mn concentration for the fish fed $Inr_{0.3}$ diet than those of fish fed Basal, $Inr_{0.15}$ and $Inr_{0.075}$ diets. Moreover, liver Cu concentration tended to increase with the corresponding increase in dietary trace mineral levels. However, there was no significant difference in Zn & Fe contents in liver tissue of fish fed different experimental diets. While, bone tissue trace minerals content showed significantly higher Zn & Mn content for the fish fed $Inr_{0.3}$ and $Inr_{0.15}$ diets than those of fish fed Basal and $Inr_{0.075}$ diets. However, there was no significant difference in Cu content of bone tissue among the fish fed different experimental diets. While, the data for whole body trace mineral contents showed significantly higher Zn concentration for

the fish fed Inr_{50} and Inr_{100} than those of fish fed $Inr_{0.075}$ and Basal diets. However, there was no significant difference in whole body Cu concentration among the groups of fish fed different experimental diets. Moreover, the non-specific enzyme copper-zinc superoxide dismutase (Cu-Zn SOD) activity was recorded to be significantly higher for the fish fed $Inr_{0.3}$ diet than those of fish fed Basal and $Inr_{0.075}$ diets. Observation from the challenge test against the bacteria *E. tarda* infection showed significantly lower cumulative mortality for the fish fed $Inr_{0.3}$ and $Inr_{0.15}$ than those of fish fed Basal and $Inr_{0.075}$ diets. Therefore, the present experimental results suggested that the inorganic trace minerals (Cu, Zn, Mn & Fe) premix dietary supplementation at a minimum level of 0.3% of the diet could be necessary for juvenile Korean rockfish. Further research are warranted to evaluate the efficacy of dietary inorganic trace minerals premix at higher than 0.3% inclusion level in the presence of mineral antagonist phytic acid, to ensure the maximum growth, tissue saturation and optimum health of marine fish, Korean rockfish.

Evaluation of the Efficacy dietary chelated trace Minerals (Cu, Zn, Mn & Fe) Premix in juvenile Korean rockfish (Experiment II)

The present experiment was conducted to evaluate the efficacy of dietary chelated trace minerals (Cu, Zn, Mn & Fe) premix in the diet supplemented with natural mineral antagonist, Phytic acid in juvenile Korean rockfish. Five semi-purified diet were formulated including a deficient basal control, and diets supplemented with the inorganic trace mineral premixes at three different levels *viz.*0.6% ($Min_{0.6}$), 0.3% ($Min_{0.3}$), 0.15 ($Min_{0.15}$) and 0.075% ($Min_{0.075}$). Each experimental diet was supplemented with 2% of dicalcium phosphate and 1% of phytic acid to match the antagonism with the commercial practical diets. Twenty four numbers of juvenile Korean rockfish averaging 9 ± 0.2 g (mean ± SD) were randomly distributed in to 20 experimental tanks as quadruplicate groups. Fish were fed at the feeding rate of 2~3% body weight per day for 16 weeks. At the end of the experiment, WG and SGR of fish fed $Min_{0.3}$ was significantly higher than those of fish fed Basal diet. However, there was no significant difference in WG and SGR among the group of fish fed Basal, $Min_{0.15}$, $Min_{0.075}$ and $Min_{0.6}$ diets and also among the group of fish fed $Min_{0.6}$, $Min_{0.3}$, $Min_{0.15}$ and $Min_{0.075}$ diets. Trace minerals content in liver tissue showed a significantly higher Cu concentration for the fish fed $Min_{0.3}$ diet than those of fish fed Basal, $Min_{0.15,}$ $Min_{0.075}$ and $Min_{0.6}$ diets. Moreover, liver Cu concentration tended to increase with the corresponding increase in dietary trace mineral levels. Moreover, Zn, Mn and Fe concentration in liver tissue was significantly higher for the group of fish fed $Min_{0.3}$ diets than those of fish fed Basal, $Min_{0.15}$ and $Min_{0.075}$ diets. Trace mineral concentration of whole body showed, significantly highest Zn and Mn content for the fish fed $Min_{0.3}$ diets than those of fish fed Basal, $Min_{0.15}$ and $Min_{0.075}$ diets. While, trace mineral content in bone tissue showed, significantly higher Cu concentration for fish fed $Min_{0.6}$ followed by $Min_{0.3}$ and $Min_{0.075}$.

Furthermore, Zn and Mn contents of whole body was recorded to be significantly highest for the fish fed $Min_{0.3}$ followed by $Min_{0.6}$ diets. Data for the whole body Fe content showed, significantly highest concentration for the fish fed $Min_{0.6}$ followed by $Min_{0.3}$ diets. The hepatic Cu-Zn SOD activity was recorded to be peaked for the fish fed $Min_{0.3}$ diet followed by those of fish fed $Min_{0.6}$, $Min_{0.15}$, $Min_{0.075}$ and Basal diets. While the cumulative mortality after the *E. tarda* infection was significantly lowest for fish fed $Min_{0.6}$ followed by $Min_{0.3}$, $Min_{0.075}$, $Min_{0.15}$ and Basal diets. Therefore, results from the present experiment suggested that, the optimum dietary inclusion level of chelated trace mineral premix could be greater than 0.075% but less than 0.3% of the diet based on growth performance, trace minerals saturation in tissues and nonspecific enzymatic activity in juvenile Korean rockfish.

Introduction

Korean rockfish, ***(****Sebastes schlegeli)* has been the subject of a vast number of fish nutrition research in last three decades and consequently the nutrients requirement has been well defined. However, the importance of trace minerals in the Korean rockfish nutrition has been outside the purview of fish nutritionists' community. Trace minerals function primarily as catalysts in enzyme systems within cells. The roles that trace minerals play in enzymatic reactions range from weak, ionic strength effects to highly specific associations known as metalloenzymes (Underwood, 1971). Deficiencies and or imbalances of trace minerals can alter the activity of certain enzymes and function of specific organs thus impairing specific metabolic pathways as well as overall immune function. Even though, trace minerals are typically required in much lower quantity in fish diet but its supplementation at optimum level is a critical issue in fish feed formulation. Scientific reports gathered over last two decades indicate, few trace mineral are quite sensitive and proper care should be taken to ensure their inclusion at optimum level in fish feed formulation. Only a few scientific review including, Davis & Gatlin (1996) and NRC 2011 summarizes the estimate of mineral requirement in few commercially important aquaculture species. However, the trace mineral requirements in several other aquaculture species are still unknown and the practical feed formulation follow the general estimation (Bai, 2014) including for the feed formulation of Korean rockfish.

The rapid growth in aquafeed production has been driving the expansion of aquaculture of Korean rockfish. A variety of quality feeds from the domestic and multinational fish feed companies are commercially available in the market. However, due to the heavy dependence on imported ingredients, particularly true in the case of fishmeal, fish feed has become the most expensive feed over other animal feeds and its sustainability has become the major challenge. A series of studies (Bai & Kim, 1997; Kim & Bai, 1997; Kim & Bai, 1999; Wang et. al., 2004; Kim et. al., 2002) conducted in our laboratory and several other research around the country investigated the efficacy of different promising alternative to dietary FM in Korean rockfish. Consequently, the inclusion level of FM in commercial feed formulation of Korean rockfish has dramatically declined. As evidenced by the recent global overview on FM articulated by Tacon & Metian (2008), in current trend the commercial fish feed formulation for Korean rockfish comprise FM only 25~35% of feed, which used to be higher than 50% of the feed previously. Worthy to note that, although there has been increased use of dietary plant protein in Korean rockfish commercial feed, the source and inclusion level of dietary trace minerals is still remain the same traditional inorganic (sulfate/nitrate) premix.

Whereas, the use of inorganic salts can result in poor bioavailability of the mineral, mainly due to the numerous nutrient and ingredient antagonisms that impair absorption (Underwood and Suttle 1999). Worth mentioning in last two decades, there has been only one published report on the efficacy of trace minerals premix in Korean rockfish (Lee et. al., 1998). The aforementioned study used a 40% fishmeal containing diet and inorganic source of trace minerals in their experiment, which is having least scientific relevance in the current scenario of commercial feed formulation for Korean rockfish.

More bioavailability of trace minerals is probably due to better absorption, which enhances its efficiency (Downs et al. 2000; Yu et al. 2000; Guo et al. 2001). Baker and Ammerman (1995) reported that relative bioavailability estimate of organic Cu sources ranged from 88% to 147% of the response to cupric sulfate in poultry, swine, sheep and cattle. The benefits of supplementation organic trace minerals in dairy diets have also been demonstrated in research and in the field. Traditionally inorganic forms of trace minerals rapidly dissociation in the rumen and are free to interact with antagonists, resulting in the loss of the trace minerals prior to absorption by the animal (Henry et. al.,1992; Ward et. al., 1996). Chelated or organic trace minerals are bound to organic ligands through coordinate covalent bonds. The bonds between the ligand and the mineral can prevent the minerals from interacting with antagonists and improve the bioavailability of the mineral (Ward et al., 1996; Bailey et. al., 2001). The basic reason for the use of organic forms of trace minerals is the increased bioavailability of organic vs inorganic sources of the minerals (El Ashry et. al., 2012). Organic minerals are important trace mineral sources, because they protect trace elements from forming insoluble complexes (such as with phytate) in the digestive tract and facilitate transport across the intestinal mucosa (Ashmead, 1993). It was confirmed that organic zinc had higher bioavailability than inorganic zinc in terrestrial vertebrates and aquatic animals, such as the chick (Wedekind et al., 1992), abalone (*Haliotis discus hannai*) (Tan and Mai, 2001), channel catfish (Paripatananont and Lovell, 1995) and rainbow trout (Apines et al., 2001). However, it was also suggested in some other studies that substitution of organic zinc for inorganic zinc did not lead to improvement in growth of pig (Swinkels et al., 1996; Wedekind et al., 1994), chick (Pimentel et al., 1991) or tilapia (Do Carmo E Sá et al., 2005; Zhao et al., 2011). Nevertheless, traditional bioavailability assays, as well as more recently developed gene expression assays for mineral bioavailability demonstrate that not all organic trace minerals are equally effective and bioavailable. These assays indicate that Mintrex® organic trace minerals, a chelate of two 2-hydroxy-4 (methylthio) butanoic acid (HMTBa) ligands per atom of trace mineral (zinc, copper, manganese or iron), provide a highly bioavailable source of trace minerals. Chelates are the organic form of trace minerals and are usually considered for use in animal diet as alternatives to inorganic trace minerals source. Nevertheless, every manufacture claims their product to be best in terms of quality, it is always challenging to confirm the ultimate efficiency of the chelated trace minerals and their premix in fish feed formulation.

Therefore, these experiments were conducted to evaluate the efficacy of dietary inorganic and chelated trace minerals (Cu, Zn, Mn & Fe) premixes on the growth performance, bioavailability and health of Korean rockfish fed diets supplemented with natural trace minerals inhibitor dicaclium phosphate and phytic acid.

Materials and Methods: (Experiment I and II)

Diet Formulation and Preparation

Table 2 shows the basal experimental diet formulation used in experiment 1 and 2. Nine isonitrogenous and isocaloric semi purified diet were formulated to contain minimum level of native mineral derived from the ingredients. Table 1 shows, analyzed mineral content of number of ingredients prior to the experimental diet formulation. One of the four semi-purified diets including a deficient basal control, and diets supplemented with the inorganic trace mineral premixes at three different levels of 0.3% ($Inr_{0.3}$), 0.15% ($Inr_{0.15}$) and 0.075% ($Inr_{0.075}$). Mineral premix at 0.3% of dietary inclusion were formulated to supply the trace minerals at zinc at 30 mg/kg, iron at 30 mg/kg, copper at 7 mg/kg and manganese at 5 mg/kg of the diet, as per the requirement level of marine fish (NRC, 2011). Each diet was supplemented with 2% of dicalcium phosphate and 1% of phytic acid to match the mineral antagonism with the commercial practical diets. One of the five semi-purified diets including a deficient basal control, and diets supplemented with the chelated trace mineral premixes at four different levels 0.6% ($Min_{0.6}$), 0.3% ($Min_{0.3}$), 0.15% ($Min_{0.15}$) and 0.075% ($Min_{0.075}$) were formulated. Mineral premix at 0.3% (100%) of dietary inclusion were formulated to supply the chelated trace minerals at zinc at 30 mg/kg of diet, iron at 30 mg/kg of the diet, copper at 7 mg/kg and manganese at 5 mg/kg of the diet, as per the requirement level of marine fish (NRC, 2011) as well as similar with the inorganic trace mineral experiment. Each diet was supplemented with 2% of dicalcium phosphate and 1% of phytic acid to match up the antagonism with the commercial practical diets. The chelated trace mineral premix (Mintrex) containing the Cu, Zn, Mn & Fe were supplied by Novus International Inc. St. Luis. USA. The trace mineral premix containing the Cu, Zn, Mn & Fe source was supplemented at the expense of cellulose in the diet. Vitamin-free casein (United

States Biochemical, Cleveland, OH, USA), fish oil (DHA + EPA enriched; refined fish oil, E-Wha oil Co. Ltd., Pusan, Korea), and corn starch (United States Biochemical, Cleveland, OH, USA) were used as the main dietary protein, lipid and carbohydrate sources, respectively. Lyophilized olive flounder muscle powder was added at 10 % to all diets to increase palatability and acceptance of the experimental diets. All dry ingredients were finely ground, weighed, mixed manually for 5 minutes and then transferred to a mixer for another 15-minute mixing. Fish oil was then added slowly while mixing was continued. All ingredients were mixed for another 10 minutes and then Inorganic trace mineral premix mixed well with the other feed ingredients. Finally, distilled water was added to the mixture to form dough and dry pellets were made from this dough using a laboratory pelleting machine (Baokyong Commercial Co., Pusan, Korea). The pellets were air dried until the moisture was reduced to <10 g/kg diet. After processing, all the diets were broken up and sieved into the appropriate pellet size (4 mm diameter), packed into small bags and stored at -24^{o}C until used.

Table 3 and 4 shows the analyzed mineral content of the experimental diets used experiment 1 and 2 respectively. Although, the analyzed Cu contained was comparatively lower than the designed Cu value for different experimental diets but it was in the requirement range for marine fish (NRC, 2011) at 0.3% supplementation. While, analyzed Mn and Zn content for different experimental diets were comparatively bit higher than the designed value.

Experimental Fish and Feeding Trial

The feeding trial was carried out at the Feeds and Foods Nutrition Research Center, Pukyong National University, Busan. Fish were transported to the experimental station and acclimated to the experimental conditions for two weeks before the feeding trial began. Twenty seven numbers of acclimatized fish averaging initial body weight 9 ± 0.2 g (mean ± SD) were allocated to 16 and 20 experimental tanks in experiment 1 and 2 respectively. Fish were fed basal diet for one week to induce the body reserved mineral depletion. After one week, three fish each tank was removed for analysis of initial mineral concentration in liver and whole body tissue. Afterwards, 24 numbers of fish in quadruplicate group were randomly assigned to one of the four treatments. All the experimental fish were fed one of the experimental diet at the feeding rates of 2 ~ 3 % twice a day for the experimental period of 16 weeks. The feeding trial was conducted in a recirculatory system with 54-L aquaria receiving filtered seawater at a rate of 0.8L min^{-1}. Supplemental aeration was provided to maintain dissolved oxygen level near saturation. Water temperature was maintained at 17 ± 1°C. Supplemental aerations were provided to maintain dissolved oxygen levels near saturation (8.8 ± 0.3 mg/L). The seawater pH, salinity, total ammonia-nitrogen and nitrites were 8.23 ± 0.13, 32.5 ± 0.7 ppt, 0.037 - 0.052 mg/L and 0.13 ± 0.09 mg/L, respectively. These values were within optimum ranges for normal growth and health of juvenile olive flounder (Wang et al., 2002). Care was taken to ensure that no uneaten food remained in the tanks during feeding, thus leaching of trace minerals into water was negligible. Tanks were siphoned an hour before the morning feeding and water in the center tank was completely replaced one hour after feeding in the evening. Mortality was checked daily. Any dead fish were removed and not replaced during the experiment. Total fish weight in each aquarium was determined every 2 weeks, and the amount of diet fed to the fish was adjusted accordingly. The aquaria were thoroughly cleaned during the time fish were removed for weighing to minimize algae and fungal growth. Fish were starved 24 h before each weighing to avoid inclusion of ingested feed in the weight measurement as well as to reduce stress.

Sample Collection and Analysis

At the end of the feeding trial, fish were starved for 24 h and the total number and weight of fish in each aquarium was determined for calculation of weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER), and survival. Three additional fish were selected from each aquarium, killed and frozen at -80^{o}C for proximate composition and whole body Cu, Zn, Mn & Fe concentration analyses. Also, two fish were randomly sampled from each tank, and then liver were removed from each fish and pooled for determining Cu-Zn superoxide dismutase (SOD) activity.

Proximate composition analyses of experimental diets and fish body were performed by the standard methods of AOAC (2000). Samples of diets and fish were dried to a constant weight at 105℃ to determine moisture content. Ash was determined by incineration at 550℃; crude lipid by soxhlet extraction using Soxtec system 1046 (Foss, Hoganas, Sweden) and crude protein by Kjeldahl method (N × 6.25) after acid digestion.

Enzyme Assay

Superoxide dismutase (SOD) activity was measured by its ability to inhibit superoxide radical dependent reactions using the Ransod Kit (Randox, Crumlin, UK). Briefly, the reaction mixture (1.7 ml) contained xanthine (0.05 mM) and 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT, 0.025 mM) dissolved in 50 mM CAPS (pH 10.2) and 0.94 mM EDTA. In the presence of xanthine oxidase (80 U l_1, 250 ml), superoxide and uric acid were produced from xanthine. The superoxide radical then reacted with INT to produce a red formazan dye. The optical density was measured at 450 nm, 37 (C, and the rate of reaction was estimated from the absorbance readings at 30 s and 3 min after adding xanthine oxidase. A reference standard SOD was supplied with the Ransod Kit. One unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50%. Specific activity was expressed as SOD units mg $^{-1}$.

Minerals Analysis

Copper, zinc, manganese and iron contents of rearing water, diet, tissue, and final whole-body were determined by digestion of samples in nitric acid (AOAC 2000). The concentrations of copper in the diluted digest solution were determined by using an Inductively Coupled Plasma Mass Spectrometer (Perkin-Elmer 3300, Waltham, MA).

Histological analysis

Total collagen analysis was carried out at NARC, Vietnam by Novus technicians. Total collagen concentration (mg/g tissue) was analyzed using a kit test Quickzyme Total Collagen Assay (QuickZyme Bioscience, CE Leiden, The Netherland).

Challenge Test

After the termination of growth trail, challenge test was carried out for 2 weeks. Five fish from each replication were injected intraperitoneally with the bacteria species *Edwardsiella tarda* at 1.3 x 10^8 CFU/ ml (LD_{50}). Pronounced mortality could be observed only after 5 ~6th day, therefore challenge test was carried out for 14 days.

Statistical Analysis

All data were analyzed by one-way ANOVA to test for the effects of the dietary treatments. When significant differences were found, Tukey's multi-comparison test was used to identify differences among experimental groups. Treatment effects were considered with the significance level at P <0.05. All statistical analyses were carried out by SAS version 9.0 software (SAS Institute, Cary, NC, USA).

Results (Exp. I)

Growth Performance

Weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER) and survival of juvenile Korean rockfish fed different levels of inorganic trace mineral premix for 16 weeks are shown in table Table 5. At the end of feeding trial, there was no significant difference in WG, SGR, FE and PER of fish fed Basal, $Inr_{0.3}$, $Inr_{0.15}$ and $Inr_{0.075}$ diets. Survival rate ranged between 73 to 81% among the group of fish fed different experimental diets but the trend was non-significant. Numerical value for WG showed a higher value in WG for the group of fish fed $Inr_{0.075}$ and $Inr_{0.3}$than those of fish fed Basal diets but without any statistical difference. The value for FE tended to increase with the corresponding increase in dietary trace mineral inclusion level without any significant difference among different groups. Overall growth performance observation suggested that the dietary inclusion level for inorganic trace minerals premix could be a minimum of 0.3% ($Inr_{0.3}$) of the diet for Korean rockfish.

Trace Minerals content in Tissue

At the end of experiment, the analyzed Cu, Zn, Mn & Fe concentration in the liver, bone and whole body tissue of Juvenile Korean rock fish fed different levels of inorganic trace mineral premix are shown in table 6, 7, & 8 respectively. Trace mineral, Mn concentration in liver tissue was recorded to be significantly highest for the fish fed $Inr_{0.3}$ than those of fish fed all other experimental diets. While, Cu concentration in liver tissue tended to increase with the increase in dietary inorganic trace mineral level and the numerical value was highest for fish fed $Inr_{0.3}$, however this value was statistically not different from fish fed all other experimental diets. Moreover, there was no significant difference in Zn & Fe concentration in liver tissue of fish fed different experimental diets.

Trace minerals content in bone tissue of fish fed different levels of inorganic trace mineral premix showed, significantly higher Zn & Mn for the fish fed $Inr_{0.3}$ and $Inr_{0.15}$ diets than those of fish fed Basal and $Inr_{0.075}$ diets. While, bone tissue Fe content data showed significantly higher concentration for fish fed $Inr_{0.075}$ and $Inr_{0.15}$ than those of fish fed Basal and $Inro_{0.3}$ diets. While, there was no significant difference in the Cu concentration of bone tissue among the fish fed different experimental diets.

Data for the whole body trace mineral contents showed, significantly higher Zn concentration for the fish fed $Inr_{0.15}$ and $Inr_{0.3}$ than those of fish fed $Inr_{0.075}$ and Basal diets. While, Fe concentration was significantly higher for fish fed $Inr_{0.075}$ than those of fish fed Basal, $Inr_{0.15}$ and $Inr_{0.3}$ diets. However, the Fe concentration in whole body was not significant difference between the groups of fish fed Basal and $Inr_{0.3}$ diets. While, there was no significant difference in whole body Cu concentration among the group of fish fed different levels of dietary inorganic trace minerals premix.

Proximate Composition

Table 9 shows the proximate composition of whole body of juvenile Korean rockfish fed different levels of inorganic trace mineral premix for 16 weeks. There was no significant difference in whole body protein, lipid, moisture and ash content among the fish fed Basal, $Inr_{0.075}$, $Inr_{0.15}$ and $Inr_{0.3}$ diets. However, the value for the whole body ash content was recorded to numerically increase with the increase in dietary inclusion level of inorganic trace mineral but the difference were not statistically different. Likewise, whole body protein and lipid were also recorded to increase non significantly with the increase in the dietary levels of inorganic trace minerals premix. On the other hand, whole body moisture was recorded to gradually decrease among the fish fed $Inr_{0.3}$, $Inr_{0.15}$ and $Inr_{0.075}$ compared to Basal diet.

Enzyme activity

Hepatic copper-zinc superoxide dismutase (Cu-Zn SOD) activity observed at the end of the experiment has been shown in Table 10. Cu-Zn SOD activity was recorded to be significantly lower for fish fed Basal diet than those of fish fed $Inr_{0.075}$, $Inr_{0.15}$ and $Inr_{0.3}$ diets. The Cu-Zn SOD activity was significantly highest for fish fed Inr50 diets than those of fish fed Inr25, inr50 and Basal diets. However, there was no significant difference in Cu-Zn SOD activity between the fish fed $Inr_{0.075}$ and $Inr_{0.075}$ diets.

Histological Analysis

Table 11 shows the collagen concentration for Juvenile Korean rockfish fed different levels of inorganic trace mineral premix for 16 weeks. Although, numerical difference were recorded no clear trend could be drawn statistically among the fish fed Basal, $Inr_{0.075}$, $Inr_{0.15}$ and $Inr_{0.3}$ diets.

Challenge Test

Figure1 Shows the cumulative mortality rate of juvenile Korean rockfish injected with *E. tarda.* Observation from the challenge test against the bacteria *E. tarda* infection showed significantly lower cumulative mortality for the fish fed $Inr_{0.3}$ and $Inr_{0.15}$ than those of fish fed Basal and $Inr_{0.075.}$

Discussion and Conclusion (Exp. I)

Results of this study demonstrated that the juvenile Korean rockfish has a distinct requirement for trace minerals, Cu, Zn, Mn and Fe that cannot be met by these trace minerals in the rearing water, and thus dietary supplementation is necessary. Moreover, in the presence of mineral inhibitor phytic acid, naturally present in dietary plant protein, the dietary inclusion level for trace minerals should be redefined. Whereas, the growth rate in the present experiment could appear to be lower than the various previous published reports on Korean rockfish. But the growth performances are comparable with our various previous experiments conducted in the same species fed semi-purified diet (Bai et al. 1996 & Bai & Lee 1996). At the end of the present experiment, there was no significant difference in WG, SGR, FE and PER of fish fed Basal, $Inr_{0.075}$, $Inr_{0.15}$ and $Inr_{0.3}$ diets contains trace mineral inhibitor phytic acid.

Similar to our observation, Li & Robinson (1996) reported no significant difference in growth performance among the Channel catfish, (*Ictalurus punctatus*) fed different levels of Zn sulfate in practical diets. These aforementioned authors concluded that, channel catfish do not need supplemental Zn for maximum growth when practical diets containing animal protein, but require supplemental Zn for maximum bone Zn deposition. Moreover, Tan et. al., 2011 could not find any significant growth difference in juvenile Yellow catfish, (*Pelteobagrus fulvidraco*) fed six graded levels of dietary Cu sulfate between 0.01 to 0.08g/kg diet. Likewise, in a recent study, dietary magnesium (Mg) supplementation at different levels could not improve the growth performance including feed intake, weight gain and feed conversion efficiency in juvenile gibel carp, *Carassius auratus gibelio* (Han et. al., 2012). Further, Gatlin and Wilson (1986) also reported that the growth rates were similar for channel catfish fed diets with copper levels ranging from 0 to 40 mg Cu/kg for 13 weeks. Worth mentioning that, none of these above mentioned studies used the phytic acid inclusion in their experimental diets. Therefore, the present study clearly demonstrate that, antinutrients present in dietary plant protein adversely effects the trace mineral metabolism and thereby the fish growth performance.

Various previous studies showed that minerals are mainly stored in bone and liver, respectively (Keen and Graham, 1989; Linder et al., 1998; Turnlund, 1998; Peretz et al., 2001; Apines et al., 2004) and therefore their levels in these organs have been reported to be promising indices for evaluating their status and bioavailability. Trace mineral, Mn concentration in liver tissue was recorded to be significantly highest for the fish fed $Inr_{0.3}$ than those of fish fed all other experimental diets. While, Cu concentration in liver tissue tended to increase with the increase in dietary inorganic trace mineral level and the value was highest for fish fed $Inr_{0.3}$, however this value was statistically not different from fish fed all other experimental diets. While, trace mineral contents in bone tissue showed, significantly higher Zn & Mn content for the fish fed $Inr_{0.3}$ and $Inr_{0.15}$ diets than those of fish fed Basal and $Inr_{0.075}$ diets. Similar to our observation, reports for fish and shrimp have shown a positive correlation between liver Cu content and dietary inclusion level (Lee & Shiau 2002; Shaw & Handy 2006). Zinc can influence bone mineralization either directly, as divalent cation acting on nucleation and mineral accumulation, or indirectly, as a co-factor of enzymes involved in the process like alkaline phosphatase (Gomez et al., 1999). In humans, the role of Zn in bone formation involves the activation of bone alkaline phosphatase, osteoblast tyrosine kinase and RNA synthetase (Yamaguchi and Hashizime, 1994). In the present experiment, data for the whole body trace mineral contents showed, significantly higher Zn concentration for the fish fed $Inr_{0.15}$ and $Inr_{0.3}$ than those of fish fed $Inr_{0.075}$ and Basal diets. Likewise, hepatopancreatic and whole body Zn concentration in shrimp generally increased as dietary Zn supplementation increased in Grass shrimp, *Penaeus monodon* (Shiau &

Jiang, 2006). On the other hand, tissue Fe content has been reported not to be very responsive parameters in trace mineral studies. For instance, Davis & Lawrence (1992) reported no significant difference in tissue Fe content with the corresponding increase in the dietary levels fed to whiteleg shrimp. However, in the present experiment, bone tissue Fe content data showed significantly higher concentration for fish fed $Inr_{0.075}$ and $Inr_{0.15}$ than those of fish fed Basal and $Inr_{0.3}$ diets in the presence of dietary phytic acid. Since, there is no report on the tissue Fe content in the fish fed phytic acid contains diet, thus the tissue Fe concentration could be perhaps misleading to consider as the promising indicator. The overall analyzed tissue mineral content in the present experiment, were responsive to the dietary inclusion level and appeared to be affected by the presence of antinutrients, phytic acid inclusion in the diet.

Although, non-significant differences were recorded in whole body proximate composition parameters including protein, lipid, moisture and ash content but no clear trend could be drawn. Since, various previous experiments (Lee & Shiau 2002; Shaw & Handy 2006; Shiau & Jiang, 2006; Lin & Shiau, 2010) have also not emphasized the importance of whole body proximate composition in trace minerals experiments. It appears, whole body proximate composition could not be a responsive indicator to dietary trace minerals. Further, this is the first study evaluating the effects of dietary trace minerals on histological analysis, particularly true for juvenile Korean rockfish. Thus, comparing the collagen concentration with other commercially important fish could also be perhaps misleading.

One family of antioxidant enzymes, the superoxide dismutase (SOD), functions to remove damaging reactive oxygen species (ROS) from the cellular environment by catalyzing the dismutation of two superoxide radicals to hydrogen peroxide and oxygen (Lin et al. 2008). Copper is involved in the antioxidant system as it is an integral part of the enzymes Cu-Zn superoxide dismutase (SOD) and ceruloplasmin molecules. The present study showed that liver Cu-Zn SOD activity significantly increased up to a peak value at dietary trace mineral premix inclusion at $Inr_{0.15}$ and afterwards gradually declined. It has been reported that inadequate and excess dietary trace mineral such as Cu reduced Cu-Zn SOD activities and destroyed SOD, respectively in fish (Lin et al. 2008). Gatlin and Wilson (1986) reported that the activity of liver Cu-Zn SOD was significantly reduced in channel catfish fed diets containing 0-2.0 mg Cu/kg as compared to those fed 4 mg Cu/kg diet. In their study, liver Cu-Zn SOD activity was taken as the most sensitive indicator of copper status, and the dietary copper requirement of channel catfish was estimated to be 5 mg Cu/kg diet based on the activity of this enzyme. Likewise, in the present experiment, significantly depressed hepatic Cu-Zn SOD activity in the juvenile Korean rockfish fed Basal diet suggests, insufficient dietary trace mineral adversely affects the enzymatic activity. Since, the present study lack the treatment of inorganic trace mineral premix at higher level, thus it is hard to conclude that at higher level of inclusion it may adversely effects the enzymatic activity in other fish. It remains an important research area needs further investigation.

Observation with the subsequent challenge test suggested the significant difference in disease resistance against the *E. tarda* infection among the fish fed different level of inorganic trace mineral premix for 16 weeks. At the end of 14 days of challenge test, significantly highest cumulative mortality was recorded for fish fed Basal diet followed by $Inr_{0.075}$, $Inr_{0.15}$ and $Inr_{0.3}$. Trace minerals being the integral part of several metalloenzymes have significantly greater impacts on preventive health management and success of an aquaculture venture. For example, Lim et al., (2001a) attributed the imbalances in Iron (Fe) would compromise the immune system and the resistance of fish to disease. The effects of dietary zinc on immune response and disease resistance in fish has also been reviewed by Lim et al. (2001). Likewise, various other authors have attributed the potential of micronutrients particularly true in the case of dietary trace mineral as the immunostimulants and thereby to increase the disease resistance. The challenge test results from this preliminary study opens a new avenue to evaluate the efficacy of dietary trace minerals and their premixes as the safe and effective alternative of unpopular antibiotic to fend off the disease.

In conclusion, the results from the experiment demonstrated that the juvenile Korean rockfish has a distinct requirement for the trace minerals, viz. Cu, Zn, Mn and Fe that cannot be met by these trace minerals in the rearing water, and thus dietary supplementation is necessary. Juvenile Korean rockfish fed a dietary trace mineral premix at 0.15 & 0.3% ($Inr_{0.15}$ & $Inr_{0.3}$) (NRC, 2011) showed a higher trace mineral saturation in tissue, disease resistance and improved nonspecific enzyme activity compared to those of fish fed basal and 0.075% ($Inr_{0.075}$) in the presence of natural mineral inhibitor phytic acid. Therefore, the present experimental results suggested that the inorganic trace minerals (Cu, Zn, Mn & Fe) premix dietary supplementation at a minimum level of 0.3% of the diet could be necessary for juvenile Korean rockfish. However, further research are warranted to evaluate the efficacy of dietary trace minerals and their premix at higher than 0.3% dietary inclusion level in marine fish juvenile Korean rockfish in the presence of natural dietary mineral inhibitor phytic acid.

Results (Exp. II)

Growth Performance

Weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER) and survival of juvenile Korean rockfish fed different levels of chelated trace mineral premix is shown in Table 13. At the end of 16 weeks of feeding trial, Final weight of fish fed $Min_{0.3}$ was significantly higher than those of fish fed basal and $Min_{0.6}$ diets. However, there was no significant difference in final weight among the group of fish fed Basal, $Min_{0.075}$, $Min_{0.15}$ and also among $Min_{0.075}$, $Min_{0.15}$ and $Min_{0.6}$. Moreover, WG and SGR of fish fed $Min_{0.3}$ was significantly higher than those of fish fed Basal diet. However, there was no significant difference in WG and SGR among the group of fish fed Basal, $Min_{0.075}$, $Min_{0.15}$ and $Min_{0.6}$ diets and also among the group of fish fed $Min_{0.6}$, $Min_{0.3}$, $Min_{0.15}$ and $Min_{0.075}$ diets. While, there was no significant difference in FE and PER of fish fed different experimental diets. Survival rate ranged between 69.79 to 84.37 among different experimental treatments group without any statistical difference.

Trace Mineral contents in Tissue

At the end of experiment, analyzed trace mineral contents in Liver, Bone and Whole body is shown in table 14, 15 and 16 respectively. Cu concentration in Liver tissue from the group of fish fed $Min_{0.3}$ showed significantly higher concentration than those of Basal, $Min_{0.15}$, $Min_{0.075}$ and $Min_{0.6}$ diets. However, there was no significant difference in Cu content in liver tissue among the group of fish fed Basal, $Min_{0.15}$ and $Min_{0.6}$ diets. While Zn, Mn and Fe concentration in liver tissue was significantly higher for the group of fish fed $Min_{0.3}$ diets than those of fish fed

Basal, $Min_{0.075}$ and $Min_{0.15}$ diets. However, there was no significant difference in Zn, Mn and Fe content from the liver tissue of fish fed Basal, $Min_{0.075}$ and $Min_{0.15}$ diets.

Trace mineral concentration of whole body showed, significantly highest Zn and Mn content for the fish fed $Min_{0.3}$ diets than those of fish fed Basal, $Min_{0.075}$ and $Min_{0.15}$ diets. However, there was no significant difference in whole body Mn content among the fish fed $Min_{0.075}$, $Min_{0.15}$ and $Min_{0.3}$ diets and also there was no significant difference in Mn content among the group of fish fed $Min_{0.6}$, $Min_{0.075}$, $Min_{0.15}$ and Basal diets. While data for whole body Cu content showed increasing trend, which was significantly higher for the group of fish fed $Min_{0.3}$ than those of fish fed $Min_{0.15}$ diets. However, there was no significant difference in whole body Cu content of fish fed $Min_{0.3}$, $Min_{0.6}$, $Min_{0.075}$ and Basal diets.

Trace mineral content in bone tissue showed, significantly highest Cu concentration for fish fed $Min_{0.6}$ followed by $Min_{0.3}$ and $Min_{0.075}$. However, there was no significant difference in whole body Cu content between fish fed Basal and $Min_{0.15}$ diets. While Zn and Mn concentration was recorded to be significantly highest for the fish fed $Min_{0.3}$ followed by $Min_{0.6}$ diets. Data for the whole body Fe content showed, significantly higher concentration for the fish fed $Min_{0.6}$ followed by $Min_{0.3}$. While, there was no significant difference in whole body Fe content among the fish fed Basal, $Min_{0.075}$ and $Min_{0.15}$ diets.

Whole-body proximate composition

Table17 Shows the proximate composition of whole body of Juvenile Korean rockfish fed different levels of chelated trace mineral premix for 16 weeks. There was no significant difference in protein, lipid, moisture and ash contents among the fish fed Basal, $Min_{0.075}$, $Min_{0.15}$, $Min_{0.3}$ and $Min_{0.6}$ diets.

Enzyme Activity

Hepatic thiobarbituric acid reactive substances (TBARS), copper-zinc superoxide dismutase (Cu-Zn SOD) Table 18. The significant effects of dietary level of chelated trace mineral premix on hepatic Cu-Zn SOD were observed. The enzyme activity for fish fed Basal diet was significantly lower than those of shrimp fed $Min_{0.075}$, $Min_{0.15}$, $Min_{0.3}$and $Min_{0.6}$ diets. The Cu-Zn SOD activity was recorded to be peaked for the fish fed Min100 diet followed by those of fish fed $Min_{0.6}$, $Min_{0.15}$, $Min_{0.075}$ and Basal diets. However, there was no significant difference in Cu-Zn SOD activity between the fish fed $Min_{0.075}$ and $Min_{0.15}$ diets.

Histological analysis

Table 19 shows the analyzed collagen concentration of the Juvenile Korean rockfish fed different level of chelated trace mineral premix for 16 weeks. Although, numerical difference were recorded among different treatment but there was no significant difference among all treatments.

Challenge Test

Figure 2 shows the cumulative mortality in juvenile Korean rockfish recorded for 14 days followed by *E.tarda* infection. Mortality began from fourth day onwards of the *E. tarda* injection and it became pronounced after 7th days. At the end of the experiment, the cumulative mortality for fish fed Basal diet was significantly higher than those of fish fed $Min_{0.075}$, $Min_{0.15}$, $Min_{0.3}$ and $Min_{0.6}$ diets. The cumulative mortality was significant lowest for fish fed $Min_{0.6}$ followed by $Min_{0.3}$, $Min_{0.075}$, $Min_{0.15}$ and Basal diets.

Discussion and Conclusion (Exp. II)

Results of this study showed that the growth response of juvenile Korean rockfish was significantly affected by the different levels of chelated trace mineral premix in diet supplemented with phytic acid. In the present experiment, WG and SGR of fish fed $Min_{0.3}$ was significantly higher than that of fish fed Basal diet. However, there was no significant difference in WG and SGR among the group of fish fed Basal, $Min_{0.075}$, $Min_{0.15}$ and $Min_{0.6}$ diets and also among the group of fish fed $Min_{0.6}$, $Min_{0.3}$, $Min_{0.15}$ and $Min_{0.075}$ diets. While, there was no significant difference in FE and PER of fish fed different experimental diets. Overall, growth performances suggested the optimum dietary inclusion level for chelated trace mineral premix in diet containing mineral antagonist could be greater than 0.075% ($Min_{0.075}$) but less than 0.3% ($Min_{0.3}$). Similar to our observation, various previous authors have reported the significant effects of chelated trace mineral on the growth performance of fish irrespective of species. For instance, growth reduction was recorded in olive flounder fed either insufficient or excess dietary chelated copper (Mohseni et. al., 2011) in agreement with results in other aquatic animals (Shaw and Handy 2006; Lin et al. 2008; Wu and Huang 2008; Tan et al. 2011; Bharadwaz et. al., 2014).On the other hand, few studies reported no significant difference among the group of fish fed different levels of chelated trace mineral, Zn (Li & Robinson, 1996; Tan & Mai, 200; Shao et al., 2010). Available information on the beneficial effects of dietary inclusion of chelated trace minerals and premix and their salts on growth performance are inconsistent and appear to vary among fish species, fish size or age. The compositions of experimental diets, native trace mineral content of dietary ingredients, culture and feeding regime used in their experiments are additional factors. Overall reports on chelated trace mineral indicate a wide scope for its application in aquafeed formulation.

Cowey (1976) suggested that besides growth, other physiological parameters such as tissue enzyme activity may also be appropriate to quantify nutrient requirement in fish. In the present study, Cu concentration in Liver tissue from the group of fish fed $Min_{0.3}$ showed significantly higher concentration than those of Basal, $Min_{0.075}$, $Min_{0.15}$ and $Min_{0.6}$ diets. However, there was no significant difference in Cu content in liver tissue among the group of fish fed Basal, $Min_{0.15}$ and $Min_{0.6}$ diets. Moreover, Zn, Mn and Fe concentration in liver tissue was significantly higher for the group of fish fed $Min_{0.3}$ diets than those of fish fed Basal, $Min_{0.075}$ and $Min_{0.15}$ diets. The liver is the major Cu storage organ in fish (Miller et al., 1993; Lorentzen et al., 1998; Shiau and Ning, 2003). Likewise, hepatic Cu concentration increased with increasing dietary Cu supplementation levels (Lin et. al., 2010; Lin et al., 2008). While, trace mineral concentration of whole body showed, significantly highest Zn and Mn content for the fish fed $Min_{0.3}$ diets than those of fish fed Basal, $Min_{0.075}$ and $Min_{0.15}$ diets. While data for whole body Cu content showed increasing trend, which was significantly higher for the group of fish fed $Min_{0.3}$ than those of fish fed $Min_{0.15}$ diets. Moreover, trace mineral content in bone tissue showed, significantly highest Cu concentration for fish fed $Min_{0.6}$ followed by $Min_{0.3}$ and $Min_{0.075}$. Apines et al. (2003) and Apines-Amar et al. (2004) indicated that trace elements chelated with amino acids, which include Cu, Zn, and Mn, seem to be more available in rainbow trout. This is because organic minerals are generally considered less sensitive to the inhibitory action of other compounds due to the different absorption pathway (Ashmead,1992). Since, in the present experiment every diet was supplemented with the mineral inhibitor, our observation well corroborated with these aforementioned reports. While, it has been also reported that, metal accumulation in fish tissues depends on exposure dose and time as well as other factors such as water chemistry and metabolic activity of the fish (Heath 1995; Fırat and Kargın 2010). These reasons could be attributed for the non-significant trend observed in tissue accumulation for the juvenile Korean rockfish fed high level of trace mineral premix ($Min_{0.6}$).

In our previous study with Olive flounder, whole-body moisture as well as muscle protein contents appeared not to be greatly influenced by dietary Cu supplementation (Mohseni et. al., 2012). The observation were similar in the present experiment, protein, lipid, moisture and ash content was found not to be significantly affected by the dietary levels of chelated trace mineral premix. Moreover, due to lack of report on the effects of trace minerals on the collagen concentration in muscle, it is hard to draw any conclusion on histological analysis.

Superoxide dismutase as an anti-oxidant enzyme plays an important role in the protection of cells from free radical damage (Fang et al. 2002). This enzyme, as one of the copper-dependent enzymes, has been shown to be an excellent indicator of copper nutrition in rat and cat (Paynter et al. 1979; Doong et al. 1983; Wang et al. 2009). Gatlin and Wilson (1986) also reported that the activity of liver Cu-Zn SOD was significantly reduced in channel catfish fed diets containing 0-2.0 mg Cu/kg as compared to those fed 4 mg Cu/kg diet. In the present study significantly highest, Cu-Zn SOD activity was recorded for the group of juvenile Korean rockfish fed Min100, followed by $Min_{0.6}$, $Min_{0.15}$, $Min_{0.075}$ and Basal diet. It clearly appeared that, insufficient or excess of dietary trace minerals from the chelated source depress the enzyme activity. The toxicity of chelated trace minerals including those of caused by trace minerals is well documented (Lin et. al., 2010). Likewise, in our previous experiment, chelated Cu toxicity and threshold level was clearly demonstrated in Olive flounder (Musheni et. al., 2012). Therefore, proper care should be taken in chelated trace mineral inclusion level to avoid the deficiency as well as toxicity to ensure an optimum health thereby optimum enzyme activity.

Like various other aquaculture species, Korean rockfish cultured commercially at very high densities, and common disease outbreaks are regarded as a major hurdle constraining industry expansion. *Vibrio*, *Edwardsiella* and *Streptococcus* species are some of the major disease-causing bacterial pathogens in the Korean aquaculture industry. Moreover, as the use of antibiotics without prescription has been banned in Korea since July 2012, there is growing consensus that the future sustainability of the industry will depend upon application of alternative preventive strategies. In the present observation in challenge test, the cumulative mortality for fish fed Basal diet was significantly higher than those of fish fed $Min_{0.075}$, $Min_{0.15}$, $Min_{0.3}$ and $Min_{0.6}$ diets. The cumulative mortality was significantly lowest for fish fed $Min_{0.6}$ followed by $Min_{0.3}$, $Min_{0.075}$, $Min_{0.15}$ and Basal diets. Overall observation suggested, inclusion of dietary chelated trace mineral premix with further refinement can provide a noble alternative to antibiotic for the health management in Korean rockfish aquaculture.

In conclusion, the present experimental results suggested that chelated trace mineral premix could be a safe, effective and bioavailable source of trace mineral for marine fish juvenile Korean rockfish. The optimum dietary inclusion level for chelated trace mineral could be greater than 0.075% ($Min_{0.075}$) but lower than 0.3% ($Min_{0.3}$) in juvenile Korean rockfish. The observation with the fish fed 0.6% ($Min_{0.6}$) suggested, proper care must be taken to avoid the adverse effects of dietary chelated trace minerals and should be supplemented at optimum level. Results from the challenge test suggested, chelated trace minerals holding a great potential as an alternative to antibiotics in sustainable health management in aquaculture, stagnating further in depth study.

Chapter 3:

Evaluation of efficacy of different levels of dietary Inorganic and chelated trace minerals (Cu, Zn & Mn) premixes in Whiteleg shrimp, *Litopenaeus vannamei*

Evaluation of the efficacy of different levels of dietary Inorganic trace minerals (Cu, Zn & Mn) premix in juvenile whiteleg shrimp (Experiment III)

Abstract

The present experiment was conducted to evaluate the efficacy of dietary inorganic trace minerals (Cu, Zn & Mn) premix in the diet supplemented with high plant protein in White leg shrimp. Five isonitrogenous and isocaloric practical diets were formulated including a deficient basal control, and diets supplemented with the inorganic trace mineral premixes at four different levels *viz.*0.25% ($Inr_{0.25}$), 0.5% ($Inr_{0.5}$), 1 ($Inr_{1.0}$) and 2% (Inr_2). Each experimental diet was formulated to contain high level of dietary soybean at 45% of the diet as the dietary protein source to match up the antagonism with the commercial practical diets. Eleven numbers of acclimatized shrimp averaging initial body weight 0.6 ± 0.01g (mean ± SD) were randomly distributed to 15 experimental tanks. Triplicate group of experimental shrimp were fed one of the experimental diets at satiation for 8 weeks. At the end of the experiment, final weight (FW), weight gain (WG) and specific growth rate (SGR) of shrimp fed Inr_2 was significantly higher than those of shrimp fed Basal diet. However, there was no significant difference in FW, WG and SGR among the shrimp fed Basal, $Inr_{0.25}$, $Inr_{0.5}$ and Inr_1 and also among Inr_2, Inr_1, $Inr_{0.5}$ and $Inr_{0.25}$ diets. Feed conversion ratio (FCR) ranged between 2.55 to 3.19, which was significantly lower for shrimp fed Inr_2 than those of shrimp fed Basal diet. However, there was no significant difference in FCR among the group of shrimp fed, Basal, $Inr_{0.25}$, $Inr_{0.5}$ and Inr_1 diets. The trace mineral Cu content in hepatopancreas of shrimp fed $Inr_{0.5}$, Inr_1, Inr_2 was significantly higher than those of shrimp fed Basal diet. Hepatopancreatic Zn content was significantly higher among the shrimp fed $Inr_{0.25}$, $Inr_{0.5}$, Inr_1 and Inr_2 diets than that of shrimp fed Basal diet. However, there

was no significant difference in hepatopancreatic Zn content between shrimp fed $Inr_{0.25}$ and Inr_2 and also between $Inr_{0.5}$ and Inr_1 diets. While, Cu-Zn SOD activity from the hepatopancreas was recorded to be significantly higher for the shrimp fed $Inr_{0.5}$ than that of shrimp fed Basal diet. However, there was no significant difference in hepatic Cu-Zn SOD activity among the group of shrimp fed Basal, $Inr_{0.25}$, $Inr_1$and Inr_2 diets also among the group of shrimp fed $Inr_{0.25}$, $Inr_{0.5}$, Inr_1 and Inr_2 diets. The CU-Zn SOD activity in serum of shrimp fed different levels of dietary inorganic trace mineral premix corroborated with the hepatopancreatic Cu-Zn SOD activity. Serum Cu-Zn SOD activity was recorded to be peaked for the shrimp fed $Inr_{0.25}$ diet, which was significantly higher than that of shrimp, fed Basal, $Inr_{0.5}$, Inr_1 and Inr_2 diets. Moreover, in hematological performance, protein content was recorded to gradually increase with the increase in dietary inclusion level of inorganic trace mineral premix. The protein content for shrimp fed Basal diet was recorded to be significantly lower than those of shrimp fed all other diets. While, plasma glucose level found to gradually increase with the increase in dietary inorganic trace mineral inclusion level and found to be highest for shrimp fed Inr_2 diets, although it was not significantly different from those of fish fed all other diets. The value for plasma cholesterol was found to be also peaked for shrimp fed $Inr_{0.5}$, and afterwards gradually declined without any statistical difference. Therefore, these results suggested that, the optimum dietary inclusion level for the inorganic trace minerals (Cu, Zn & Mn) premix in plant protein shrimp diet could be greater than 0.5% but less than 2% to ensure the optimum growth, tissue mineral saturation and enzymatic activities in Whiteleg shrimp, *L. vannamei*.

Evaluation of the efficacy of different levels of dietary Chelated trace minerals (Cu, Zn & Mn) premix in whiteleg shrimp (Experiment IV)

Abstract

The present experiment was conducted to evaluate the efficacy of dietary chelated trace minerals (Cu, Zn & Mn) premix in the diet supplemented with high plant protein in White leg shrimp. Five isonitrogenous and isocaloric practical diets were formulated including a deficient basal control, and diets supplemented with the chelated trace mineral premixes at four different levels *viz.*0.25% ($Min_{0.25}$), 0.5% ($Min_{0.5}$), 0.75 ($Min_{0.75}$) and 1% (Min_{1}). Each experimental diet was formulated to contain high level of dietary soybean at 45% of the diet as the dietary protein source to match the antagonism with the commercial practical diets. Eleven numbers of acclimatized shrimp averaging initial body weight 0.6 ± 0.01g (mean ± SD) were randomly distributed to 15 experimental tanks. Triplicate group of experimental shrimp were fed one of the experimental diets at satiation for 8 weeks. At the end of the experiment, final weight (FW), weight gain (WG) and specific growth rate (SGR) of shrimp fed Basal diet was significantly lower than those of shrimp fed $Min_{0.25}$, $Min_{0.5}$ and $Min_{0.75}$ diets. While, there was no significant difference in FW, WG and SGR among the shrimp fed Basal and Min_{1} diets also between the group of shrimp fed Basal, $Min_{0.75}$ and Min_{1} diets. Survival ranged between 84 to 93% among different treatments without any statistical difference. The analyzed trace mineral Cu content in hepatopancrease of shrimp fed Basal diet was significantly lower than those of shrimp fed $Min_{0.5}$, $Min_{0.75}$ and Min_{1} diets. The value for hepatopancreatic Zn content among the shrimp fed $Min_{0.5}$, $Min_{0.75}$ and Min_{1} diets was significantly higher than those of shrimp fed Basal and $Min_{0.25}$ diets. While, whole body Cu content showed a significantly higher value for shrimp fed $Min_{0.5}$ than

those of shrimp fed Basal diet, however, there was no significant difference in whole body Cu content among the group of shrimp fed $Min_{0.25}$, $Min_{0.5}$, $Min_{0.75}$ and Min_1 diets. Whole body Zn concentration was significantly higher for shrimp fed $Min_{0.75}$ than those of shrimp fed Basal and Min_1 diets. The hepatopancreatic Cu-Zn SOD activity was found to be significantly higher for the shrimp fed $Min_{0.25}$ and $Min_{0.5}$ than those of shrimp fed $Min_{0.75}$ and Min_1 diets. However, there was no significant difference in Cu-Zn SOD activity among the shrimp fed Basal, $Min_{0.25}$, $Min_{0.5}$ diets and also among the shrimp fed $Min_{0.75}$ and Basal diets. The Cu-Zn SOD activity in the serum of shrimp fed $Min_{0.5}$ diets was significantly higher than those of shrimp all other diets. However, there was no significant difference in serum Cu-Zn SOD activity among the shrimp fed $Min_{0.25}$, $Min_{0.75}$ and Min_1 diets. Hematological performance showed significantly higher plasma protein content for the shrimp fed $Min_{0.5}$ than those of shrimp fed Basal diets. Furthermore, plasma glucose level also found to be significantly highest for the shrimp fed $Min_{0.5}$ diet than those of shrimp fed all other experimental diets. Meanwhile, plasma cholesterol level was recorded to be highest for shrimp fed $Min_{0.25}$ followed by $Min_{0.75}$ and Min_1 diets. However, there was no significant difference in plasma cholesterol content between shrimp fed $Min_{0.75}$ and Min_1, and also between $Min_{0.5}$ and $Min_{0.75}$ diets. Therefore, these results suggested that the optimum dietary chelated trace minerals (Cu, Zn & Mn) premix inclusion level in plant protein based shrimp diet could be greater than 0.25% but less than 0.75% for Whiteleg shrimp.

Introduction

A massive expansion of farming areas along with the intensification has led to a dramatic increase in the annual production of whiteleg shrimp, *Litopenaeus vannamei* (Boone) in the Republic of Korea from 0 metric ton (mt) in 2005 to 2,844 (mt) in 2011 (MOMAF, 2012). The global production of whiteleg shrimp increased from 146,362 (mt) in 2000 to 2,720,929 (mt) in 2010 (FAO, 2012). This species is recieving increasing attention worldwide as a potential species of intensive farming in ponds (Hopkins et al., 1993; Burford et al., 2003), raceways (McAbee et al., 2003; Browdy and Moss, 2005) and floating cages (Zarain et al., 2010). Farmers prefer raising the whiteleg shrimp, because the species is easier, quicker, and more profitable to grow than other commonly farm-raised shrimp (Hedlund, 2007). The excellent disease resistant quality compared to other shrimp species has encouraged several shrimp farmers along the coast line to shift to *L. vannamei* farming. Paralleling the growth of the shrimp industry has been an expansion in feed production. While, growth rates of tank-reared shrimp that depend on prepared diets for 100% of their nutritional needs do not match the growth rates that are commonly observed in natural pond environments. To understand the proper feed inputs, it is needed to have a clear understanding of nutrients requirement including those of dietary trace minerals. Furthermore, shrimp aquaculture has gone under phenomenal intensification worldwide, where the diseae outbreak has become a major threat. To ensure the optimum growth and health of shrimp, it is needed to have a clear understanding of nutrient requirement, particualry true in the case of micro nutrients such as trace mienrals.

Whereas, the trace mineral requirements in several commercialy important aquaculture species are still unknown and the practical feed formulation follow the general estimation. Particularly true in the case of whiteleg shrimp, *Litopenaeus vannamei*, where the aquafeed formulation for this species follow the estimation recommended for grass shrimp, *Penaeus monodon*. Although, the significance of tarce minerals in shrimp nutrition and health has been well recognized and a few studies have evlauted the efficacy of chelated Zn (Lin et. al., 2013) and chelated Cu (Bharadwaz et. al., 2014). However, to the best of our knowledge there has been absolute lack of information on the efficacy of inorganic trace mineral premix in whiteleg shrimp.

The overall scientific reports gathered over last three decades suggest there is an absolute requirement for dietary tarce minerals in crustaceans including the whiteleg shrimp, *L. vannamei*. Worthy to note that, aquafeed formulated to serve as shrimp feed has been reported to consuming the highest fishmeal quantity in aquaculture (Tacon, 2008). However, historical high price of FM and sustainability issue has exerted pressure to replace the fishmeal with some promising alternative dietary protein source. Consequently, numerous fishmeal replacement studies have been conducted in different shrimp species including whiteleg shrimp. Whereas, plant protein contain a wide array of antinutrients, among them the presence of antagonists factors such as phytic acid has been acknowledges as the major barrier hindering the bioavailability of trace mineral. Phytic acid (myo-inositol 1,2,3,4,5,6- hexakisphosphate) is the major phosphorus (P) storage compound in plant seeds and can account for up to 80 percent of total phosphorus. Phytic acid binds with divalent cationic trace minerals rendering them unavailable to the animal and these are consequently lost to the environment as waste (Cheryan, 1980; Davis and Gatlin, 1996; Davis et al., 1993; Li and Robinson, 1997). Whereas, fish nutritists and economic experts predicts the trend to be continue and dietary plant protein will have a substantial role in the sustainable development of shrimp aquaculture. Thus, the limited bioavilability and potential dietary deficiency of trace minerals are serious concern as adequate trace mineral ensuring the optimum growth and health of cultured species in aquaculture. Fortunately, a few studies conducted very recently have vouched the efficacy of dietary chelated trace mineral, Zn (Lin et. al., 2013) and Cu (Bharadwaj et. al., 2014) in Whiteleg shrimp. However, there has been an absolute lack of report on the efficacy chelated trace minerals premix in this species. Therefore, these present studies were conducted to evaluate the efficacy of different levels of dietary inorganic and chelated trace mineral (Cu, Zn & Mn) premix on the growth performance, bioavilbility and non specific immune response in Whiteleg shrimp, *L. vannamei*.

Materials and Methods (Exp. III & IV)

Diet Formulation and Preparation

Nine isonitrogenous and isocaloric practical diets were formulated to contain minimum level of native mineral derived from the feed ingredients. The composition of the basal diet as shown in Table 27. One of the five practical diets including a deficient basal control, and diets supplemented with the inorganic trace mineral premixes at four different levels (0.25%, 0.5%, 1% and 2% of requirement levels) were formulated. One of the five practical diets including a deficient basal control, and diets supplemented with the chelated trace mineral premixes at four different levels (50%, 100%, 150%, and 200% of requirement levels) were formulated. Mineral premix at 0.5% (100%) of dietary inclusion were formulated to supply the trace minerals at zinc at 60 mg/kg of diet, copper at 40 mg/kg and manganese at 50 mg/kg of the diet, as per the requirement level of marine shrimp (NRC, 2011) from either source. Unlike, Korean rockfish experiment, none of the experimental diet was supplemented with either dicalcium phosphate or phytic acid. To match the antagonism with the commercial practical shrimp diets, high level of dietary soybean at 45% of the diet as the dietary protein source was added in each experimental diet. Trace minerals premix was added to the each experimental diet at graded level at the the expense of dietary cellulose. Soybean meal, fish oil (DHA + EPA enriched; refined fish oil, E-Wha oil Co. Ltd., Pusan, Korea), and wheat floor (United States Biochemical, Cleveland, OH, USA) were used as the main dietary protein, lipid and carbohydrate sources, respectively. All dry ingredients were finely ground, weighed, mixed manually for 5 minutes and then transferred to a mixer for another 15-minute mixing. Fish oil was then added slowly while mixing was continued. All ingredients were mixed for another 10 minutes and then Inorganic trace mineral premix mixed well with the other feed ingredients. Finally, distilled water was added to the mixture to form dough and dry pellets were made from this dough using a laboratory pelleting machine (Baokyong Commercial Co., Pusan, Korea). The pellets were air dried until the moisture was reduced to <10 g/kg diet. After processing, all the diets were broken up and sieved into the appropriate pellet size (4 mm diameter), packed into small bags and stored at -24^{o}C until used. Table 28 shows the analyzed trace mineral content of the five experimental diets.

Experimental Shrimp and Feeding Trial

The feeding trial was carried out at the Feeds and Foods Nutrition Research Center, Pukyong National University, Busan. Juvenile Whiteleg shrimp were transported to the experimental station and acclimated to the experimental conditions for one week before the feeding trial began. Eleven numbers of acclimatized shrimp averaging initial body weight 0.6 ± 0.01g (mean ± SD) were allocated to 15 experimental tanks in both experiments 3 and 4. Shrimp were fed basal diet for one week to induce the body reserved mineral depletion. After one week, 11 numbers of shrimp in triplicate group were randomly assigned to one of the five treatments in experiment 3 as well as experiment 4. All the experimental shrimp were fed one of the experimental diets at approximately satiation assuming a FCR of around 2. The feeding trial was conducted in a recirculatory system with 54-L aquaria receiving filtered seawater at a rate of 0.8L min^{-1}.

Supplemental aeration was provided to maintain dissolved oxygen level near saturation. Water temperature was maintained at 28 ± 1°C. Supplemental aerations were provided to maintain dissolved oxygen levels near saturation (6.5 ± 0.3 mg/L). The seawater pH, salinity, total ammonia-nitrogen and nitrites were 8.23 ± 0.13, 32.5 ± 0.7 ppt, 0.037 - 0.052 mg/L and 0.13 ± 0.09 mg/L, respectively. Tanks were siphoned an hour before the morning feeding and water in the center tank was completely replaced one hour after feeding in the evening. Mortality was checked daily. Any dead shrimp were removed and feeding amount to respective tank was adjusted accordingly since dead shrimp was not replaced during the experiment.

Sample Collection and Analysis

At the end of the feeding trial, shrimp were starved for 24 h and the total number and weight of shrimp in each aquarium was determined for the calculation of weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), and survival. Three additional shrimp were selected from each aquarium, killed and frozen at -80°C for proximate composition, whole body and liver trace mineral viz. Cu, Zn & Mn concentration analyses. Also, three shrimp were randomly sampled from each tank, then liver was removed from each shrimp and pooled for determining Cu-Zn superoxide dismutase (SOD) activity, shrimp used for hepatic enzyme analysis, were also subjected plasma and serum collection. Those three shrimps per tank were randomly captured and about 0.3 ml of haemolymph samples were withdrawn from the ventral sinus in the first abdominal segment using a 26-gauge hypodermic needle on a 1-ml syringes and then, plasma was separated by centrifugation at 3000 ×g for 20 min and stored at −70 ℃ for determination of haemolymph biochemical parameters including plasma total protein, cholesterol, triglyceride and glucose. Meanwhile, each syringe was pre-filled with 0.3 ml of anticoagulant (113 mM glucose, 27.2 mM sodium citrate, 2.8 mM citric acid, and 71.9 mM NaCl).

Proximate composition analyses of experimental diets and fish body were performed by the standard methods of AOAC (2000). Samples of diets and fish were dried to a constant weight at 105℃ to determine moisture content. Ash was determined by incineration at 550℃; crude lipid by soxhlet extraction using Soxtec system 1046 (Foss, Hoganas, Sweden) and crude protein by Kjeldahl method (N × 6.25) after acid digestion.

Enzyme Assay

Superoxide dismutase (SOD) activity was measured by its ability to inhibit superoxide radical dependent reactions using the Ransod Kit (Randox, Crumlin, UK). Briefly, the reaction mixture (1.7 ml) contained xanthine (0.05 mM) and 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT, 0.025 mM) dissolved in 50 mM CAPS (pH 10.2) and 0.94 mM EDTA. In the presence of xanthine oxidase (80 U l_1, 250 ml), superoxide and uric acid were produced from xanthine. The superoxide radical then reacted with INT to produce a red formazan dye. The optical density was measured at 450 nm, 37 (C, and the rate of reaction was estimated from the absorbance readings at 30 s and 3 min after adding xanthine oxidase. A reference standard SOD was supplied with the Ransod Kit. One unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50%. Specific activity was expressed as SOD units ml_1.

Mineral Analysis

Copper, zinc, and manganese contents of rearing water, diet, tissue, and final whole-body were determined by digestion of samples in nitric acid (AOAC 2000). The concentrations of trace minerals in the diluted digest solution were determined by using an Inductively Coupled Plasma Mass Spectrometer (Perkin-Elmer 3300, Waltham, MA).

Statistical Analysis

All data were analyzed by one-way ANOVA to test for the effects of the dietary treatments. When significant differences were found, Tukey's multi-comparison test was used to identify differences among experimental groups. Treatment effects were considered with the significance level at P <0.05. All statistical analyses were carried out by SAS version 9.0 software (SAS Institute, Cary, NC, USA).

Results (Exp. III)

Growth Performance

Table 29 shows the growth performance of shrimp fed different levels of inorganic trace minerals premix for 8 weeks. At the end of experiment, final weight (FW) of shrimp fed Basal diet was significantly lower than those of shrimp fed Inr_2 diets. However, there was no significant difference in FW among the shrimp fed Basal, $Inr_{0.25}$, $Inr_{0.5}$, Inr_1 diets, also there was no significant difference in FW of shrimp fed Inr_2, Inr_1, $Inr_{0.5}$ and $Inr_{0.25}$ diets. The trend was similar in terms of WG and SGR with the trend of FW. FCR ranged between 2.55 to 3.19, which was significantly lower for shrimp fed Inr_2 than those of shrimp fed Basal diet. However, there was no significant difference in FCR among the group of shrimp fed, Basal, $Inr_{0.25}$, $Inr_{0.5}$ and Inr_1 diets. Survival rate ranged between 75 to 93%, which was significantly higher for shrimp fed Inr_2 than those of shrimp fed $Inr_{0.5}$ diet.

Tissue Trace Mineral content

Table 30 & 31 shows the analyzed trace mineral content at the end of experiment in hepatopancreas and whole body respectively. The Cu concentration tended to increase in hepatopancreas with the corresponding increase in dietary inorganic trace mineral content. The hepatic Cu concentration was significantly higher for shrimp fed $Inr_{0.5}$, Inr_1, Inr_2 diets than those of shrimp fed Basal diet. However, there was no significant difference in Cu content between shrimp fed $Inr_{0.5}$ and Inr_1 and also between $Inr_{0.25}$ and $Inr_{0.5}$. Hepatopancreatic Zn content was significantly higher among shrimp fed $Inr_{0.25}$, $Inr_{0.5}$, Inr_1 and Inr_2 diets than that of shrimp fed Basal diet. However, there was no significant difference in hepatopancreatic Zn content between shrimp fed $Inr_{0.25}$ and Inr_2 and also between $Inr_{0.5}$ and Inr_1 diets. While hepatopancreatic Mn content for shrimp fed Inr_1 diet was significantly lower than those of shrimp fed $Inr_{0.25}$ diet. However, there was no significant difference in hepatopancreatic Mn content among the shrimp fed Basal, $Inr_{0.25}$, $Inr_{0.5}$ and Inr_2 diets. Trace minerals content in whole body of shrimp fed different levels of inorganic trace mineral premix showed significantly lowest Mn content for shrimp fed Inr_1 diets than those of shrimp fed all other experimental diets. However, there was no significant difference in whole body Mn content among shrimp fed Basal, $Inr_{0.25}$, Inr_1 and Inr_2 diets and also among the group of shrimp fed $Inr_{0.5}$, $Inr_{0.25}$, Basal and Inr_2 diets.

Enzyme Assay

The activities of hepatopancreatic and serum copper-zinc superoxide dismutase (Cu-Zn SOD) activities of shrimp fed different level of inorganic trace mineral premix is shown in table 33. Cu-Zn SOD activity in heptaopanrease was recorded to gradually increase with dietary trace mineral premix inclusion level which was peak for the shrimp fed $Inr_{0.5}$ diets and afterwards gradually declined. The Cu-Zn SOD activity for shrimp fed $Inr_{0.5}$ diet was significantly higher than those of shrimp fed Basal diet. However, there was no significant difference in hepatopancreatic Cu-Zn SOD activity among the group of shrimp fed Basal, $Inr_{0.25}$, Inr_1 and Inr_2 diets also among the group of shrimp fed $Inr_{0.25}$, $Inr_{0.5}$, Inr_1 and Inr_2 diets. The CU-Zn SOD activity in serum of shrimp fed different levels of dietary inorganic trace mineral premix corroborated with the hepatopancreatic Cu-Zn SOD activity. Serum Cu-Zn SOD activity was recorded to be peaked for the shrimp fed $Inr_{0.25}$ diet, which was significantly higher than that of shrimp fed Basal, $Inr_{0.5}$, Inr_1 and Inr_2 diets. However, there was no significant difference in serum Cu-Zn SOD activity between the shrimp fed $Inr_{0.5}$ and Basal diet.

Hematological Characteristics

Table 32 shows the recorded hematological characteristics for the shrimp fed different levels of inorganic trace mineral premix for 8 weeks. At the end of experiment, hematological protein content was recorded to gradually increase with the increase in dietary inclusion level of inorganic trace mineral premix. The protein content for shrimp fed Basal diet was recorded to be significantly lowest for shrimp fed Basal diet than those of shrimp fed all other diets. However, there was no significant difference in plasma protein content among the shrimp fed $Inr_{0.25}$, $Inr_{0.5}$, Inr_1 and Inr_2 diets. While, plasma glucose level found to gradually increase with the increase in dietary inorganic trace mineral inclusion level and found to be highest for shrimp fed Inr_2 diets, however it was not significantly different from those of fish fed all other diets. The value for plasma cholesterol was found to be also peaked at shrimp fed $Inr_{0.5}$, and afterwards gradually declined without any statistical difference.

Discussion and Conclusions (Exp. III)

The results of this study indicate that juvenile Whileleg shrimp has a distinct dietary requirement for trace minerals (Cu, Zn & Mn) which cannot be met by the ambient rearing water. Moreover, the present experiment also demonstrates that the inclusion level for dietary inorganic trace minerals must be increased in the context of increasing use dietary plant protein in marine shrimp feed formulation. The overall growth performance recorded to significantly increase with the corresponding increase in the level of dietary inorganic trace mineral premix inclusion. Final weight (FW), WG and SGR of shrimp fed Basal diet was significantly lower than those of shrimp fed Inr_2 diets. However, there was no significant difference in these parameters among the shrimp fed Basal, $Inr_{0.25}$, $Inr_{0.5}$, Inr_1 diets, and also among those of shrimp fed Inr_2, Inr_1, $Inr_{0.5}$ and $Inr_{0.25}$ diets. Reports for fish and shrimp have shown a positive correlation between the dietary inclusion level of trace minerals and growth performance (Lee and Shiau 2002; Shaw and Handy 2006; Tan et al. 2011). While, duration of the trials could also be a discrepancy factor, as the Whiteleg shrimp in the present study were fed diets for 8 weeks, which is similar to those of the (Lee and Shiau 2002; Bharadwaz et. al., 2014) and almost half of the study that of (Lin et. al., 2013) studies on shrimp. Since the experimental shrimp were fed at approximately satiation in the present experiment, thus the FCR might appear higher than the various previous reports for the same species. But our value for FCR is similar with several other authors including that of Browdy et. al., (2012) for whiteleg shrimp.

The effects of trace mineral inhibitor phytic acid on reduced mineral intake are not fully understood. In a preliminary study with *L. vannamei* where shrimp were fed semipurified diets containing different sources of copper (copper sulfate and chelated copper), a copper requirement of approximately 54 mg/kg (37 mg/kg supplemental copper) was estimated using chelated copper while no significant performance response was observed in copper sulfate fed shrimp (Bharadwaj et al.; 2014). They hypothesized that the presence of antagonists such as phytic acid, calcium and phosphorus in the diets may have inhibited the absorption and availability of inorganic copper. Phytic acid has been shown to negatively influence mineral availability, growth, body composition and nutrient utilization in fish (Laining et al., 2010; Richardson et al., 1985). On the other hand, no adverse effect of phytic acid was observed in *P. japonicus* but growth was depressed in *L. vannamei* fed diets containing phytic acid (Civera and Guillaume, 1989). A dietary excess of zinc was required to overcome the presence of dietary phytic acid in *L. vannamei* (Davis et al., 1993). The overall growth performances in the present experiment suggested the optimum inclusion level for dietary trace mineral (Cu, Zn & Mn) in plant protein shrimp diet could be higher than 0.5% ($Inr_{0.5}$) but lower than 2% (Inr_2) of diet.

Whole body and hepatopancreas tissue trace minerals contents were responsive to dietary supplementation of trace minerals, numerically increasing with increasing dietary level. High variability was observed due to small sample sizes, use of pooled samples, limitations in mineral analysis precision and changes in total copper concentrations associated with differences in animal size and physiological state (molt cycle etc.) as reported by Bharadwaz et. al., (2014). In general, hepatopancreas copper concentrations were similar than those reported in shrimp in the study by Davis et al. (1993) but lower than those reported by Lin et. al., (2013) and Bharadwaz et. al., (2014). An increase from 30 μg/g to 170 μg/g was observed in shrimp hepatopancreas with an increase in dietary copper from 2 to 130 mg/kg of copper. Davis et al. (1993a) also observed increases in hemolymph and carapace copper concentrations with increasing dietary copper levels. In *P. monodon* there was an increase in whole body copper concentrations with increasing dietary copper supplementation, from 36 μg/g in the basal control group to approximately 50 μg/g in treatment groups fed 80 and 160 mg/kg of copper (Lee and Shiau, 2002). Since in the present experiment, high level of dietary soybean was used as the protein source in the dietary formulation, our results are in agreement with few others that dietary inclusion level for trace mineral premix from inorganic source should increase.

Moreover, the data for Cu-Zn SOD activity from hepatopancreas and serum well corroborated with the results of shrimp growth performance and tissue mineral contents. The Cu-Zn SOD activity for shrimp fed $Inr_{0.5}$ diet was significantly higher than those of shrimp fed Basal diet. However, there was no significant difference in hepatopancreatic Cu-Zn SOD activity among the group of shrimp fed Basal, $Inr_{0.25}$, Inr_1 and Inr_2 diets also among the group of shrimp fed $Inr_{0.25}$, $Inr_{0.5}$, Inr_1 and Inr_2 diets. Meanwhile, the CU-Zn SOD activity in serum of shrimp fed different levels of dietary inorganic trace mineral premix corroborated with the hepatopancreatic Cu-Zn SOD activity. Serum Cu-Zn SOD activity was recorded to be peaked for the shrimp fed $Inr_{0.25}$ diet, which was significantly higher than that of shrimp, fed Basal, $Inr_{0.5}$, Inr_1 and Inr_2 diets. However, there was no significant difference in serum Cu-Zn SOD activity between the shrimp fed $Inr_{0.5}$ and Basal diet. Likewise, several studies have reported higher enzymatic activity in trace minerals supplemented diets irrespective of fish species (Apines-Amar et al., 2003; Apines-Amar et al., 2004; Tan and Mai, 2001). Moreover, the plasma protein content for shrimp fed Basal diet was recorded to be significantly lowest for shrimp fed Basal diet than those of shrimp fed all other diets. However, there was no significant difference in plasma protein content among the shrimp fed $Inr_{0.25}$, $Inr_{0.5}$, Inr_1 and Inr_2 diets. While, plasma glucose level found to gradually increase with the increase in dietary inorganic trace mineral inclusion level and found to be highest for shrimp fed Inr_2 diets, Thus, it was evident from the present experiment, higher level of dietary trace mineral inclusion from inorganic source has no adverse effects on the enzymatic activity and thereby physiological condition and shrimp health.

In conclusion, the present experimental results demonstrate a comparatively higher requirement than the recommended level for dietary trace minerals (NRC, 2011) for shrimp, particularly when the shrimp feed formulation substantially contain dietary plant protein. The optimum dietary level for trace minerals (Cu, Zn & Mn) premix could be greater than 0.5% ($Inr_{0.5}$) but lower than 2% (Inr_2) in the form of inorganic source to ensure the optimum growth, tissue mineral saturation and enzymatic activities in Whiteleg shrimp, *L. vannamei*.

Results (Exp. IV)

Growth Performance

Table 35. Shows the growth performance and survival rate of shrimp fed different levels dietray chelated trace mineral premix for 8 weeks. Significant effects of trace mineral inclusion level on shrimp growth was recorded, the final weight (FW) of shrimp fed Basal diet was significantly lower than that of shrimp fed $Min_{0.25}$, $Min_{0.5}$ and $Min_{0.75}$ diets. While, there was no significant difference in FW among the shrimp fed Basal and Min_1 diets also between the groups of shrimp fed Basal, $Min_{0.75}$ and Min_1 diets. The similar trend with FW was recorded for WG and SGR among the different treatments group. The FCR value ranged between 2.5 to 3.19 among different treatment groups, the FCR value for shrimp fed $Min_{0.25}$ and $Min_{0.5}$ was significantly lower than those of shrimp fed basal diets. However, there was no significant difference in FCR among the groups of shrimp fed Basal, $Min_{0.75}$ and Min_1 diets and also among the $Min_{0.25}$, $Min_{0.5}$, $Min_{0.75}$ and Min_1 diets. Survival ranged between 84 to 93% among different treatments without any statistical difference.

Trace Mineral content in Tissue

Table 36 & 37 shows the analyzed trace mineral content in liver and whole body of shrimp fed different levels of chelated trace mineral premix for 8 weeks respectively. The hepatopancreatic Cu content for shrimp fed Basal diet was significantly lower than those of shrimp fed $Min_{0.5}$, $Min_{0.75}$ and Min_1 diets. However, there was no significant difference in hepatopancreatic Cu content among the shrimp fed $Min_{0.25}$, $Min_{0.5}$, $Min_{0.75}$ and Min_1 diets. While, hepatopancreatic Zn content was found to be significantly lowest for the shrimp fed $Min_{0.25}$ diet than those of shrimp fed all other experimental diets. Furthermore, the value for hepatopancreatic Zn content among the shrimp fed $Min_{0.5}$, $Min_{0.75}$ and Min_1 diets was significantly higher than those of shrimp fed Basal and $Min_{0.25}$ diets, however there was no significant difference among the group of shrimp fed $Min_{0.5}$, $Min_{0.75}$ and Min_1 diets. While, hepatopancreatic Mn content was found to gradually increase with the increase in dietary inclusion level of chelated trace mineral premix up to $Min_{0.75}$ and afterwards sudden declined, without any statistical difference.

Whole body Cu content showed a consistent increasing trend up to $Min_{0.5}$ and afterwards gradually declined. The value for whole body Cu content was significantly higher for shrimp fed $Min_{0.5}$ than those of shrimp fed Basal diet, however there was no significant difference in whole body Cu content among the group of shrimp fed $Min_{0.25}$, $Min_{0.5}$, $Min_{0.75}$ and Min_1 diets. Whole body Zn concentration as recorded to gradually increase with the increase in dietary inclusion level of chelated trace mineral premix, which was significantly higher for shrimp fed $Min_{0.75}$ than those of shrimp fed Basal and Min_1 diets. However, there was no significant difference in whole body Zn content among the shrimp fed $Min_{0.25}$, $Min_{0.5}$ and $Min_{0.75}$ diets and also between the shrimp fed $Min_1$and Basal diets. The value for whole body Mn content was found to gradually increase with the increase in inclusion level of dietary chelated trace mineral without any statistical difference.

Enzyme Activity

Table 39 shows the analyzed hapatopancreatic and serum copper-zinc superoxide dismutase (Cu-Zn SOD) activity for the shrimp fed different levels dietary chelated trace mineral premix for 8 weeks. The hepatopancreatic Cu-Zn SOD activity was found to be significantly higher for the shrimp fed $Min_{0.25}$ and $Min_{0.5}$ than those of shrimp fed $Min_{0.75}$ and Min_1. However, there was no significant difference in Cu-Zn SOD activity among the shrimp fed Basal, $Min_{0.25}$, $Min_{0.5}$ diets and also among the shrimp fed $Min_{0.75}$ and Basal diets. The Cu-Zn SOD activity in serum of shrimp fed $Min_{0.5}$ diets was significantly higher than those of shrimp all other diets. However, there was no significant difference in Serum Cu-Zn SOD activity among the shrimp fed $Min_{0.25}$, $Min_{0.75}$ and Min_{1} diets.

Hematological Parameters

Table 38 shows the hematological characteristics of shrimp fed different levels of chelated trace mineral premix for 8 weeks. Plasma protein content was found to be significantly higher for shrimp fed $Min_{0.5}$ than those of shrimp fed Basal diets, however, there was no significant different in plasma protein content among the shrimp fed $Min_{0.25}$, $Min_{0.5}$, $Min_{0.75}$ and Min_{1} diets. Plasma glucose level also found to be significantly highest for the shrimp fed Min100 diet than those of shrimp fed all other experimental diets. However, there was no significant difference in plasma glucose level among the shrimp fed Basal, $Min_{0.25}$, $Min_{0.75}$ and Min_{1} diets. Meanwhile, plasma cholesterol level was recorded to be highest for shrimp fed $Min_{0.25}$ followed by $Min_{0.75}$ and Min_{1} diets. However, there was no significant difference in plasma cholesterol content between shrimp fed $Min_{0.75}$ and Min_{1}, and also between $Min_{0.5}$ and $Min_{0.75}$ diets.

Discussion and Conclusion (Exp. IV)

This study is the first report on the safe and toxic dietary chelated trace minerals (Cu, Zn & Mn) premix in marine shrimp, *Litopenaeus vannamei*. The significant effects of different levels of dietary chelated minerals premix on shrimp growth performance was clearly observed. Overall observation demonstrated the trace minerals requirement level as recommended in NRC (2011), could be optimum in plant based shrimp diet, if the trace minerals are specifically derived from the chelated sources. In the present experiment, the final weight (FW), weight gain (WG), specific growth rate (SGR) of shrimp fed Basal diet was significantly lower than that of shrimp fed $Min_{0.25}$, $Min_{0.5}$ and $Min_{0.75}$ diets. While, there was no significant difference in these parameters among the shrimp fed Basal and Min_1 diets also between the groups of shrimp fed Basal, $Min_{0.75}$ and Min_1 diets. The current study demonstrates the benefit of dietary chelated trace minerals premix on the growth of *L. vannamei*. In other aquatic animals, the use of chelated trace elements has resulted in improved growth (Apines-Amar et al., 2004; Paripatananont and Lovell, 1995; Satoh et al., 2001; Tan and Mai, 2001). Micronutrients particularly Cu, Zn and Mn are known to be essential for growth both in animals and humans (Apines-Amar et al., 2004; Sharif et al., 2012). Deficiency of Zn reduced the levels of IGF-I, growth hormone receptor and rowth hormone binding protein mRNA (Clegg et al., 1995; McNall et al., 1995). Like IGF-I, Zn can increase the protein component of bone and play a role in bone growth in collaboration with IGF-I (Ma and Yamaguchi, 2001a,b). Worthy to note that, the observed growth performance in the present experiment also indicated that the inclusion of dietary chelated trace mineral premix at higher levels is unnecessary and ineffective. Thus to ensure the optimum growth at optimum feed cost, chelated trace minerals should be included at optimum level. Similar to our observation, various previous authors have reported the significant effects of chelated trace mineral on the growth performance of fish irrespective of species. For instance, growth reduction was recorded in olive flounder fed either insufficient or excess dietary chelated copper (Mohseni et. al., 2011) in agreement with results in other aquatic animals (Shaw and Handy 2006; Lin et al. 2008; Wu and Huang 2008; Tan et al. 2011; Bharadwaz et. al., 2014).

Cowey (1976) suggested that besides growth, other physiological parameters such as tissue enzyme activity may also be appropriate to quantify nutrient requirement in fish. In the present study, the hepatopancreatic Cu content for shrimp fed Basal diet was significantly lower than those of shrimp fed $Min_{0.5}$, $Min_{0.75}$ and Min_1 diets. Moreover, the value for hepatopancreatic Zn content among the shrimp fed $Min_{0.5}$, $Min_{0.75}$ and Min_1 diets was significantly higher than those of shrimp fed Basal and $Min_{0.25}$ diets, however there was no significant difference among the group of shrimp fed $Min_{0.5}$, $Min_{0.75}$ and Min_{1} diets. While, hepatopancreatic Mn content was found to gradually increase with the increase in dietary inclusion level of chelated trace mineral premix up to $Min_{0.75}$ and afterwards sudden declined, without any statistical difference. The observation for the hepatopancreatic Zn content was unexpected which was found to be significantly lowest for the shrimp fed $Min_{0.25}$ diet than those of shrimp fed all other experimental diets. While, trace mineral contents in the whole body showed, a consistent increasing trend in Cu concentration up to $Min_{0.5}$ and afterwards gradually declined. Whole body Zn concentration as recorded to gradually increase with the increase in dietary inclusion level of chelated trace mineral premix, which was significantly higher for shrimp fed $Min_{0.75}$ than those of shrimp fed Basal and Min_{1} diets. While, the value for whole body Mn content was found to gradually increase with the increase in inclusion level of dietary chelated trace mineral without any statistical difference. Apines-Amar et al. (2003) and Apines-Amar et al. (2004) indicated that trace elements chelated with amino acids, which include Cu, Zn, and Mn, seem to be more available in rainbow trout. This is because organic minerals are generally considered less sensitive to the inhibitory action of other compounds due to the different absorption pathway (Ashmead,1992). Whereas, the present experimental diets were formulated to contain high level of dietary plant protein to supply the adequate trace mineral inhibitor. The results with tissue minerals concentration supported those previous reports that chelated trace minerals are comparatively less sensitive to mineral inhibitor present protein.

Furthermore, observation with hepatopancreatic and serum enzymatic activities well corroborated with the findings of growth performance and tissue minerals contents. The hepatopancreatic Cu-Zn SOD activity was found to be significantly higher for the shrimp fed

$Min_{0.25}$ and $Min_{0.5}$ than those of shrimp fed $Min_{0.75}$ and Min_1. Furthermore, the serum Cu-Zn SOD activity for shrimp fed $Min_{0.5}$ diets was significantly higher than those of shrimp all other diets. Similarly, Gatlin and Wilson (1986) also reported that the activity of liver Cu-Zn SOD was significantly reduced in channel catfish fed diets containing 0-2.0 mg Cu/kg as compared to those fed 4 mg Cu/kg diet. Free Cu has been reported to induce the oxidation and destruction of SOD in vitro (Cecconi et al. 2002). Trace minerals such as copper plays both pro-and anti-oxidation roles in biological system. The antioxidant enzymes such as Cu-Zn SOD and CAT have been extensively used as biomarkers of oxidative stress (Kopecka-Pilarczyk and Correia 2009). Similarly, depressed hepatic Cu-Zn SOD activity in beluga was observed when the dietary Cu was insufficient (≤3.5 mg Cu/kg diet) or in excess >50 mg Cu/kg diet, also depressed muscle Cu-Zn SOD activity in fish was observed when the dietary Cu was insufficient (≤3.5 mg Cu/kg diet) (Mohseni et. al., 2011). The reason could be attributed due to the increase in production of reactive oxygen species, possibly due to lower rate of fish metabolism. Indeed, some authors found a decrease in the activity of metabolism-related enzymes at low or high Cu concentration (Lin et al. 2008; Wang et al. 2009), which would later return to the original state as the fish acclimatised to the Cu. However, there is little information available on the enzyme assay in *L. vannamei*, more studies are required to clarify the role of trace minerals particularly from chelated source in the enzyme system of shrimp. In conclusion, results from the present experiment suggested the optimum dietary chelated trace minerals (Cu, Zn & Mn) inclusion level in plant protein based shrimp diet could be greater than 0.25% ($Min_{0.25}$) but less than 0.75% ($Min_{0.75}$).

Chapter 4. General Discussion and Conclusions

Juvenile Korean rockfish and whiteleg shrimp have a distict requirement for dietary trace minerals which can not be met by the trace mienrals from the amibinet water. Therefore, trace minerals Cu, Zn, Mn & Fe must be supplied through diet in adequate quantity. Furthermore, the overall obseravations from these experimnets support the hypothesis that mineral antagonist, phytic acid naturally present in dietary plant protein binds with divalent cationic trace minerals and adversely affect the bioavilbility. Thus, with the increase in dietary plant protein in fish and shrimp feed formulation, the dietary inclusion level for trace minerals should also be redefined.

Chelated trace mienrals premix used in the present experiments, Mintrex trace minerals are relatively new type of organically bound trace minerals that has become available on the market. It is a one molecule of trace minerals chelated with 2-hydroxy-4 (methylthio) butanoic acid (HMTBa), which is the hydroxy analog of methionine. In previous studies, it was found that HMTBa from this Zn source was fully available as a methionine source in broiler chicks (Yi et al., 2007) and hybrid striped bass (Morone chrysops × Morone saxatilis) (Savolainen and Gatlin, 2010). Meanwhile, Zn from Mintrex Zn was more bioavailable than Zn from ZnSO4 in chicks and poults (Dibner, 2005; Yuan et al., 2011). Mintrex® Cu shows the same pattern as copper sulfate in increasing liver copper content in chickens for fattening when added to a basal diet in supplementations between 10 and 500 mg/kg diet. Mntrex Cu was reported to be four to five times more effective than inorganic source in terms of bioavlibility of Cu in whiteleg shrimp, *L. vannamei* (Bharadwaj et. al., 2014). Observation from the present experimnets together with afore mentioned experiments, could be taken as encouraging demonstration of bioavailability of trace minerals derived from chelated source. Neverthless, every manufacture claim their product to be the best and it is always doubtful to to believe the true value and efefciency of those products. A cross comparison between the inorganic and chelated source of tarce minerals could provide a more concrete efficiency of these two sources of trace minerals. Overall data suggested, 2~ 4 times higher efficiency of chelated trace minerals (Cu, Zn, Mn & Fe) premix in juvenile Korean rockfish and 3~6 times higher efficiency of chelated premix (Cu, Zn & Mn) to promote the similar growth, trace mineral bioavilbility as well as to ensure the optimum enzymatic activity and health. Worthy to note that, chelated trace minerals and their premix should also be supplemented at optimum level, high level of dietary inclusion could also be toxic. Overall trend shows, chelated trace minerals and premix is holding a great potential as safe and effective alternative to traditional inorganic source of trace minerals in aquaculture. Even though despite of potential benefit, the high cost of chelated trace mineral often acknowledged as the major factor limiting its use in aquafeed industry. Thus, complementary inclusion of chelated with inorganic trace minerals could be logical step to encourage the inclusion of alternative dietary mineral source in aquafeed formulation.

Exp. I – Evaluation of the efficacy of dietary Inorganic Trace Minerals (Cu, Zn, Mn & Fe) Premix in Juvenile Korean rockfish:

Juvenile Korean rockfish has a distinct requirement for the trace minerals, *viz*. Cu, Zn, Mn and Fe which cannot be met by these trace minerals present in the rearing water. The dietary supplementation of at least 0.3% of the diet is obligatory. Moreover, the trace minerals inclusion level should further increase in the presence of minerals inhibitor phytic acid, which is abundantly present in the dietary plant protein. Further research are warranted to evaluate the efficacy of dietary inorganic trace minerals premix at higher than 0.3% (NRC, 2011) inclusion level in the presence of mineral inhibitor phytic acid to ensure the maximum growth, tissue saturation and optimum health.

Exp. II- Evaluation of the efficacy of dietary Chelated Trace Minerals (Cu, Zn, Mn & Fe) Premix in Juvenile Korean rockfish:

The optimum dietary inclusion level of chelated trace mineral premix could be greater than 0.075% but less than 0.3% of the diet based on growth performance, trace minerals saturation in tissues and nonspecific enzymatic activity in juvenile Korean rockfish. The observation with the fish fed 0.6% of diet suggested, proper care must be taken to avoid the adverse effects of dietary chelated trace minerals and should be supplemented at optimum level. Results from the challenge test suggested, chelated trace minerals holding a great potential to improve the disease resistance and thereby fend off the disease. However, stringent further in depth studies to clearly understand the efficacy of chelated trace minerals in sustainable health management in marine finfish aquaculture.

Exp. III - Evaluation of the efficacy of dietary Inorganic Trace Minerals (Cu, Zn & Mn) Premix in Juvenile Whiteleg shrimp:

The present study demonstrated a comparatively higher requirement than the level recommended for dietary trace minerals in NRC, 2011 for marine shrimp, particularly when the shrimp feed formulation substantially contain dietary plant protein. The optimum dietary inclusion level for the inorganic trace minerals (Cu, Zn & Mn) premix in plant protein diet, could be greater than 0.5% but less than 2% to ensure the optimum growth, tissue minerals saturation and enzymatic activities in Whiteleg shrimp, *L. vannamei*.

IV- Evaluation of the efficacy of dietary Chelated Trace Minerals (Cu, Zn, & Mn) Premix in Juvenile Whiteleg shrimp:

Results from the present experiment suggested that the optimum dietary chelated trace minerals (Cu, Zn & Mn) inclusion level in plant protein based shrimp diet could be greater than 0.25% but less than 0.75%. Chelated trace minerals could be safe, effective and more bioavailable source of trace minerals to ensure the maximum growth, tissue mineral saturation and optimum enzymatic activity in Whiteleg shrimp. However, proper care must be taken in chelated trace minerals supplementation at optimum level to avoid any adverse effects due to higher level of inclusion.

Table 1. Trace Mineral contents of feed ingredients[1]

Ingredients /Mineral	Mn	Cu	Fe	Zn
Muscle Powder	1.35	3.4	59.2	56.0
Wheat Flour	4.29	7.25	61.9	13.3
Casein	0.67	11.7	94.6	78.9
Corn Starch	0.10	1.03	58.9	19.0
Fish Meal	9.09	0.75	341.2	86.8
Dehulled Soybean Meal	39.37	15.5	127.7	62.4
ISP	11.92	30.5	144.5	77.2

[1]Values are the means from duplicate groups of samples

Table 2. Basal diet composition used in experiment 1 and 2

Ingredient	% in diet
Casein[1]	38.6
Muscle Powder	10
Wheat flour	16.95
Corn Starch	12.21
Corn Starch	4.9
α-*Cellulose*	4.5
Fish Oil	10.2
Vitamin Premix[2]	3
Mineral Premix (Inorganic/Chelate)	0
Phytic acid[1]	1.0
Dicalcium Phosphate[1]	2
MeraMet[3] (1/10)	0.54

[1] Sigma Chemicals

[2] Vitamin mixtures (contains as mg/kg diet): DL-alpha tocopherol acetate. 60 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; ribo- flavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 5 mg; ascorbic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium pantothenate, 50 mg; choline chloride, 2000 mg.

[3] Supplied by Novus International Inc. Canada

Table 3. Analyzed Mineral Content (mg kg^{-1} ; DM) of different experimental diets[1]

Diets/Minerals	Basal	$Inr_{0.3}$	$Inr_{0.15}$	$Inr_{0.075}$
Cu	3.53	10.4	6.03	4.2
Zn	48.0	79.2	64.9	53.5
Mn	3.89	9.45	5.76	6.57
Fe	95.5	176	124.7	65.9

[1]Values are means from triplicates groups of samples

Table 4. Growth Performance of Juvenile Korean rockfish fed different levels of Inorganic Trace Mineral Premix for 16 weeks[1]

Diet/Parameters	Final weight (g)	WG (%)[2]	SGR (%/day)[3]	FE (%)[4]	PER[5]	Survival (%)
Basal	20.4^{a}	103.2^{a}	2.54^{a}	46.6^{a}	0.98^{a}	76.0^{a}
$Inr_{0.075}$	25.0^{a}	122.5^{a}	2.82^{a}	54.3^{a}	1.10^{a}	81.2^{a}
$Inr_{0.15}$	21.2^{a}	108.9^{a}	2.64^{a}	47.9^{a}	1.05^{a}	73.9^{a}
$Inr_{0.3}$	23.0^{a}	118.3^{a}	2.76^{a}	50.7^{a}	1.19^{a}	78.1^{a}
Pooled SEM[6]	0.64	6.39	0.06	2.48	0.05	1.51

[1]Values are means from groups (n = 4) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Weight gain (%): (final wt. - initial wt.) × 100 / initial wt.

[3]Specific growth rate (%/day): 100× (Ln final wt. - Ln initial wt.)/days

[4]Feed efficiency (%): (wet weight gain (g) / dry feed intake (g)) × 100

[5]Protein efficiency ratio: (wet weight gain / protein intake)

[6]Pooled standard error of means: SD/√n

Table 5. Trace Mineral Contents in Liver tissue (mg/kg) of juvenile Korean rockfish fed different leve ls inorganic trace mineral premix for 16 weeks[1]

Diet/Minerals	Cu	Zn	Mn	Fe
Basal	2.96[a]	315.9[b]	1.72[b]	58.4[a]
$Inr_{0.075}$	2.91[a]	451.7[a]	1.13[b]	64.8[a]
$Inr_{0.15}$	2.81[a]	330.9[b]	1.29[b]	56.3[a]
$Inr_{0.3}$	3.62[a]	267.9[b]	5.12a	57.0[a]
Pooled SEM[2]	0.21	23.31	0.44	2.24

[1]Values are means from groups (n = 4) of fish where the means in each column with different superscripts are significantly different (*P* < 0.05).

[2]Pooled standard error of means: SD/√n

Table 6. Trace Mineral Contents in bone tissue (mg/kg) of juvenile Korean rockfish fed different levels of inorganic trace mineral premix for 16 weeks[1]

Diet/Minerals	Cu	Zn	Mn	Fe
Basal	2.89^{a}	2756^{b}	7.22^{c}	103.3^{c}
$Inr_{0.075}$	3.12^{a}	1824^{c}	6.63^{c}	158.1^{ab}
$Inr_{0.15}$	2.62^{a}	3322^{ab}	9.81^{b}	180.8^{a}
$Inr_{0.3}$	2.43^{a}	3588^{a}	22.7a	143.7^{b}
Pooled SEM[2]	0.14	207.4	1.72	0.179

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different (P < 0.05).

[2]Pooled standard error of means: SD/√n

Table 7. Trace Mineral Contents in whole body (mg/kg) of juvenile Korean rockfish fed different leve ls inorganic trace mineral premix for 16 weeks[1]

Diets/Minerals	Cu	Zn	Mn	Fe
Basal	1.53[a]	443.0[d]	2.09[b]	47.1[c]
$Inr_{0.075}$	1.59[a]	704.5[c]	3.22[a]	83.2[a]
$Inr_{0.15}$	1.65[a]	1022.3[a]	2.13[b]	65.4[b]
$Inr_{0.3}$	1.80[a]	829.4[a]	2.12[b]	47.3[c]
Pooled SEM[2]	0.09	56.9	0.20	4.24

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: $SD/\sqrt{n}$

Table 8. Whole body proximate composition of Juvenile Korean rockfish fed different levels of Inorganic Trace Mineral Premix for 16 weeks[1]

Diets/Minerals	Moisture	Protein	Lipid	Ash
Basal	74.3^{a}	59.2^{a}	17.9^{a}	46.6^{a}
Inr$_{0.075}$	72.6^{a}	59.2^{a}	17.8^{a}	54.3^{a}
Inr$_{0.15}$	73.35^{a}	60.3^{a}	17.4^{a}	47.9^{a}
Inr$_{0.3}$	72.28^{a}	60.0^{a}	17.0^{a}	50.7^{a}
Pooled SEM[6]	0.33	0.24	0.25	0.20

Table 9. Hepatic Cu-Zn superoxide dismutase (Cu- Zn SOD) activity of juvenile Korean rockfish fed different levels of Inorganic Trace Mineral Premix[1]

Diets/Parameters	Cu-Zn SOD Units/g tissue	TBARS (μmol MDA/L)
Basal	47.8[c]	98.7[a]
$Inr_{0.075}$	102.6[b]	91.0[b]
$Inr_{0.15}$	127.9[a]	93.0[b]
$Inr_{0.3}$	112.6[b]	89.7[b]
Pooled SEM[2]	8.01	2.26

[1]Values are means from groups (n = 4) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: SD/√n

Table 10. Histological analysis (Total collagen, mg/kg) of juvenile Korean rockfish fed different levels of inorganic trace mineral premix for 16 weeks[1]

Diets/Parameters	Total Collagen
Basal	1.64[a]
$Inr_{0.075}$	1.49[a]
$Inr_{0.15}$	1.6[a]
$Inr_{0.3}$	1.21[a]
Pooled SEM[2]	0.10

Table 11. Mineral Content (mg kg^{-1} ; DM) of different experimental diets[1]

Diets/Minerals	Basal	$Min_{0.075}$	$Min_{0.15}$	$Min_{0.3}$	$Min_{0.6}$
Cu	3.53	4.15	6.62	10.28	16.79
Zn	48.06	46.55	67.99	77.41	210.07
Mn	3.89	6.57	6.83	10.65	16.02
Fe	95.56	101.6	126.05	177.45	317.8

[1]Values are average of three replicates samples

Table 12. Growth Performance of Juvenile Korean rockfish fed different levels of chelated Trace Mineral Premix for 16 weeks[1]

Diets/Parameters	Final weight (g)	WG (%)[2]	SGR (%/day)[3]	FE (%)[4]	PER[5]	Survival (%)
Basal	20.4^{b}	103.2^{b}	2.54^{b}	46.6^{a}	0.98^{a}	76.0^{a}
$Min_{0.075}$	20.6^{ab}	129.0^{ab}	2.87^{ab}	56.2^{a}	1.23^{a}	69.7^{a}
$Min_{0.15}$	23.7^{ab}	132.7^{ab}	2.89^{ab}	57.4^{a}	1.24^{a}	73.9^{a}
$Min_{0.3}$	28.5^{a}	171.1^{a}	3.12^{a}	65.0^{a}	1.37^{a}	84.3^{a}
$Min_{0.6}$	22.9^{b}	129.0^{ab}	2.87^{ab}	56.6^{a}	1.23^{a}	72.9^{a}
Pooled SEM[6]	0.92	9.4	0.06	3.02	0.06	2.33

[1]Values are means from groups (n = 4) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Weight gain (%): (final wt. - initial wt.) × 100 / initial wt.

[3]Specific growth rate (%/day): 100× (Ln final wt. - Ln initial wt.)/days

[4]Feed efficiency (%): (wet weight gain (g) / dry feed intake (g)) × 100

[5]Protein efficiency ratio: (wet weight gain / protein intake)

[6]Pooled standard error of means: SD/√n

Table 13. Trace Mineral Contents in Liver tissue (mg/kg) of juvenile Korean rockfish fed different levels Chelated trace mineral premix for 16 weeks[1]

Diets/Minerals	Cu	Zn	Mn	Fe
Basal	2.96^{bc}	315.9^{b}	1.72^{b}	58.4^{c}
$Min_{0.075}$	2.89^{c}	549.3^{a}	1.45^{b}	60.9^{bc}
$Min_{0.15}$	3.35^{b}	256.7^{b}	1.29^{b}	72.5^{bc}
$Min_{0.3}$	4.51^{a}	478.9^{a}	10.1^{a}	96.2^{ab}
$Min_{0.6}$	3.58^{b}	287.9^{b}	1.45^{b}	123.8^{a}
Pooled SEM[2]	0.16	28.85	0.81	7.43

[1]Values are means from groups (n = 4) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: SD/√n

Table 14. Trace Mineral Contents in Whole body (mg/kg) of juvenile Korean rockfish fed different le vels Chelated trace mineral premix for 16 weeks[1]

Diets/Minerals	Cu	Zn	Mn	Fe
Basal	1.53[a]	443[c]	2.09[c]	47.1[d]
$Min_{0.075}$	2.15[ab]	848[b]	2.90[abc]	88.9[b]
$Min_{0.15}$	1.97[b]	222[d]	3.02[abc]	63.9[cd]
$Min_{0.3}$	3.08[a]	1095[a]	3.54[a]	88.9[b]
$Min_{0.6}$	3.15[a]	667[b]	2.20[bc]	136[a]
Pooled SEM[2]	0.20	74.39	0.17	7.58

[1]Values are means from groups (n = 4) of fish where the means in each column with different supscripts are significantly different (*P* < 0.05).

[2]Pooled standard error of means: SD/√n

Table 15. Trace Mineral Contents in Bone tissue (mg/kg) of juvenile Korean rockfish fed different lev els Chelated trace mineral premix for 16 weeks[1]

Diets/Minerals	Cu	Zn	Mn	Fe
Basal	2.89^{c}	2756^{c}	7.22^{d}	103.38^{c}
$Min_{0.075}$	5.43^{b}	2743^{c}	6.70^{d}	178.91^{c}
$Min_{0.15}$	2.29^{c}	3507^{b}	10.8^{c}	152.14^{c}
$Min_{0.3}$	5.50^{b}	4255^{a}	48.0^{a}	322.79^{b}
$Min_{0.6}$	8.67^{a}	3628^{b}	14.4^{b}	425.37^{a}
Pooled SEM[2]	0.57	154.1	3.57	29.7

[1]Values are means from groups (n = 4) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: SD/√n

Table 16. Whole body proximate composition (DM basis) of juvenile Korean rockfish fed different lev els of chelated trace mineral premix for 16 weeks[1]

Diets/Parameters	moisture	protein	lipid	ash
Basal	74.39[a]	59.27[a]	17.99[a]	16.9[a]
$Min_{0.075}$	72.97[a]	60.50[a]	16.11[a]	17.28[a]
$Min_{0.15}$	73.27[a]	59.02[a]	16.98[a]	17.26[a]
$Min_{0.3}$	72.90[a]	59.31[a]	17.72[a]	17.55[a]
$Min_{0.6}$	74.80[a]	60.21[a]	15.59[a]	17.31[a]
Pooled SEM[6]	0.89	0.21	0.25	0.35

[1]Values are means from groups (n = 4) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: SD/√n

Table 17. Hepatic Cu-Zn superoxide dismutase (Cu- Zn SOD) activity of juvenile Korean rockfish fed different levels of ChelatedTrace Mineral Premix[1]

Diets/Parameters	Cu-Zn SOD Units/g tissue	TBARS (μmol MDA/L)
Basal	47.86^{d}	98.78^{a}
$Min_{0.075}$	108.84^{c}	93.47^{b}
$Min_{0.15}$	119.6^{c}	74.34^{c}
$Min_{0.3}$	163.98^{a}	67.86^{d}
$Min_{0.6}$	144.54^{b}	71.76^{c}
Pooled SEM[2]	5.03	2.26

[1]Values are means from groups (n = 4) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: SD/√n

Table 18. Histological analysis (Total collagen, mg/kg) of juvenile Korean rockfish fed different levels of Chelated trace mineral premix for 16 weeks[1]

Diets/Parameters	Collagen Concentration
Basal	1.64[a]
$Min_{0.075}$	1.62[a]
$Min_{0.15}$	1.76[a]
$Min_{0.3}$	1.72[a]
$Min_{0.6}$	1.66[a]
Pooled SEM[2]	0.10

[1]Values are means from groups (n = 4) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: SD/√n

Table 19. Composition of the basal diet

Ingredients	% in diet
Soybean meal	45.00
Wheat flour	27.40
Squid Liver powder	15.00
Cholesterol	0.20
Lecithin	4.00
Fish oil	2.00
Vitamin premix[2]	2.00
Magnesium Oxide[1]	0.35
Potassium Chloride	0.75
Potassium Phosphate[1]	0.50
Cellulose[1]	2.00
MeraMet[3]	0.80
Inorganic mineral	0.00
Mintrex mineral	0.00

1 Sigma Chemicals

2 Vitamin mixtures (contains as mg/kg diet): DL-alpha tocopherol acetate. 60 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; ribo- flavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 5 mg; ascorbic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium pantothenate, 50 mg; choline chloride, 2000 mg.

3 Supplied by Novus International Inc. Canada

Table 20. Mineral Content (mg kg^{-1} ; DM) of different experimental diets[1]

Diets/Parameters	Basal	$Inr_{0.25}$	$Inr_{0.5}$	Inr_1	Inr_2
Cu	12.81	34.88	63.34	99.98	185.47
Zn	29.39	50.30	90.06	136.65	182.74
Mn	25.92	41.66	56.01	85.34	132.57

[1]Values are average of two replicates samples

Table 21. Growth Performance of Juvenile Whiteleg shrimp fed different levels of Inorganic Trace Mineral Premix for 8 weeks[1]

Diets/Parameters	Final weight (g)	WG (%)[2]	SGR (%/day)[3]	FCR[4]	Survival (%)
Basal	3.29^{b}	486.51^{b}	2.27^{b}	3.19^{a}	84.84^{ab}
$Inr_{0.25}$	3.53^{ab}	519.40^{b}	2.45^{ab}	3.08^{a}	90.9^{a}
$Inr_{0.5}$	3.44^{b}	502.88^{ab}	2.77^{a}	3.23^{a}	75.75^{b}
Inr_{1}	3.59^{ab}	530.22^{ab}	2.49^{ab}	2.95^{ab}	84.84^{ab}
Inr_{2}	3.95^{a}	606.38^{a}	2.77^{a}	2.55^{b}	93.93^{a}
Pooled SEM[6]	0.08	14.90	0.06	0.08	2.14

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Weight gain (%): (final wt. - initial wt.) × 100 / initial wt.

[3]Specific growth rate (%/day): 100× (Ln final wt. - Ln initial wt.)/days

[4]Feed efficiency: wet weight gain (g) / dry feed intake (g)

[5]Protein efficiency ratio: (wet weight gain / protein intake)

[6]Pooled standard error of means: SD/√n

Table 22. Trace Mineral Contents in hepatopancreas tissue (mg/kg) of juvenile Whiteleg shrimp fed different levels of inorganic trace mineral premix for 8 weeks[1]

Diets/Minerals	Cu	Zn	Mn
Basal	106.3^{d}	146.8^{c}	2.02^{ab}
$Inr_{0.25}$	127.8^{dc}	272.2^{a}	2.46^{a}
$Inr_{0.5}$	177.7^{bc}	204.9^{b}	2.35^{a}
Inr_{1}	216.3^{ab}	287.7^{b}	1.97^{b}
Inr_{2}	249.5^{a}	299.1^{a}	2.12^{ab}
Pooled SEM[2]	15.48	11.84	0.09

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: $SD/\sqrt{n}$

Table 23. Trace Mineral Contents in whole body (mg/kg) of juvenile Whiteleg shrimp fed different levels of inorganic trace mineral premix for 8 weeks[1]

Diets/Minerals	Cu	Zn	Mn
Basal	18.88[a]	18.01[a]	0.559[ab]
$Inr_{0.25}$	21.24[a]	16.81[a]	0.546[ab]
$Inr_{0.5}$	19.40[a]	24.17[a]	0.710[a]
Inr_{1}	19.44[a]	23.43[a]	0.398[b]
Inr_{2}	25.59[a]	14.79[a]	0.827[a]
Pooled SEM[2]	1.23	1.74	0.06

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: SD/√n

Table 24. Hematological characteristics of Juvenile whiteleg shrimp fed different levels of Inorganic Trace Minerals premix for 8 weeks[1]

Diets/Parameters	Protein (g $dL^{-1)}$	Glucose (g $dL^{-1)}$	Cholesterol (mmol L^{-1})
Basal	2.3^{b}	24^{a}	5.1^{a}
$Inr_{0.25}$	4.85^{a}	31^{a}	14^{a}
$Inr_{0.5}$	4.4^{ab}	26^{a}	22^{a}
Inr_{1}	4.3^{ab}	30.5^{a}	15^{a}
Inr_{2}	5^{a}	40.5^{a}	8.5^{a}
Pooled SEM[2]	0.39	2.51	2.65

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different (P < 0.05).

[2]Pooled standard error of means: SD/√n

Table 25. Hepatopancreatic thiobarbituric acid reactive substances (TBARS) value, Cu-Zn superoxide dismutase (Cu-Zn SOD) of Juvenile whiteleg shrimp fed different levels of Inorganic Trace Minerals premix for 8 weeks[1]

Diet	SOD Liver Units/g tissue	TBARS (μmol MDA/L)	SOD(Serum) Units/g tissue	TBARS (μmol MDA/L)
Basal	91.88[b]	12.42[b]	95.63[b]	59.85[d]
$Inr_{0.25}$	96.41[ab]	17.95[b]	106.38[a]	135[b]
$Inr_{0.5}$	99.67[a]	41.29[a]	91.16[b]	185[a]
Inr_{1}	94.91[ab]	38.63[a]	69.02[d]	85.9[c]
Inr_{2}	95.09[ab]	10.29[b]	83.2[c]	99.8[c]
Pooled SEM[2]	1.1	3.65	4.2	14.6

Table 26. Mineral Content (mg kg^{-1} ; DM) of different experimental diets[1]

Diets/Parameters	Basal	$Min_{0.25}$	$Min_{0.5}$	$Min_{0.75}$	Min_{1}
Cu	12.81	39.30	64.07	86.25	102.57
Zn	29.39	53.41	93.87	135.66	138.78
Mn	25.92	43.61	58.83	73.75	87.87

[1]Values are average of two replicates samples

Table 27. Growth Performance of Juvenile Whiteleg shrimp fed different levels of Chelated Trace Mineral Premix for 8 weeks[1]

Diets/Parameters	Final weight (g)	WG (%)[2]	SGR (%/day)[3]	FCR[4]	Survival (%)
Basal	3.29^{c}	486.51^{c}	2.27^{c}	3.19^{a}	84.84^{ab}
$Min_{0.25}$	4.1^{ab}	646.09^{a}	2.9^{ab}	2.53^{b}	90.9^{ab}
$Min_{0.5}$	4.18^{a}	631.26^{ab}	2.91^{a}	2.55^{b}	87.87^{ab}
$Min_{0.75}$	3.93^{ab}	590.24^{ab}	2.74^{ab}	2.80^{ab}	93.93^{a}
Min_{1}	3.69^{bc}	545.27^{bc}	2.58^{bc}	2.78^{ab}	87.87^{ab}
Pooled SEM[6]	0.10	19.63	0.07	0.08	1.31

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Weight gain (%): (final wt. - initial wt.) × 100 / initial wt.

[3]Specific growth rate (%/day): 100× (Ln final wt. - Ln initial wt.)/days

[4]Feed efficiency: wet weight gain (g) / dry feed intake (g)

[5]Protein efficiency ratio: (wet weight gain / protein intake)

[6]Pooled standard error of means: SD/√n

Table 28. Trace Mineral Contents in hepatopancreas tissue (mg/kg) of juvenile Whiteleg shrimp fed different levels of Chelated trace mineral premix for 8 weeks[1]

Diets/Parameters	Cu	Zn	Mn
Basal	106.35^{b}	146.83^{c}	2.02^{a}
$Min_{0.25}$	132.49^{ab}	98.60^{d}	2.04^{a}
$Min_{0.5}$	206.89^{a}	231.73^{b}	2.13^{a}
$Min_{0.75}$	194.84^{a}	252.09^{ab}	2.35^{a}
Min_{1}	208.31^{a}	275.43^{a}	1.92^{a}
Pooled SEM[2]	12.36	18.52	0.082

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: SD/√n

Table 29. Trace Mineral Contents in whole body (mg/kg) of juvenile whiteleg shrimp fed different levels of Chelated Trace mineral premix for 8 weeks[1]

Diets/Parameters	Cu	Zn	Mn
Basal	18.88^{b}	18.01^{c}	0.559^{a}
$Min_{0.25}$	21.89^{ab}	27.32^{ab}	0.546^{a}
$Min_{0.5}$	27.69^{a}	26.03^{ab}	0.685^{a}
$Min_{0.75}$	24.94^{ab}	30.59^{a}	0.665^{a}
Min_{1}	24.18^{ab}	27.84^{bc}	0.759^{a}
Pooled SEM[2]	15.48	1.34	0.04

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: SD/√n

Table 30. Hematological characteristics of Juvenile whiteleg shrimp fed different levels of Chelated Trace Minerals premix for 8 weeks[1]

Diets/Parameters	Protein ($g\ dL^{-1}$)	Glucose ($g\ dL^{-1}$),	Cholesterol ($mmol\ L^{-1}$)
Basal	2.3^{b}	24^{b}	5.1^{d}
$Min_{0.25}$	4.75^{a}	28^{b}	24^{a}
$Min_{0.5}$	4.65^{a}	40^{a}	9^{cd}
$Min_{0.75}$	4.4^{a}	30.5^{b}	14^{bc}
Min_{1}	3.4^{ab}	26.5^{b}	18^{ab}
Pooled SEM[2]	0.36	2.03	2.31

[1]Values are means from groups (n = 3) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: $SD/\sqrt{n}$

Table 31. Hepatopancreatic and Serum, Cu-Zn superoxide dismutase (Cu-Zn SOD) and TBARS activity of Juvenile whiteleg shrimp fed different levels of Chelated Trace Minerals premix for 8 weeks[1]

Diets/Parameters	SOD Units/g tissue	TBARS (µmol MDA/L)	SOD (Serum) Units/g tissue	TBARS (µmol MDA/L)
Basal	91.88^{ab}	12.42^{b}	95.63^{b}	59.8^{bc}
Min$_{0.25}$	97.3^{a}	40.63^{a}	77.32^{c}	74.5^{b}
Min$_{0.5}$	100.5^{a}	47.06^{a}	110.11^{a}	169.9^{a}
Min$_{0.75}$	85.42^{b}	41.9^{a}	76.90^{c}	65.3^{bc}
Min$_{1}$	72.83^{c}	13.4^{b}	74.77^{c}	48.6^{c}
Pooled SEM[2]	2.07	4.1	4.6	14.7

[1]Values are means from groups ($n = 3$) of fish where the means in each column with different superscripts are significantly different ($P < 0.05$).

[2]Pooled standard error of means: SD/√n

Fig. 1. Challenge test against bacteria *E.tarda*

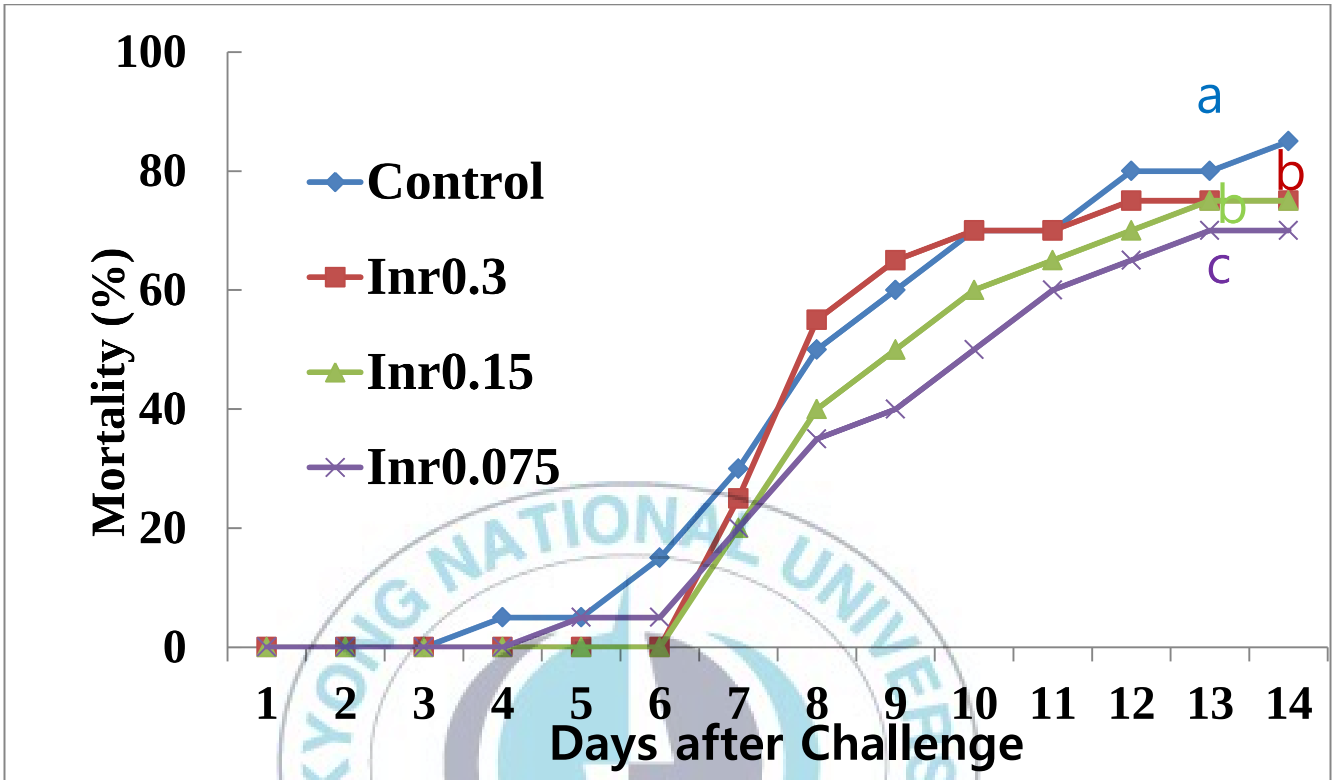
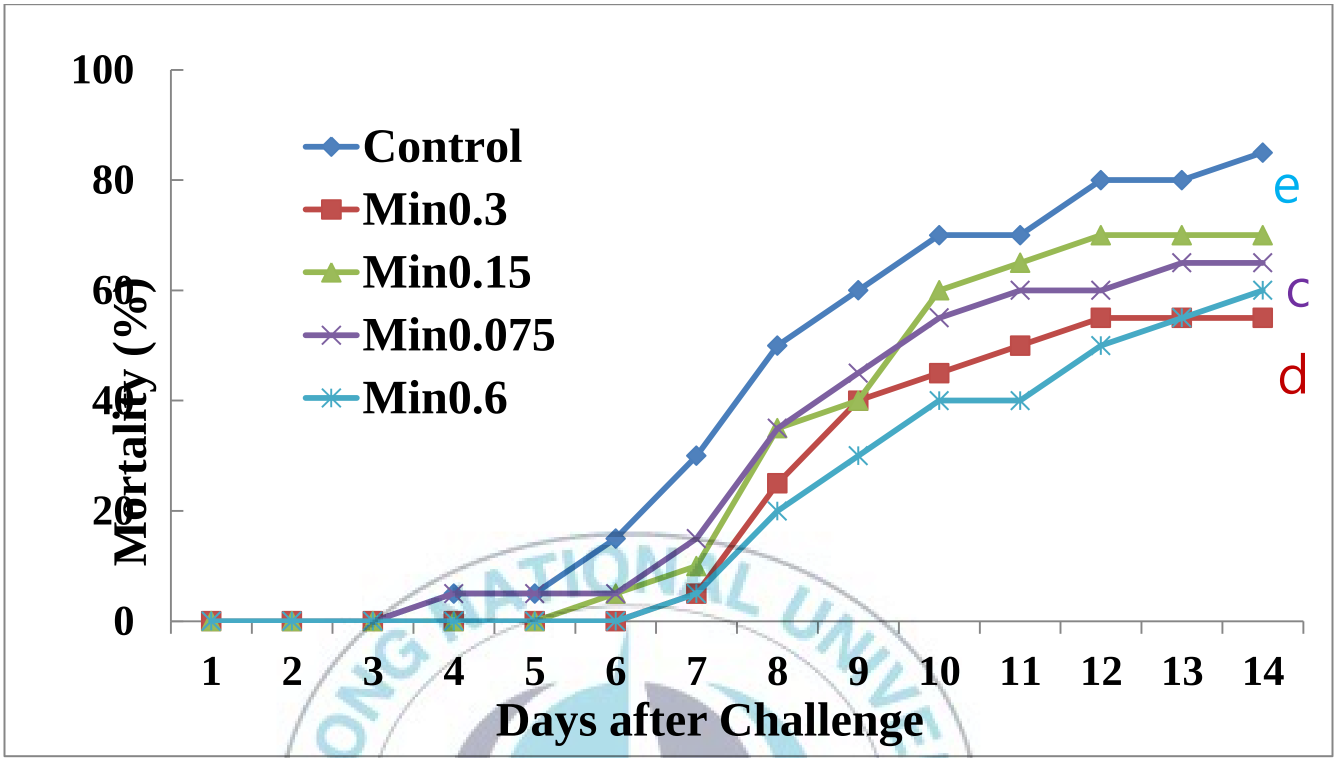


Fig. 2. Challenge test against bacteria *E.tarda*



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Appendix (Raw data)

Exp. 1.

10.27.2012	Tank	Total Weight	Fish Number	1- Weight
Diet 1	3	244.6	24	10.2
	12	238.4	24	9.9
	18	243.9	24	10.2
	27	240.7	24	10.0
Diet 2	2	241.7	24	10.1
	17	243.6	24	10.2
	23	239.4	24	10.0
	30	240.9	24	10.0
Diet 3	6	238.4	24	9.9
	11	236.0	24	9.8
	21	244.0	24	10.2
	29	240.4	24	10.0
Diet 4	1	243.5	24	10.1
	16	243.4	24	10.1
	19	244.0	24	10.2
	25	244.8	24	10.2

Exp. 1

03.15.2012	Tank	Final Weight	Fish Number	1- Weight
Diet 1	3	389.9	19	20.5
	12	315.0	19	16.6
	18	364.1	15	24.3
	27	412.6	20	20.6
Diet 2	2	451.8	19	23.8
	17	419.9	19	22.1
	23	420.4	18	23.4
	30	353.5	19	18.6
Diet 3	6	312.9	18	17.4
	11	404.8	16	25.3
	21	400.4	19	21.1
	29	353.9	18	19.7
Diet 4	1	451.2	18	25.1
	16	419.8	19	22.1
	19	448.7	20	22.4
	25	435.7	21	20.7

Exp 2.

03.15.2012	Tank	Initial Weight	Fish Number	1- Weight
Diet 1	3	244.6	24	10.2
	12	238.4	24	9.9
	18	243.9	24	10.2
	27	240.7	24	10.0
Diet 2	5	236.7	24	9.9
	9	238.0	24	9.9
	15	239.8	24	10.0
	24	243.5	24	10.1
Diet 3	8	243.3	24	10.1
	14	244.1	24	10.2
	20	240.1	24	10.0
	26	240.1	24	10.0
Diet 4	4	244.5	24	10.2
	7	244.1	24	10.2
	28	244.6	24	10.2
	31	241.8	24	10.1
Diet 5	13	244.8	24	10.2
	10	242.5	24	10.1
	22	241.4	24	10.1
	32	243.3	24	10.1

Exp. 2.

03.15.2012	Tank	Final Weight	Fish Number	1- Weight
Diet 1	3	389.9	19	20.5
	12	315.0	19	16.6
	18	364.1	15	24.3
	27	412.6	20	20.6
Diet 2	5	480.6	21	22.9
	9	569.4	15	38.0
	15	543.5	22	24.7
	24	519.3	23	22.6
Diet 3	8	500.1	18	27.8
	14	421.9	20	21.1
	20	357.8	16	22.4
	26	384.1	17	22.6
Diet 4	4	330.6	16	20.7
	7	329.5	14	23.5
	28	464.5	20	23.2
	31	435.5	17	25.6
Diet 5	13	451.9	20	22.6
	10	408.1	16	25.5
	22	333.1	16	20.8
	32	429.1	18	23.8

Exp. 3

03-02- 2014

	Tank No	Total Weight	number	Individual weight	DM	Total feeding	Individual feeding
Diet 1	1	6.18	11	0.6	0.930	1.89	0.16
	20	6.35	11	0.6	0.930	1.89	0.16
	30	6.03	11	0.5	0.930	1.89	0.16
Diet 2	3	6.18	11	0.6	0.930	1.89	0.16
	18	6.32	11	0.6	0.930	1.89	0.16
	22	6.33	11	0.6	0.930	1.89	0.16
Diet 3	5	6.14	11	0.6	0.930	1.89	0.16
	24	6.32	11	0.6	0.930	1.89	0.16
	31	6.32	11	0.6	0.930	1.89	0.16
Diet 4	8	6.33	11	0.6	0.930	1.89	0.16
	13	6.16	11	0.6	0.930	1.89	0.16
	27	6.33	11	0.6	0.930	1.89	0.16
Diet 5	10	6.22	11	0.6	0.930	1.89	0.16
	11	6.10	11	0.6	0.930	1.89	0.16
	29	6.16	11	0.6	0.930	1.89	0.16

25-03- 2014

	Tank No	Total Weight	number	Individual Final weight	DM	Total feeding	Individual feeding
Diet 1	1	30.15	10	3.0	0.930	1.89	0.16
	20	30.69	9	3.4	0.930	1.89	0.16
	30	31.19	9	3.5	0.930	1.89	0.16
Diet 2	3	23.80	7	3.4	0.930	1.89	0.16
	18	30.88	9	3.4	0.930	1.89	0.16
	22	31.40	9	3.5	0.930	1.89	0.16
Diet 3	5	32.50	10	3.3	0.930	1.89	0.16
	24	37.56	10	3.8	0.930	1.89	0.16
	31	35.75	10	3.6	0.930	1.89	0.16
Diet 4	8	30.89	10	3.1	0.930	1.89	0.16
	13	34.55	9	3.8	0.930	1.89	0.16
	27	34.62	9	3.8	0.930	1.89	0.16
Diet 5	10	39.77	10	4.0	0.930	1.89	0.16
	11	42.25	11	3.8	0.930	1.89	0.16
	29	40.50	10	4.1	0.930	1.89	0.16

Exp. 4

03-02- 2014

	Tank No	Total Weight	number	Individual weight	DM	Total feeding	Individual feeding
Diet 1	1	6.18	11	0.6	0.930	1.89	0.16
	20	6.35	11	0.6	0.930	1.89	0.16
	30	6.03	11	0.5	0.930	1.89	0.16
Diet 2	2	6.30	11	0.6	0.930	1.89	0.16
	19	6.18	11	0.6	0.930	1.89	0.16
	21	6.40	11	0.6	0.930	1.89	0.16
Diet 3	4	6.25	11	0.6	0.930	1.89	0.16
	17	6.02	11	0.6	0.930	1.89	0.16
	23	6.06	11	0.6	0.930	1.89	0.16
Diet 4	6	6.23	11	0.6	0.930	1.89	0.16
	15	6.68	11	0.6	0.930	1.89	0.16
	25	6.00	11	0.6	0.930	1.89	0.16
Diet 5	7	6.22	11	0.6	0.930	1.89	0.16
	14	6.10	11	0.6	0.930	1.89	0.16
	26	6.16	11	0.6	0.930	1.89	0.16

25-03- 2014

	Tank No	Total Weight	number	Individual Final weight	DM	Total feeding	Individual feeding
Diet 1	1	30.15	10	3.0	0.930	1.89	0.16
	20	30.69	9	3.4	0.930	1.89	0.16
	30	31.19	9	3.5	0.930	1.89	0.16
Diet 2	2	44.65	10	4.5	0.930	1.89	0.16
	19	37.44	9	4.2	0.930	1.89	0.16
	21	39.20	10	3.9	0.930	1.89	0.16
Diet 3	4	39.93	10	4.0	0.930	1.89	0.16
	17	41.16	10	4.1	0.930	1.89	0.16
	23	43.16	10	4.3	0.930	1.89	0.16
Diet 4	6	40.80	10	4.1	0.930	1.89	0.16
	15	39.38	11	3.6	0.930	1.89	0.16
	25	41.50	10	4.2	0.930	1.89	0.16
Diet 5	7	36.27	10	3.6	0.930	1.89	0.16
	14	31.59	9	3.5	0.930	1.89	0.16
	26	39.40	10	3.9	0.930	1.89	0.16