Organic iron requirements of gilthead sea bream (*Sparus aurata*)

by

ALEXANDROS SAMARTZIS

A thesis submitted to Plymouth University

In partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences

In collaboration with

Hellenic Centre of Marine Aquaculture

May 2013

Organic iron requirements of gilthead sea bream (*Sparus aurata*)

by

ALEXANDROS SAMARTZIS

A thesis submitted to Plymouth University

In partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences

In collaboration with

Hellenic Centre of Marine Aquaculture

May 2013

Title: Organic iron requirements of gilthead sea bream *(Sparus aurata)*.

Name: Alexandros Samartzis

Abstract

The aim of the current study was to determine the organic iron (Fe) requirements of gilthead sea bream (*Sparus aurata*). A total number of four experiments have been carried out each one for 12 weeks, in order to address and extend the knowledge on nutritional issues and challenges related with the culture of the gilthead sea bream in the Greek aquaculture industry and therefore enhance the fish health status under intensive culture conditions. These experiments aimed to determine the optimum level of organic Fe supplemented in commercial type diets of sea bream, the comparison between an organic Fe form and two inorganic Fe forms added in the diet of the fish, the effect of supplemented organic Fe on sea bream species exposed to oxygen deprivation stressors related to poor aquaculture husbandry practices and finally the interaction of organic Fe in the diet of sea bream with various levels of other trace minerals (Zn, Cu). The parameters evaluated were the growth performance of the fish, the Fe concentration in three selected tissues (the spleen, the liver and the muscle), the haematological status of the fish (the haematocrit, the red blood cell count, and the haemoglobin) and both the humoral and cellular immunology of the fish (the antibacterial activity of serum and the respiratory burst respectively). 150 mg/Kg of added organic Fe appears to be the recommended level as well as the minimum amount on fish exposed to overstocking

conditions. The comparison between the two inorganic Fe forms (Ferrous Sulphate and Ferrous Carbonate) added in the diets show no significant effect on the fish. While, the fish fed the diets with 150 mg/Kg organic Fe and Cu levels lower that 5 mg/Kg had higher Hb values.

Table of Contents

List of Tables

List of Figures

Fig. 3.8 The Hb on the gilthead sea bream fed the experimental diets for 12 weeks (g/dL). Values are the means (n = 18) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 3.9 The respiratory burst activity in the blood of the gilthead sea bream fed the experimental diets for 12 weeks (RLU). Values are the means (n = 18) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 3.10 The antibacterial activity of serum in the gilthead sea bream fed the experimental diets for 12 weeks (Units/mL). Values are the means (n = 18) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 4.1 The initial and final weight of the gilthead sea bream treated with the experimental diets for 12 weeks. Values are the means (n = 15) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 4.2 The SGR and FCR of the gilthead sea bream treated with the experimental diets for 12 weeks. Values are the means (n = 15) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 4.3 The Fe tissue concentration in the muscle of gilthead sea bream fed the experimental diets for 12 weeks (mg/Kg). Values are the means (n = 15) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig.4.4 The Fe tissue concentration in the liver of gilthead sea bream fed the experimental diets for 12 weeks (mg/Kg). Values are the means (n = 15) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig.4.5 The Fe tissue concentration in the spleen of gilthead sea bream fed the experimental diets for 12 weeks (mg/Kg). Values are the means (n = 15) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 4.6 The effects on the Htc of the seven experimental diets in the gilthead sea bream. Values are the means (n = 18) of three replicate tanks expressed with the

standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 4.7 The effects on the RBC of the seven experimental diets in the gilthead sea bream (10^5mL^{-1}). Values are the means (n = 18) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 4.8 The effects on the Hb of the seven experimental diets in the gilthead sea bream. Values are the means (n = 18) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 5.1 The initial and final weight for both A.S. and C.S. exposed gilthead sea bream trials (gr.). Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 5.2 The SGR for both A.S. and C.S. exposed gilthead sea bream trials. Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 5.3 The FCR for both A.S. and C.S. exposed gilthead sea bream trials. Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 5.4 The Fe concentration in the spleen of the gilthead sea bream fed the three experimental diets and exposed to the acute stressor (A.S.) (mg/Kg). Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 5.5 The Fe concentration in the muscle of the gilthead sea bream fed the three experimental diets and exposed to the acute stressor (A.S.) (mg/Kg). Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 5.6 The Fe concentration in the liver of the gilthead sea bream fed the three experimental diets and exposed to the acute stressor (A.S.) (mg/Kg). Values are the

means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p < 0.05$.

Fig. 5.7 The Fe concentration in the spleen of the gilthead sea bream fed the three experimental diets and exposed to the chronic stressor (C.S.) (mg/Kg). Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p < 0.05$.

Fig. 5.8 The Fe concentration in the muscle of the gilthead sea bream fed the three experimental diets and exposed to the chronic stressor (C.S.) (mg/Kg). Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p < 0.05$.

Fig. 5.9 The Fe concentration in the liver of the gilthead sea bream fed the three experimental diets and exposed to the chronic stressor (C.S.) (mg/Kg). Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p < 0.05$.

Fig. 5.10 The Htc of the gilthead sea bream fed the three experimental diets and exposed to the acute stressor (A.S.) (%). Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p < 0.05$.

Fig. 5.11 The RBC counts of the gilthead sea bream fed the three experimental diets and exposed to the acute stressor (A.S.) ($10^5 mL^{-1}$). Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p < 0.05$.

Fig. 5.12 The Hb of the gilthead sea bream fed the three experimental diets and exposed to the acute stressor (A.S.) (g/dL). Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p < 0.05$.

Fig. 5.13 The Htc of the gilthead sea bream fed the three experimental diets and exposed to the chronic stressor (C.S.) (%).Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p < 0.05$.

standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 6.3 Fe concentration in the spleen of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 6.4 Fe concentration in the muscle of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 6.5 Fe concentration in the liver of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 6.6 Cu concentration in the spleen of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 6.7 Cu concentration in the muscle of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 6.8 Cu concentration in the liver of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 6.9 Zn concentration in the spleen of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 6.10 Zn concentration in the muscle of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 6.11 Zn concentration in the liver of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

Fig.6.12 Effects on the red blood cell count in gilthead sea bream fed the experimental diets for 12 week ($10^5 mL^{-1}$). Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

Fig.6.13 Effects on the haematocrit in gilthead sea bream fed the experimental diets for 12 week. Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

Fig. 6.14 Effects on the haemoglobin in gilthead sea bream fed the experimental diets for 12 week. Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

Fig.6.15 The chemiluminescence in the blood of gilthead sea bream fed the experimental diets for 12 week. Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

Fig. 6.16 The antibacterial activity of serum in gilthead sea bream fed the experimental diets for 12 week. Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

Dedicated to my grandmother Anna

and the loving memory of my grandfather Alekos

Acknowledgements

The completion of my PhD degree is one of the most challenging and important activities I have done in my whole life so far. It was a long voyage full of adventure and discoveries. In addition I had the privilege to meet and work with a number of people that the least I could do is acknowledge their support and help and pay my gratitude to them.

First of all I would like to thank my supervisors Prof. Simon Davies and Dr. Ioannis Nengas. My first debt of gratitude must be offered to Prof. Simon Davies who believed in me and gave me the opportunity to start my academic life having a great advisor and an elite scientist beside me. Furthermore I would like to thank Prof. Davies for his support and to say that it was a true honor meeting him. Exceptional gratitude must be given to Dr Nengas who was beside me through my experiments helping and guiding me, not only as a great mentor but also as a good friend.

I would like to thank the people consisting the institutions of Plymouth University and Hellenic Centre of Marine Aquaculture. My friends and colleagues sharing the same office during the beginning of the PhD while I was in Plymouth. In addition, I would like to thank the UoP staff for their help and understanding while I was in Greece. A special thank to my colleagues in the H.C.M.R., Fotini Kokou, George Rigos, Morgan Henry and Antigoni Vasillaki who helped and supported me on a daily basis.

Moreover I would like to give my appreciation to Alltech and especially to John Sweetman for his trust and help at the beginning of my PhD

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

Work submitted for this research degree at the Plymouth University has not formed part of any other degree either at PlymouthUniversity or at another establishment

This study was financed with the aid of a studentship form the Natural Environment Research Council and carried out in collaboration with Plymouth Marine Laboratory.

A programme of advanced study was undertaken, which included a final year honours course in colloid chemistry, supervised information technology instruction and a postgraduate course on analysis and hazard assessment of marine pollution.

Relevant scientific seminars and conferences were regularly attended at which work was often presented; external institutions were visited for consultation purposes and several papers prepared for publication

Publications (or presentation of other forms of creative and performing work):

- *Samartzis, A. Nengas, I. Kokou, Davies, S. (2011)* Determination of the Organic iron levels and its bioavailability in semi-purified dietsfor the gilthead sea bream (*Sparus aurata*). *Aquaculture Europe 2011 (Oral Presentation)*
- Rigos, G. Samartzis, A. Henry M. Fountoulaki E. Cotou E. Sweetman J. Davies S. Nengas I. (2010) Effects of additive iron on growth, tissue

distribution, haematology and immunology of gilthead sea bream *Sparus aurata. Aquaculture Int. (Scientific Paper)*

- Rigos, G. Samartzis, A. Henry M. Fountoulaki E. Cotou E. Sweetman J. Davies S. Nengas I. (2010) Effects of additive iron on growth, tissue distribution, haematology and immunology of gilthead sea bream *Sparus aurata. EAFP International Conference (Poster Presentation)*

External Contacts:

Word count of main body of thesis: 33.216

Signed: ....

15/06/2014

Date: ....

Chapter 1 General Introduction

Over the last few decades aquaculture has developed into a highly productive and efficient industry (New, 1999). Up to 60% of the production costs in farms with Mediterranean species are due to feeds (Lucas & Southgate, 2003). This is why the research in the field of nutrition is so intense. Fish nutrition is one of the key factors for the economy of the farms due to the relation of fish health and growth. In particular, minerals are very important for the normal life processes of fish, and as a result, the determination of mineral requirement in cultured fish diets is a key factor. Minerals (or inorganic elements) are needed by animals in order to maintain many of the metabolic processes, and provide material for major structural elements. The minerals can be divided into two types; the major minerals, which are those required in large quantities in the diets of the fish, the main of which are: calcium, phosphorus, magnesium, sodium, potassium, chlorine and sulphur, and the trace minerals, which are required in trace amounts. Some trace minerals are iron, iodine, manganese, copper, cobalt, zinc, selenium, molybdenum, flurine, aluminium, nickel, vanadium, silicon, tin and chromium. The mineral requirements in the fish diets depend on the fish species. But generally the fish meals are rich sources of iron, magnesium, zinc, iodine, selenium, calcium and phosphorum and the plant meals contain manganese and copper (Lucas & Southgate, 2003).

1.1 The gilthead sea bream (*Sparus aurata*)

In taxonomical terms, gilthead sea bream belongs to the subphylum of *Vertebrata*, the class of *Osteicthyes*, the subdivision of *Teleostei*, the order of *Perciformes* and finally the family of *Sparidae* (Papanastasiou, 1976). It has an oval body, with a curved head profile, small eyes and a low mouth slightly oblique with thick lips. It has four to six canine-like teeth interiorly and two to four blunter teeth posterior in each jaw. It has an eleven spine dorsal fin and a three spine anal fin. There are 73 to 85 scales along the lateral line. Gilthead sea bream have a silvery grey colour with a black blotch at the origin of lateral line (Fig. 1.1) (Bauchot *et al.*, 1981; Pavlidis & Mylonas, 2011; FAO, 2010). The species is commonly found throughout the Mediterranean region, less frequently in eastern, south-eastern Mediterranean and rarely in the Black sea. It can also be found from the British Isles to Cape Verde and around the Canary Islands (Fig. 1.2) (Koutsogiannopoulos, 2010). Gilthead sea bream is a benthopelagic with deferral behaviour. It is a coastal species, inhabiting sea grass beds, sandy and rocky bottoms as well as in the surf zone in between 30 to 150 meters depth. The species are protandric (most of the individuals are first male and then become females). Spawning occurs from October to December. The maturity for males is reached at 1-2 years (20-30cm) and for females at 2-3 years (33-40cm). They are carnivorous species, mainly feed on molluscs, mussels, crustaceans and fish; although they can also be herbivorous in some cases (Koutsogiannopoulos, 2010; Papanastasiou, 1976; Pavlidis & Mylonas, 2011).

In aquaculture, they are fed with formulated 'dry' diets (pelleted feeds). The benefits of the pellet feeds are the lower dust content, the consistent pellet size, the

higher digestibility, the prolonged pellet integrity in water, the variable sinking rate, and finally, the ability to control the levels of a variety of ingredients like minerals (Lucas & Southgate, 2003). Over and above, feed benefits can reduce the problems with water quality, nutritional deficiencies and feed distribution (Barnable, 1986; Lucas & Southgate, 2003). Sea bream is of high economic importance especially for Greece, which accounts for more than half the European production. According to the Food and Agriculture Organization of the United Nations (2012) global production in 2011 was 110,000 mt. 93% of the gilthead sea bream harvest coming from aquaculture production while only the 7% coming from capture fisheries from Mediterranean countries. Gilthead sea bream is considered to be a traditional cultured species in Mediterranean countries, and especially in Greece where there is a close relation with the European sea bass (*Dicentrarchus labrax*). In Greek aquaculture industry the two species are cultured separately, although their production in most cases is undertaken in the same farms. Gilthead sea bream is an aquaculture species farmed intensively in sea cages at an average density of 15–25 Kg m^{-3} with a FCR (food conversion ratio) between 1.5 and 2.0 The culture period of the gilthead sea bream takes between 18 to 24 months (from larvae up to 400g fish, which is the market table size). However this varies with location and water temperatures. The commercial size of gilthead sea bream can vary from 250g to more than 1500g. The nutritional characteristics of the aquaculture sea bream diets are normally extruded pellets with a 45-50% protein and about 20% lipid, with fish meal and fish oil the main raw materials used in their diets in accordance with the most carnivorous marine fishes (Pavlidis & Mylonas, 2011; Petridis & Rogdakis, 1996; Mayer *et al,.* 2008; Lupatsch *et al.,* 2003).
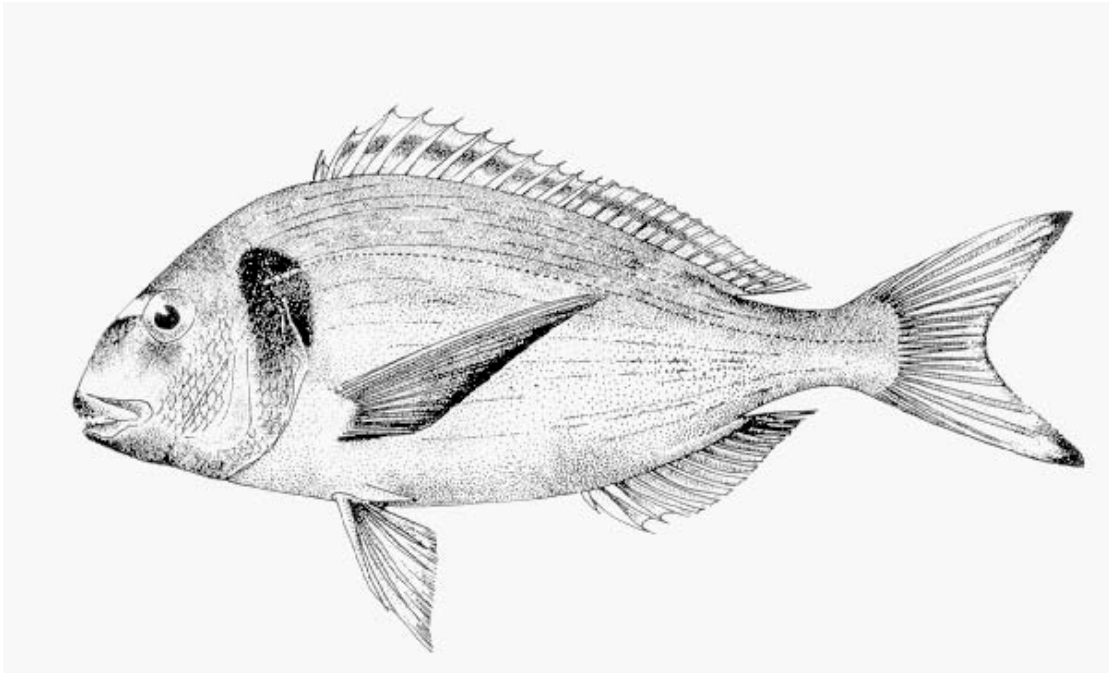
Fig. 1.1 Gilthead sea bream (available at www.FAO.org).
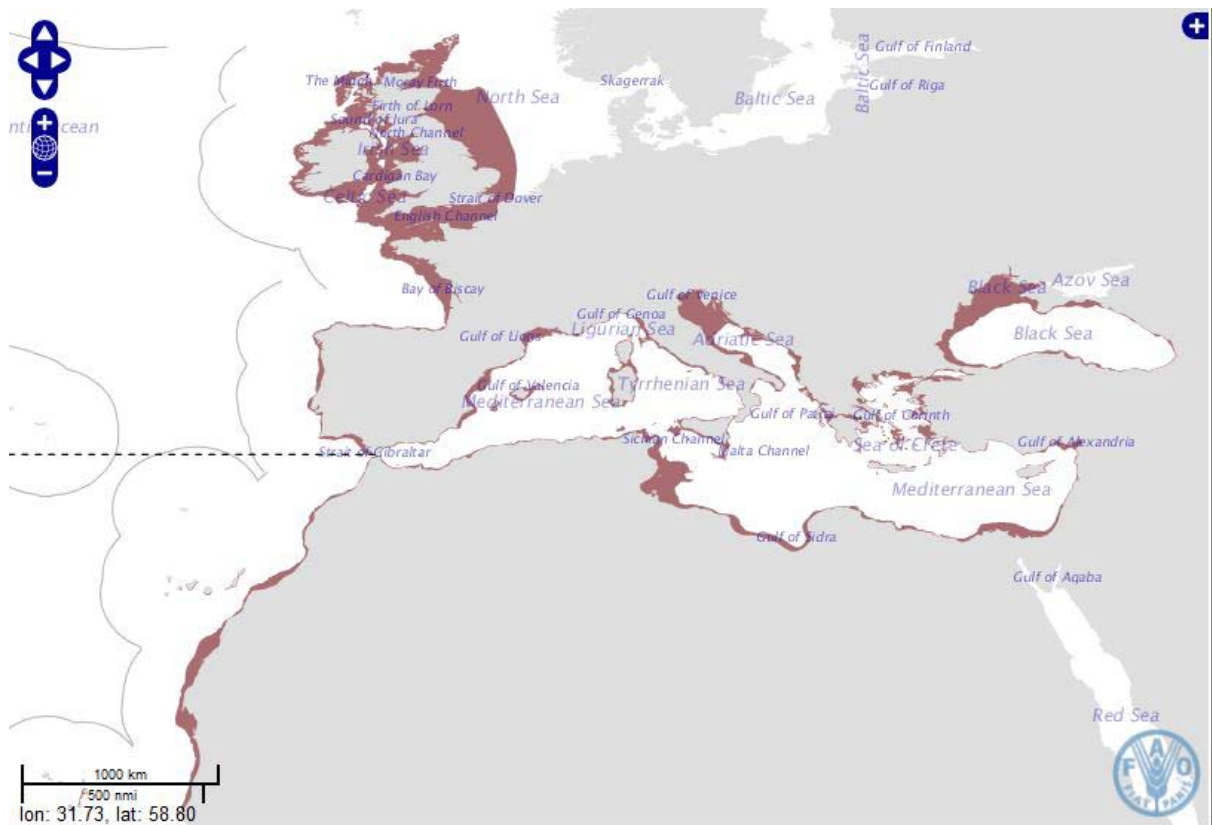
Fig. 1.2 Gilthead sea bream distribution (available at www.FAO.org).

1.2 Trace minerals

All animals need minerals or inorganic elements in their normal lives, in order to develop appropriately. In contrast to terrestrial animals, fish have the ability to absorb elements from their external environment and not only from their diets. The requirements of some of these essential elements, which are mainly absorbed from the external environment, are minimal. As a result, the artificial diets must be very precise and accurate, because it is difficult to formulate diets for species that live in an artificial environment that is limited in minerals. A great threat is mineral deficiencies that can cause health and growth problems (Halver & Hardy, 2002). The mineral functional forms and characteristic concentrations have to be maintained within narrow ranges of ordinary metabolic activities in the cells, and the tissues of the fish. The homeostatic mechanism facilitates this process in fish, by catering for the fluctuation in dietary intake. The minerals in fish diets are important for many reasons like, skeletal formation, maintenance of colloidal systems, regulation of acid-base equilibrium, and for biological important compounds, such as hormones and enzymes. On the other hand, mineral deficiencies can cause problems such as biochemical, structural and functional pathologies. The main factors that can cause these deficiencies depend on several factors, including the duration and degree of mineral deprivation (Watanabe *et al.,* 1997; Reilly, 2004; Halver & Hardy, 2002). Generally, the trace mineral requirements in fish are characterised by the small quantities that are required. In most cases, the requirements are less than 100 mg/Kg dry diet. The efficiency with which the body utilises the dietary minerals is the key factor for the biological availability of a mineral in the fish diets. Their variety depends on the feedstuff and the composition of the diets. The bioavailability can be

influenced by a number of factors like the level and the form of the nutrients, the size of the particle, the digestibility of the diet, the nutrient interactions (either synergistic or antagonistic), the physiological as well as the pathological conditions of the fish, the waterborne mineral concentrations and finally and more importantly the actual species (Watanabe *et al.*, 1997). The main trace minerals in fish diets are: iodine, manganese, copper, cobalt, zinc, selenium, molybdenum, flurine, aluminium, nickel, vanadium, silicon, tin and chromium and iron (Reilly, 2004). Iron, zinc and copper are discussed in detail below, with particular emphasis given on the iron.

1.3 Iron

Iron (Fe) is one of the most important trace minerals in fish. It has an active role in oxidation/reduction reactions and electron transport associated with cellular respiration (Watanabe *et al.*, 1997). Information on the iron requirements of fish and more specifically in absorption and metabolism is limited, and particularly in Mediterranean fish information is almost nonexistent. However, there are reports showing that species, like Atlantic salmon (*Salmo salar*) which has been studied extensively, has a limited capacity to regulate iron absorption and metabolism leading to excess iron in blood related organ tissues. Also, the excessive dietary iron intake may cause a heavy infestation with lice and winter ulcers (Andersen *et al.*, 1998; Lall, 2002). The requirements of this mineral have been determined for several species. The minimum dietary iron requirement has been reported to be between 60 and 100 mg/Kg in Atlantic salmon smolts (Andersen *et al.*, 1998), 30 mg/Kg in channel catfish (*Ictalurus punctatus*) (Gatlin & Wilson, 1986), 170 mg/Kg in

Japanese eel (*Anguilla japonica*) (Nose & Arai, 1979) and 150 mg/Kg in red sea bream (*Chrysophrys major*) (Sakamoto & Yone, 1976) (Table 1.1). Deficiency of iron induces haematological suppression, reduced growth and poor feed conversion in farmed fish (Tacon, 1992).

Table 1.1 Known Fe requirements of aquaculture species.

Species common name	Species scientific name	Fe requirements	Source
Atlantic salmon	*Salmo salar*	60-100 mg/Kg	Andersen *et al.,* 1996
Channel catfish	*Ictalurus punctatus*	30 mg/Kg	Gatlin & Wilson, 1986
Japanese eel	*Anguilla japonica*	170 mg/Kg	Nose & Arai, 1979
Red sea bream	*Pagrus major*	150 mg/Kg	Sakamoto & Yone, 1976
Gilthead sea bream	*Sparus aurata*	150-200 mg/Kg	Rigos et al., 2010

Iron is considered to be a fascinating challenge for nutritional scientist due to the existence of unanswered questions on how the body manages its iron econonomy, inspite of two centuries of scientific investigations (Buttriss, 2001). The iron chemistry is complex. Iron is one of the d-block transition elements and can exist in oxidation states ranging from -2 to +6. Although in biological systems, these are limited to the ferrous (+2), ferric (+3) and ferryl (+4) and it dissolves in acids to form

salts (Reilly, 2004). In humans there is about 50 mg of iron per kg of body weight, while 60% of it is present as haemoglobin in erythrocytes (red blood cells) and 10% in myoglobin of muscle. The final 30% occurs in a variety of iron-containing proteins such as the iron storage and transporting proteins, iron-sulfur enzymes and cytochromes (Reilly, 2004). The iron absorption from ingested feeds takes place in the proximal small intestine. The three stages of iron transfer from the lumen of the intenstine in to the mucosal cell and then on to the plasma pool starts with the uptake from the gut lumen across the pical mucosa, then the intracellular processing takes place an transported in the enterocytes and finally it is stored and extranterocyte transport across the basolateral membrane and then into the plasma. It should be mentioned that the regulation of dietary absorption of iron is considered the major factor in the maintenance of iron hemeostasis in the body (Pietrangelo, 1998; Reilly, 2004).

Iron is the second most abundant metal (following aluminium), in the earth's crust and it is included in almost all foods. Some key factors influencing iron absorption is: the proportion of organic and inorganic components of the diet, the amount increase and the conditions of the digestive tract. Iron present in foods in inorganic form or as iron-protein complex must be reduced to the ferrous state to be available for absorption (Ewing & Charlton, 2007; Lall, 2002). While, organic form of iron occurs in food in combination with proteins, such as hemoglobin, myoglobin and other complexes, it has been noted that the presence of reducing substances in the diet like ascorbic acid can enhance the ability of fish to absorb iron. Generally, the organic forms of iron can be absorbed better compared to the inorganic forms (Watanabe *et al.*, 1997). The iron deficiency can cause microcytic anemia, or even low haemaglobin levels in brook trout (*Salvelinus fontinalis*) and rainbow trout

(*Oncorhynchus mykiss*). The iron deficiency anaemia is the final stage in fish, of a progressive negative iron balance. There are two stages prior to the depletion of functional iron. The first is the depletion of iron stores in the spleen, liver and head kidney and secondly the diminished erythropoiesis leading to anaemia and reduced activity of the iron dependent enzymes. This leads to a negative iron balance, serum ferritin levels fall and storage iron is taken up by plasma transferring for delivery to iron requiring tissues, the saturation of plasma transferring is reduced below the 16% required for normal erythropoiesis resulting the beginning of iron deficiency erythropoiesis leading to anaemia (Beard *et al.,* 1996; British Nutrition Foundation, 1995). On the other hand, the iron deficiency cannot affect the growth of the fish. However, the major effect of iron toxicity includes reduced growth, poor feed utilization, feed refusal, increased mortality, diarrhoea and histopathological damage to liver cells (Lall, 2002).

1.4 Copper and Zinc

The nutritional importance and role of copper (Cu) was firstly identified in animals and especially in cattle in the early twentieth century, when observed a form of anaemia on the animals grazing on pastures with a low soil copper level and related to deficiency of the element (Reilly, 2004). Copper is an essential trace element for fish. It is involved in the activity of enzymes such as cytochrome oxidase, superoxide dismutase, lysyl oxidase, dopamine hydroxylase and tyrosinase. More over copper (copper-proteins and chelates) has a very important and vital metabolic

role. Some of the first studies in the subject using rats show that copper is necessary for haemoglobin formation (Harte *et al.,* 1928). In addition it was also shown that anaemia in humans was also considered to be related to copper deficiency (Mills, 1930). The majority of copper in the diets is in organic form, bound to proteins, amino acids and other large molecules while inorganic iron forms usually are absorbed through water. In humans, between 30-60% of ingested copper is absorbed while the efficiency of the absorption varies inversely with the levels of copper in the diet (Turnland *et al.,* 1989). The eyes are the area that copper can be found in high levels generally, along with melanins, bound to protein. In addition, other organs where copper is found in comparatively large amounts are the liver, the brain and the heart. The absorption of ingested copper is known to compete with other divalent metals in the lumen of the gut. High levels of dietary zinc are known to be antagonistic to copper absorption. In animal studies iron was observed to interfere with copper absorption in those fed with a pre-existing low copper diet. While in human, infants fed iron-enriched diets absorbed less copper compared to the ones fed a lower iron diet (Reilly, 2004; Haschke *et al.,* 1986; Boobis, 1999). In cases of copper toxicity, it can cause damages to the gills and it is possible to cause necrosis to the liver and the kidney of the fish (Watanabe *et al.,* 1997; Lall, 1989). According to Murai *et al.* (1981) the high levels of copper in the diets of Channel catfish can depress growth and impair feed conversion. Further studies show that a deficiency of cooper can affect the activities of cytochrome c oxidase in heart and copper-zinc superpxide dismutase in liver. The optimum level of copper in the diets of finfish which is applied in many fish ranges from 3 to 5 mg of copper per kg of diet (Watanabe *et al.,* 1997).

Zinc (Zn) is one of the main trace elements in the nutrition of fish due to its involvement in various metabolic pathways and its ubiquitous in the cellular metabolism. Mainly it works as a specific cofactor of several enzymes. Moreover zinc is an integral part of about 20 metalloenzymes. The main ones are alkaline phosphatase, alcohol dehydrogenase and carbonic anhydrase. There are more than 200 zinc enzymes known, of which more that 25% play metabolic roles in animals. Important functions of zinc are the connection with prostaglandin metabolism and in some cases the structural role in nucleoproteins. Research on zinc-gene interactions has assigned a basic role for this element in order to control the growth of the fish (Chesters, 1991). The absorption of dietary zinc from the feeds is determined, partly by the chemical form of the element and more importantly by the presence or absence of other components in the diets that can either inhibit or enhance uptake (Reilly, 2004). Because of the zinc ubiquity in the environment and in the ingredients of feeds, the possibility of zinc deficiency to cause significant problems to fish is very low. Due to the many important roles of zinc in a great variety of metabolic activities and its wide distribution in tissues and in every cell of the body, possible zinc deficiencies can have wide range of consequences. Many zinc deficiencies are difficult to identify due to the overlapping with the effects of deficiencies of other nutrients especially in cases of mild zinc deficiencies (Reilly, 2004). In aquaculture the artificial environment that fish are cultivated, zinc deficiency can cause cataract disease in the eye lens of the fish as well as growth depression, high mortality, short body dwarfism and erosion to the fins and the skin of the fish. Generally the optimum level of zinc in the diets of fish ranges from 15 to 40 mg of zinc per Kg of diet (Lall, 1989).

1.5 Aquaculture stressors

In aquaculture, one of the most important considerations in relation with the successful husbandry of fish, is stress. The development of modern aquaculture practises raise an increasing concern about stress shown in fish farms, because stressors are almost unavoidable in intensive aquaculture practices (Fagerlun, 1995). According to Brett (1958), stress can be defined as 'a state produced by an environmental or other factor which extends the adaptive responses of an animal beyond the normal range or which disturbs the normal functioning to such an extent that, in either case, the chances of survival are significantly reduced'. The individual fish as well as the fish population can be affected by stress at all levels of organization, from the biochemical perturbations and even changes in community structure. As a result the detrimental stress effects can be considered either as direct effects when they are manifested at the whole-organism or suborganismally level or as indirect effect when operating in population may exert their influence on organism-dependent energy or trophic pathways (Adams, 1990a, b). In finfish aquaculture it is usually the important effects of stress are more direct and involve an acute physiological response to the aquaculture related stressor or stressors. On the other hand the indirect effects have a high ecological relevance to fish communities and are more difficult to define and measure (Iwama *et al.,* 2012). There is a number of culture practises in commercial aquaculture that usually impose stress to the cultivated species, some are handling, sorting, grading, transport and water quality (Tort *et al.,* 2011; Schreck, 1982; Barton & Iwama, 1991). These stressors can result in massive mortalities in severe stress cases while sublethal stress can compromise various physiological and behavioural functions, it can also lead to suppressed

disease resistance and growth rate which in all cases leads to suboptimal production (Iwama *et al,.* 2012). Consequently, the stressful situations that the fish experience in commercial aquaculture after exposed to either abiotic or biotic noxious stimuli are in many cases closely related with welfare in fish and good husbandry practises (Tort *et al.,* 2011). Fish welfare is not a very easy term to define. It was often used in literature, where the concept of welfare status should mean a lack of stress and vice versa. However there is no justification for the assumption that the expression of stress situations can always compromise the fish health (Korte *et al.,* 2007). In Aquaculture all stages of the production cycle of intensive rearing of fish are exposed to a number of disturbances. Two of the most common ones are the overcrowding and the transport between units, usually with nets. These disturbances may cause physical injuries, stress and impair fish quality, health and welfare. When fish handled by skilled personnel following established protocols as well as emphasising in actions that will prevent the above, their welfare and health will improved in the long run (Tort *et al.,* 2002, 2003). So it is considered that, iron's active role in oxygen transportation can prevent stressors arise due to poor husbandry and management in oxygen deprivation disturbances.

1.6 The aim of the current study

The current study was designed in order to identify the optimum organic iron levels in the diet of gilthead sea bream and consequently to evaluate its effect on a series of commercial aquaculture parameters affecting the Greek sector. These were

the comparison with inorganic iron forms that are widely used in the Greek aquaculture fish feeds due to its inexpensiveness, the formulation of the three experimental diets supplemented with different levels of organic iron and the effects on gilthead sea bream exposed to two specific oxygen deprivation stressors associated with poor husbandry (overstocking and netting) common in aquaculture practices and finally the interactions of organic iron with two organic forms of zinc and copper (essential and available in fish feeds) in the diets of gilthead sea bream. The experimental approach containing the previously mentioned (four) experimental feeding trials in gilthead sea bream, focuses on the selected trace element (organic Iron) will be conducted and described in detail, namely:

The first experiment was designed to give an understanding in the determination of the optimum level of organic iron in commercial gilthead sea bream diets in relation to growth, mortalities, muscle, liver and spleen iron concentration, as well as hematocrit, hemoglobin and red blood cell count along with the immunological parameters. It will be a dose response experiment using commercial type semi purified diet formulations. Preliminary laboratory experiments will take place in order to find the appropriate protein with the minimum iron amount to identify the nutritional organic iron requirements of sea bream. The treatment level will include seven experimental diets, a Control (no addition of organic iron), OFe50 (50 mg/Kg addition of organic iron), OFe100 (100 mg/Kg addition of organic iron, OFe150 (150 mg/Kg addition of organic iron), OFe200 (200 mg/Kg addition of organic iron), OFe250 (250 mg/Kg addition of organic iron), OFe300 (300 mg/Kg addition of organic iron), OFe600 (600 mg/Kg addition of organic iron).

The second experiment will be conducted in order to attempt to investigate the possible differences between organic iron and inorganic iron (ferrous sulphate

and ferrous carbonate). According to Watanabe *et al.* (1997), the major factor that can influence iron absorption is the relative proportion of organic and inorganic forms of the metal in the diet, so that organic iron forms can be absorbed more efficiently compared to inorganic forms. The treatments will test a suggested organic iron level as control, three diets of each commercial source at 50 mg/Kg lower than the suggested, the suggested, and 50 mg/Kg higher than the suggested iron level respectively.

The objective of the third experiment is to investigate the organic iron requirements in gilthead sea bream under the exposure of two oxygen deprivation stressors (overstocking, netting) which are very common in culture conditions. Intensive culture is closely related to a number of husbandry procedures that can induce a number of stressors in fish. Typically, chronic and acute oxygen deprivation stressors are a common state that fish can endure under culture conditions (Iwama *et al.,* 2012). It is feasible that iron status in fish such as the sea bream may have a prevention role to these harmful for the fish health conditions. The experiment aims to evaluate the effects of three levels of organic iron in the tissue concentration, growth, haematological and immunological parameters in the fish exposed to both acute (netting) and chronic (overstocking) oxygen deprivation stressors. The three dietary treatments will be the suggested level of organic iron supplementation (150 mg/Kg) as control the OFe50 (100 mg/Kg organic iron lower than the suggested) and the OFe250 diet (100 mg/Kg organic iron lower than the suggested).

In the final experiment the aim was to investigate the possible interactions between organic Fe with organic forms of dietary Zinc and Copper in the diets of gilthead sea bream. Zinc (Zn) is also a trace mineral that can function either synergistically or antagonistically to Fe. In this respect, low dietary Fe status can

result in the elevation of Zn absorption, or if there is an excess of Fe in the diet this can induce Zn deficiencies in fish with resulting pathological results. Moreover, excess zinc can influence Fe utilisation leading to the induction of anaemia in fish. High dietary levels of Copper (Cu) can also decrease Fe absorption in the intestine on the other hand dietary Fe excess can induce Cu deficiency and affect its availability in the diet (Bodiga & Krishnapillai, 2007; Ewing & Charlton, 2007; Reilly, 2004; Watanabe *et al.*, 1997). The dietary experiment will have five semi-purified diets (Control,OFeCuMin, OFeCuMax, OFeZnMin, OFeZnMax). The control diet will have the optimum levels of organic Fe, Zn and Cu, while the rest will consist of the lower possible levels of Cu and Zn (OFeCuMin, OFeZnMin) and the highest allowed levels by legislation (with some modifications based on the Greek aquaculture practises) of Cu and Zn respectively (OFeCuMax, OFeZnMax).

Chapter 2 General materials and methods

2.1 Growth and performance

In order to evaluate the growth response of the gilthead sea bream used in the experiments, the supplementation of organic dietary Fe in their diets the specific growth rate (SGR) and feed conversion ratio (FCR) were calculated. Both parameters were calculated as described in the formulas below. Finally, the mortality rate of the fish was calculated in order to give a percentage of the mortalities occurred in the experiment. The formulas used are presented below.

$$\text{SGR (\%/day)} = \left(\frac{\text{ln Final weight} - \text{ln Initial weight}}{\text{Total Days}}\right) \times 100$$

$$\text{FCR} = \frac{\text{Dry weight of feed consumend (g)}}{\text{Wet fish weight gain (g)}}$$

$$\text{Mortality rate (\%)} = \left(\frac{\text{Initial number of fish} - \text{Final number of fish}}{\text{Initial number of fish}}\right) \times 100$$

2.2 Hematological analysis

The methodologies used to analyse the haematological values were based on standard method of analysis (Blaxhall and Daisley 1973; Lojek et al. 1997). Any anesthesia occurred before blood sampling is described in detail in the specific methodology chapter. The blood was drawn from the caudal vein using a 25 gauge needle and 1 ml syringe.

2.2.1 Hemoglobin

From each individual fish specimen 20uL of heparinised blood was sampled and diluted in 5 ml of Drabkin's reagent (Sigma, Greece). The Drabkin's reagent dilution consist 1g of Sodium bicarbonate 50mg of Potassium cyanide 200 mg of Potassium ferricynida and 1L of dH_2O. Then they were mixed thoroughly by gentle inversion and allowed to stand for at least 15 minutes for full conversion of haemoglobin (Hb) to cyanomethaemoblobin. Then 200uL was distributed in triplicate wells of a 96-well microplate. Absorbance was read at 540nm using as blank 200uL drabkin's reagent alone. Hb concentration was expressed as g of hemoglobin per diluted blood and was deduced from a standard curve. Standard curve was calculated by absorbance at 540nm using a solution of 45mg of bovine hemoglobin per mL of Drabkin reagent measured in a number of different concentrations (160uL, 2mL and 1mL of bovine Hb solution diluted in 10mL, 1mL and 2mL of Drabkin reagent, respectively).

2.2.2 Red blood cell count

The methodology followed in order to measure the red blood cell count (RBC) in the gilthead sea bream blood was based on the model introduced by Blaxhall (1972). Heparinised blood was diluted 1:500 in Dacie's solution and 20uL of suspension were introduced in a Neubauer counting chamber and the cells were counted in 5 squares (all corners and central square) of the counter by eye using a

mechanical cell counter under the optical microscope. Then the concentration per ml of blood was calculated.

2.2.3 Hematocrit

The well mixed blood was drawn into a microhaematocrit tube (Hawkesley Ltd) 75mm long heparinised (Art No 110.690 Kebo-Lab) filled to less than 3/4 and one end was sealed with plasticine. Then the tube was centrifuged in a microhaematocrit centrifuge (HETTRICH Micro 12-24) at 6000rpm for 10 minutes. The readings were made with the aid of a microhaematocrit reader and expressed as the volume of the erythrocytes per 100cm^3.

2.3 Tissue concentration analysis

There were three specific organs used for the measurements of Fe concentrations. The selected tissues were the liver, the spleen and the muscle of the gilthead sea bream species used in the experiments. For the liver and spleen sampling the whole organs were used, while for muscle a sample (without skin or bones) from above the lateral line was collected. Approximately 500 mg of sample accurately weight was placed in Teflon vessels, specially designed for microwave oven and 5mL of HNO_3 (65%) suprapure and 1mL of H_2O_2 (30%) were added. The samples were digested with MARS X-Press (CEM Corporation, NC, USA) microwave oven with a preselected program (Table 2.1). After closed vessel

microwave digestion, they were diluted to a final volume of 50mL with ultrapure water.

Table 2.1 Digestion preselected program.

	Power (W)		Ramp time (min)	^{0}C	Hold time (min)
Stage	Max	%			
1	1600	100	2	165	0
2	1600	100	3	175	5

Fe was determined by electrothermal atomic absorption spectrometry with longitudinal Zeeman-effect background correction. Quantification was performed with matrix-matched calibration curves. The calibration curves were constructed by injecting 20uL of the sample into the graphite tube together with 20uL of four standard solutions containing 10-20-40-50ug/L or blank solutions and 5uL of matrix modifier (Mg 50ug). Temperature programme for the determination of Fe using THGA graphite tube is shown in Table 2.2.

Table 2.2 Temperature program for the determination of Fe.

Step	τ (^{0}C)	Ramp time (sec)	hold time (sec)	Gas flow (mL/min)
1	110	1	15	250
2	130	15	25	250
3	1400	10	20	250
4	2300	0	5	0
5	2500	1	3	250

2.4 Immunological analysis

2.4.1 Antibacterial activity of serum

The antibacterial activity of serum was assessed against a strain of *Escherichia coli* transformed with a luciferase gene as modified by Nikoskelainen *et al.* (2002). Fish serum were diluted with Phosphate Buffer Saline (PBS) and 50 mM $Mg^{2+}Ca^{2+}$ (Mg Ca, pH 7.4) and increasing amount (0-100uL) of the diluted serum were added to 12 wells of a white 96-wells flat-bottomed microplate (Nunc) giving final serum concentration ranging from 0 to 40uL / mL for each fish. The volume was adjusted in each well to 100uL with PBS and Mg Ca so that equal amount of Mg Ca was present in all wells. Bacteria (*E. coli* K12pEGFLPLucTet; kindly provided by S. Verho, University of Turku, Finland) were grown overnight to log-phase at 37 ^{0}C in LB-broth containing 10ug tetracycline/ml, and bacterial concentration was then adjusted to OD_{450nm} of 0.1 and 50uL were added to the sera. A 3h - incubation at 23

^{0}C allowed bacterial killing. 100uL of D-luciferin (Synchem, Germany) at 0.5 mM (in 0.1 M citrate buffer, pH 5.0) were added to each well and the emitted luminescence (RLU) was measured by GeniosPro luminometer (Tecan, Austria) was proportional to the number of live bacteria. Curves representing RLU as a function of serum concentration (uL /mL) starting by a plateau and rapidly decreased to a final plateau were plotted using the Origin software, and IC50 (concentration of serum giving 50% of bacterial killing) were calculated and bactericidal activity of serum in Units/ml was deduced (1000/IC50).

2.4.2 Chemiluminescence

The induction of the respiratory burst (RB) activity in blood leucocytes was measured directly from heparinized blood following the method described by Nikoskelainen *et al.,* (2002), with some modifications. There were 275 μL of diluted blood (1:200) in g HBSS-hep-luminol (Hanks' Balanced Salt Solution containing 0.1% gelatin, 5U/mL of heparin, 100 I.U./mL of penicillin streptomycin, 5ug/ mL of oxytetracycline, 0.19mM luminol, pH 7.4) were dispensed in triplicate wells of a white flat-bottomed 96-well plates. In addition the background luminescence was allowed to stabilise for 10 min. The reaction started with the addition of 25uL of zymosan at 5mg/mL or PMA at 1ug/mL in each well and luminescence was recorded for 2 h every 3 min at 23 ^{0}C. The peak chemiluminescence was determined and expressed in relative luminescent units (RLU).

Chapter 3 Determination of organic iron requirements of gilthead sea bream in commercial type diets.

3.1 Introduction

Fish need minerals in their normal lives in order to develop appropriately. They can either absorb elements from their external environment or their diets. As it is suggested, these essential elements are mainly absorbed from the diets (Halver & Hardy, 2002).The minerals in fish diets are important for many reasons like, skeletar formation, maintenance of colloidal systems, regulation of acid-base equilibrium and for biological important compounds such as hormones and enzymes (Lall, 1989). Iron (Fe) is one of the most important trace minerals in fish. It has an active role in oxidation/reduction reactions and electron transport associated with cellular respiration (Watanabe *et al.,* 1997; Reilly, 2004; Lee *et al.,* 1981). Information in iron requirements in fish and more specific in absorption and metabolism is limited and as far as Mediterranean fish are concerned, there is almost no information at all (Rigos *et al.,* 2010; Lall, 1989). However, there are reports showing that species, like Atlantic salmon which have been studied a lot, have a limited capacity to regulate iron absorption and metabolism leading to excess iron in blood tissue. Also, the excessive dietary iron intake may cause a heavy infestation with lice and winter ulcers (Andersen *et al.,* 1998; Lall, 2002). Tacon (1992) observed that iron deficiency can induce haematological suppression, reduce growth and lead to poor feed conversion in farmed fish. Studies show that lack of dietary iron can cause microcytic anaemia or even low haemaglobin levels in red sea bream, Japanese amberjack

(*Seriola quinqueradiata*), Japanese eel, channel catfish, Atlantic salmon and brook trout (Kawatsu, 1972; Andersen *et al.,* 1996; Gatlin & Wilson 1986; Nose & Arai, 1979; Ikeda *et al.,* 1973; Rigos *et al.,* 2010). However, the iron deficiency is not considered to affect the growth of the fish. Although, the major effect of iron toxicity includes reduced growth, poor feed utilization, feed refusal, increased mortality, diarrhea and histopathological damage to liver cells (Lall, 2002). The requirements of this mineral have been determined for a number of species. The minimum dietary iron requirement has been reported to be around 150 mg/Kg for red sea bream (Sakamoto & Yone, 1978a), between 60 and 100 mg/kg in Atlantic salmon (Andersen *et al.,* 1996), at 30 mg/Kg in channel catfish (Gatlin & Wilson, 1986) and 170 mg/Kg in Japanese eel (Nose & Arai, 1979).

In late '00 Greek aquaculture sea bream production suffers mortalities from a gill parasite (*Microcotyle* spp.), which caused anaemia and substantial losses in the cultured population (Athanassopoulou *et al.,* 2005). Several small scale industrial results show that the incorporation of organic iron in the diet of gilthead sea bream had an improvement of fish blood parameters (Nengas personal communication 2007). Consequently the determination of the optimum level was a necessity since an increased interest occurred in the Greek aquaculture sector in using iron supplementation in gilthead sea bream feeds in order to improve the fish health and disease resistance.

The aim of the study was to determine the optimum level of dietary organic iron for cultured sea bream in relation to growth, mortalities, iron concentration in selective tissues (muscle, liver and spleen), hematocrit, hemoglobin and red blood cell count as well as selected immunological parameters.

3.2 Materials and methods

3.2.1 Experimental conditions

The feeding trial was conducted in Greece, at the aquaculture laboratories of the Hellenic Centre of Marine Research (H.C.M.R.), in Athens. Gilthead sea bream juveniles of an average 14g in weight were obtained from a local farm. The fish were acclimatized for 4 weeks period fed in an unsupplemented commercial type diets (without Fe addition in the diet, while using the composition of the types and quantities of the ingredients widely used in the Greek aquaculture industry for gilthead sea bream production) in order to reduce the body stores of iron (Baker, 1986). All fish within the system were graded to ensure uniformity of fish size at the start of the trial period. Air was supplied by a liquid oxygen tank at a level of 7 ppm ±0.5. Triplicate groups of 50 gilthead sea bream were distributed randomly in 21 fibre glass tanks (150L) using an open flow system with a flow rate around 1.7L per minute. The photoperiod was 10:14, light:dark starting at 8.00 in the morning and ending at 18.00 in the afternoon. The water temperature was maintained at 21°C ±2 and the salinity of the water in the experimental tanks was 38‰.

3.2.2 Diet formulation

The experimental treatments were seven practical diets prepared in the facilities of H.C.M.R., containing fish meal, soybean meal, wheat meal and casein as main ingredients (all ingredients were provided by Biomar Hellas, while casein was provides by Sigma Hellas). Diets were supplemented with four levels of organic iron, at 50, 100, 150, 200 300 and 600 mg/Kg diet inclusion level. A control diet was also

used where no added iron was included in the premix. The organic iron source is presented in Appendix 1. The triplicate gilthead sea bream groups fed on the experimental diets in a constant level close to satiation. Satiation was determined as the point where fish stopped feeding actively. In all cases the daily feed consumption was strictly monitored and recorded. The experiment lasted for a period of 12 weeks. Before the beginning of the experiment the fish pellets were crumbled and sieved of the appropriate size required for the fish specific life stage (between 3 and 4 mm). Before the beginning of the experiment an extended analysis took place. The analysis was focused in the iron concentration in each individual ingredient that would potentially consist the gilthead sea bream diets. For each diet the organic iron amount was added in order to meet the levels of the six treatments with the tested mineral (the control diet did not have any organic iron addition), at the levels ranging from 50 mg/Kg up to 600 mg/Kg. The pre-existing iron in the diet ingredients was quite high at around 62 mg/Kg but in was the lower possible regarding keeping the experimental diet as similar as possible to the commercial type fish feeds used in the Greek aquaculture sector for the production of sea bream. A 5% mineral fee casein from bovine milk used in the diet in order to lower the amount of fish meal and soybean meal which were high in iron and increase the protein levels in the diet. Table 3.1 shows the type and the percentage of each ingredient used for the formulation of the seven experimental treatments. Table 3.2 demonstrated the total iron (both the iron found in the ingredients and the addition of the organic iron) concentration found in each experimental diet. Apart from the organic Fe inclusion, the experimental diets were identical and as a result the chemical composition (%) was the same for all seven of them. The experimental diets had levels of crude protein at 47.2±0.7%, crude fat at 17.5±0.4% and ash at 6.9±0.4%.

Table 3.1. The composition of the seven experimental diets fed to gilthead sea bream for 12 week (%).

	Control	OFe50	OFe100	OFe150	OFe200	OFe300	OFe600
		Iron	Iron	Iron	Iron	Iron	Iron
Fish meal	15	15	15	15	15	15	15
Soybean meal	15	15	15	15	15	15	15
Wheat meal	7	7	7	7	7	7	7
Wheat gluten	40	40	40	40	40	40	40
Fish oil	16	16	16	16	16	16	16
Casein	5	5	5	5	5	5	5
Premix	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Cellulose	1.7	1.6	1.6	1.6	1.5	1.7	1.1
Organic Iron	0	0.05	0.1	0.2	0.2	0.3	0.6
Total	100	100	100	100	100	100	100

Table 3.2 The iron concentration of each of the seven experimental diets.

	Control	OFe50	OFe100	OFe150	OFe200	OFe300	OFe600
Fe concentration (mg/Kg)	62.17	108.92	174.23	217.81	278.46	370.49	643.04

3.2.4 Sampling

At the end of the experiments the total number of the fish of each tank were collected, anesthetised in phenoxyethanol at 50 uL/L for 5 minutes, counted and weighted. From each experimental tanks six samples (18 in total for each experimental diet group) were collected for haematological (haematocrit, haemoglobin and red blood cell count) and immunological analysis (respiratory burst

activity and serum antibacterial activity) from the caudal vein using 1 ml syringe. For the concentration analysis in the selected tissues [muscle (above the lateral line), liver and spleen] a total number of 10 samples from each tank were collected (thirty in total for each experimental diet group). Finally the FCR and SGR of the fish were evaluated by calculating the formulas shown in section 2.1.

3.2.5 Data analysis

The evaluation of the data collected from each measured parameter was statistically analysed using the software SPSS 11.5.1. The triplicate tanks for each experimental diet were pooled for each parameter analysed. Statistical analysis between dietary groups was performed by One-Way ANOVA followed by a Tukey's multiple range comparison test. In addition some secondary tests, such as normality and post hoc tests, have been performed. The level of significance for all tests was set at $p < 0.05$. Finally the graphics prepared in order to present the findings, were created using the Microsoft excel software.

3.3 Results

3.3.1 Growth

The results regarding the gilthead sea bream groups fed the experimental diets growth performance is presented in detail below (Fig 3.1, 3.2). There was no significant difference in the fish groups fed each of the experimental diets in the final

weight of the fish (p=0.218). Similar results occurred to the SGR rate (p=0.187) calculation where the SGR was not affected by the different iron supplementation in the seven experimental diets. The Fig. 3.1 and 3.2 demonstrate the initial and final weight as well as the SGR and FCR (p=0.316) of the fish fed the experimental diets for 12 weeks, from April until July. In the current experiment there were no mortalities occurred in any of the treated groups.

Fig. 3.1 The initial and final weight of the gilthead sea bream fed the experimental diets for 12 weeks. Values are the means (n = 30) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 3.2 The SGR and FCR of the gilthead sea bream fed the experimental diets for 12 weeks. Values are the means (n = 30) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

3.3.2 Iron tissue concentration

For the tissue concentration analysis the gilthead sea bream organs that were analysed were the liver, the spleen and the muscle of the fish. Fig. 3.3, 3.4, 3.5 presents graphically the concentration of iron found in the selected tissues of each fish group fed on the experimental diets. The fish fed the different level of added organic iron show no significant differences in the iron tissue concentration in each of the three organs. The Fe concentration in the muscle of the gilthead sea bream fed of experimental diets has a p value of 0.614. The statistical analysis on the Fe concentration on liver and spleen samples of the same fish had p values 0.354 and 0.379 respectively. However in all analysed tissues there was a gradual increase in the iron concentration from the control diets to the diets between OFe150 and

OFe200 diets where reached the higher level and then slightly decrease (muscle, liver) or stays in almost similar levels until the OFe300 and OFe600 diets (spleen).
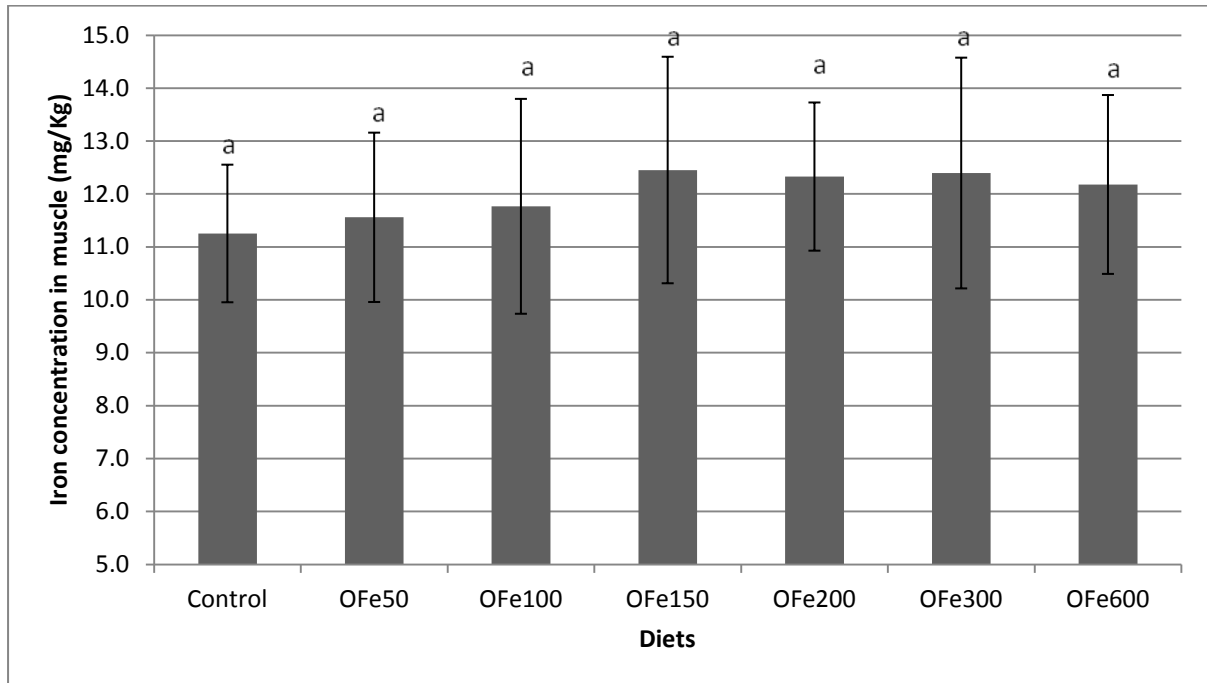
Fig. 3.3 The iron concentration in the muscle of the gilthead sea bream fed the experimental diets for 12 weeks. Values are the means (n = 30) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.
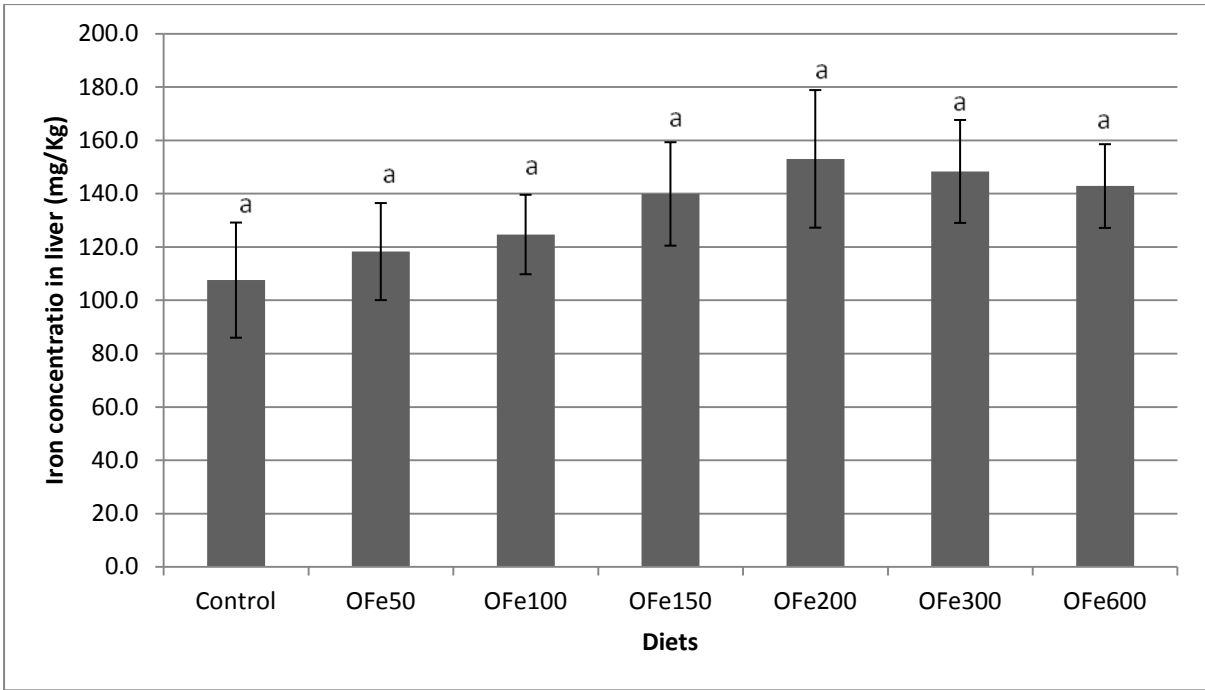
Fig. 3.4 The iron concentration in the liver of the gilthead sea bream fed the experimental diets for 12 weeks. Values are the means (n = 30) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.
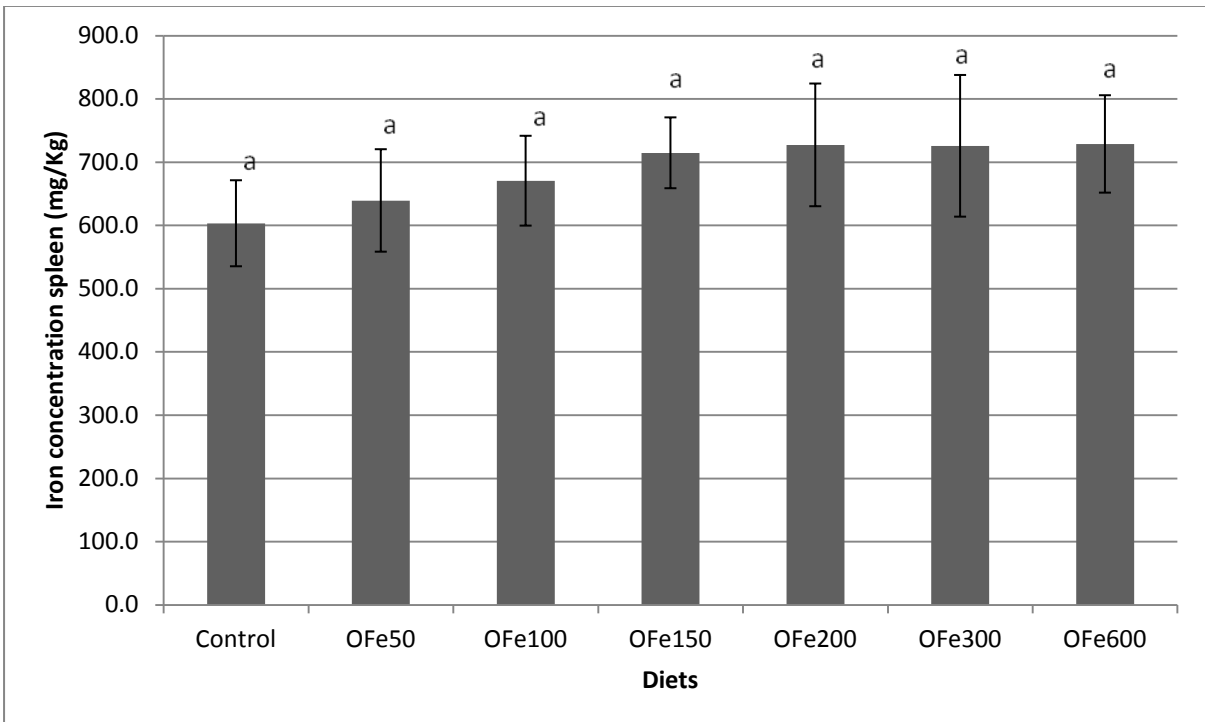
Fig. 3.5 The iron concentration in the spleen of the gilthead sea bream fed the experimental diets for 12 weeks. Values are the means (n = 30) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

3.3.3 Haematological analysis

The haematological parameters that were analysed were the haematocrit (Htc), the haemoglobin (Hb) and finally the red blood cell (RBC) count. In all three analyses there was no significant difference on the values on the fish blood. As a result the gilthead sea bream groups were not affected by the different organic iron levels in each of the seven experimental diets. Even there was no significant difference in the haematological analysis ($p>0.05$) it is worth mentioning some noticeable trends in the values presented. In all the haematological analysis results there is an incensement in the values of the fish groups as the addition of organic iron was increased in the diets. The RBC count ($p=0.062$) showed an increasing trend as the addition of organic iron increased in the diets. While the haematocrit had $p=0.411$ and the haemoglobin had a $p=0.392$. Fig. 3.6, 3.7 and 3.8 present graphically the haematocrit, haemoglobin and RBC counts in the gilthead sea bream fed the experimental diets for 12 weeks.
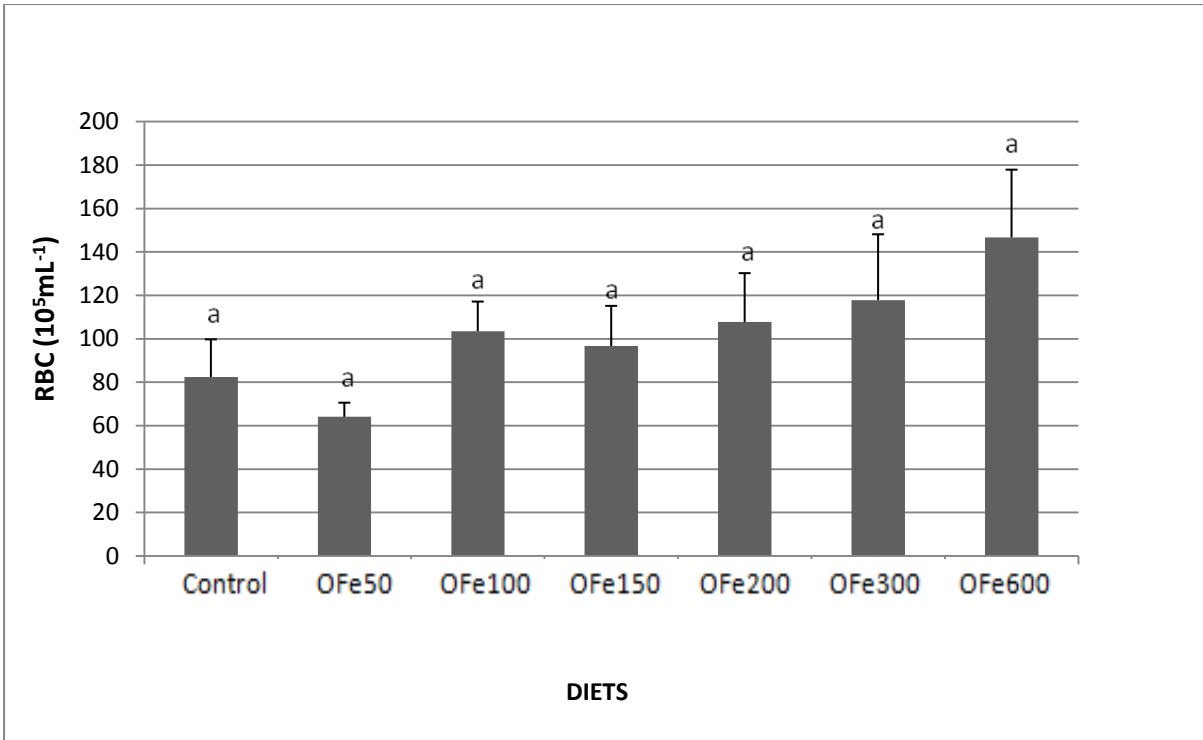
Fig. 3.6 The RBC counts on the gilthead sea bream fed the experimental diets for 12 weeks (10^5mL^{-1}). Values are the means (n = 18) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.
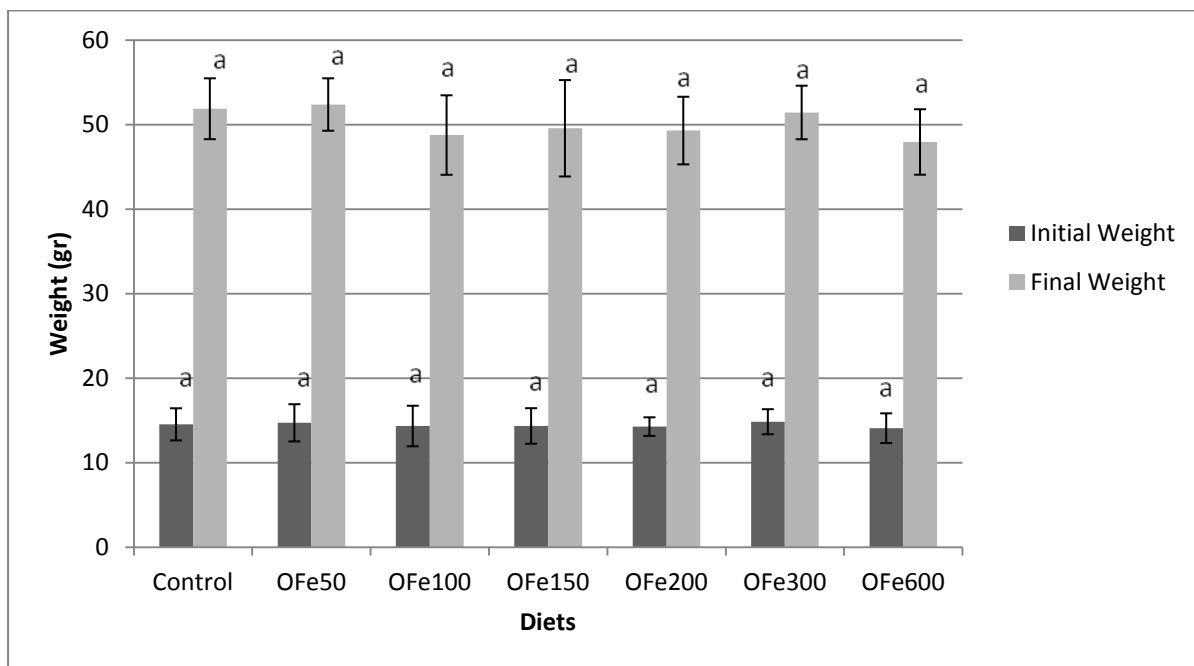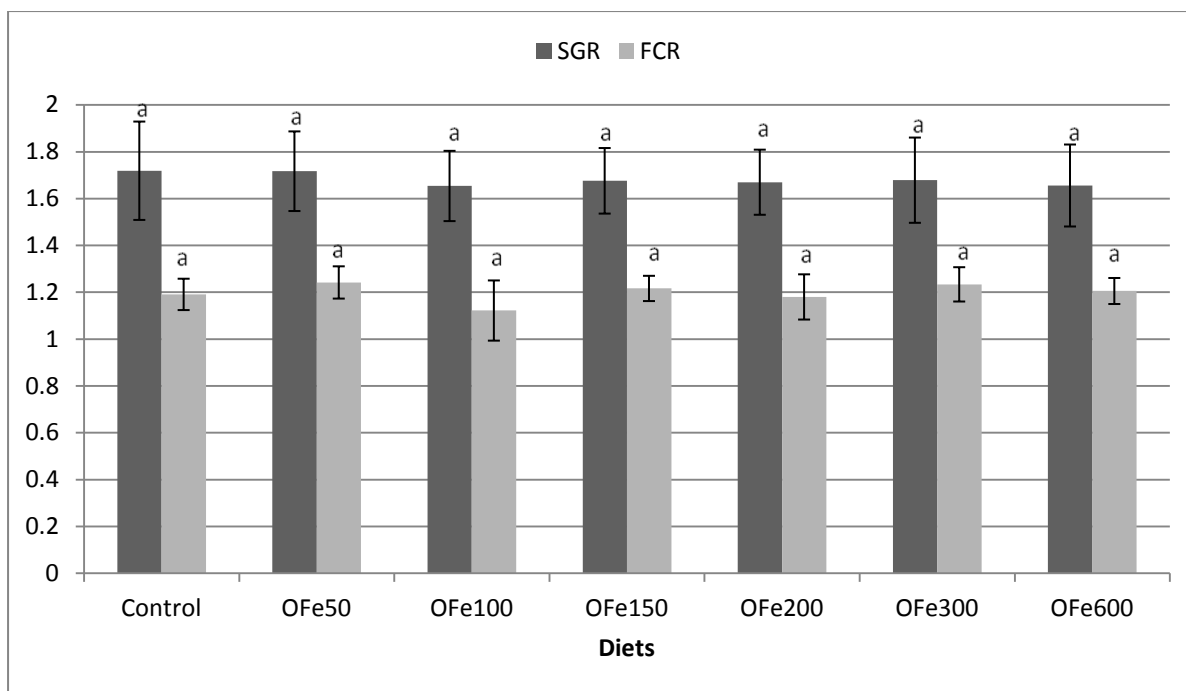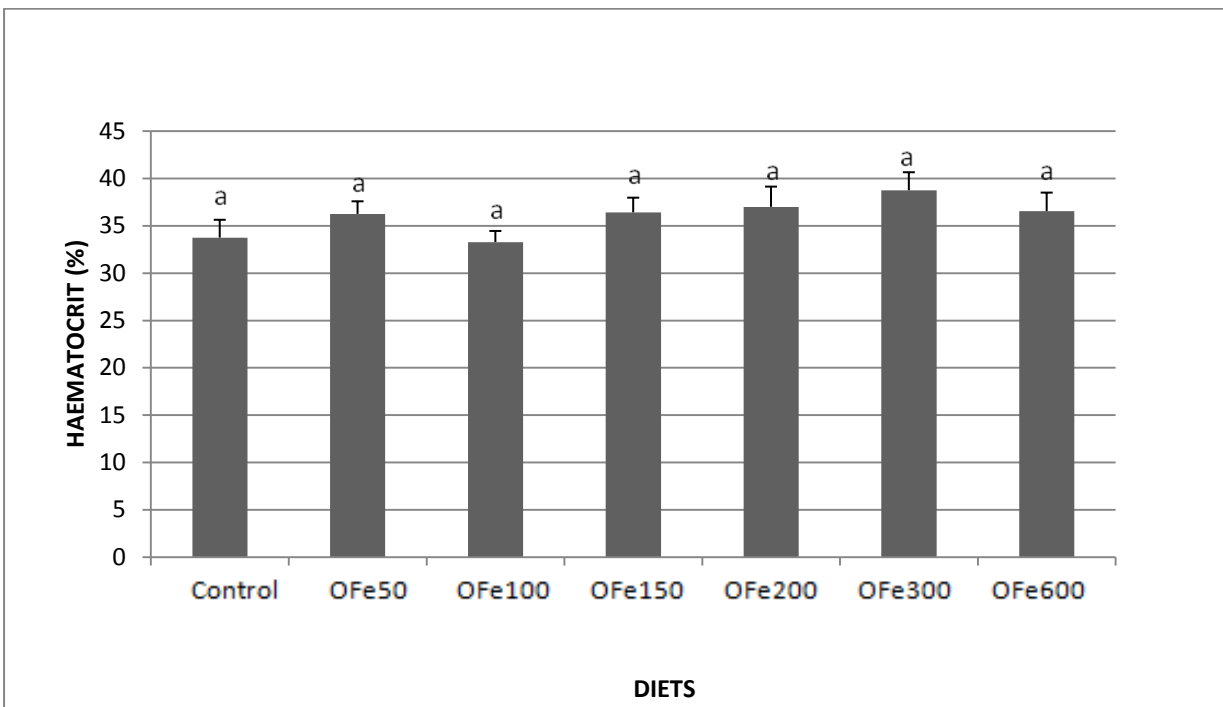

Fig. 3.7 The Htc on the gilthead sea bream fed the experimental diets for 12 weeks (%).Values are the means (n = 18) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.
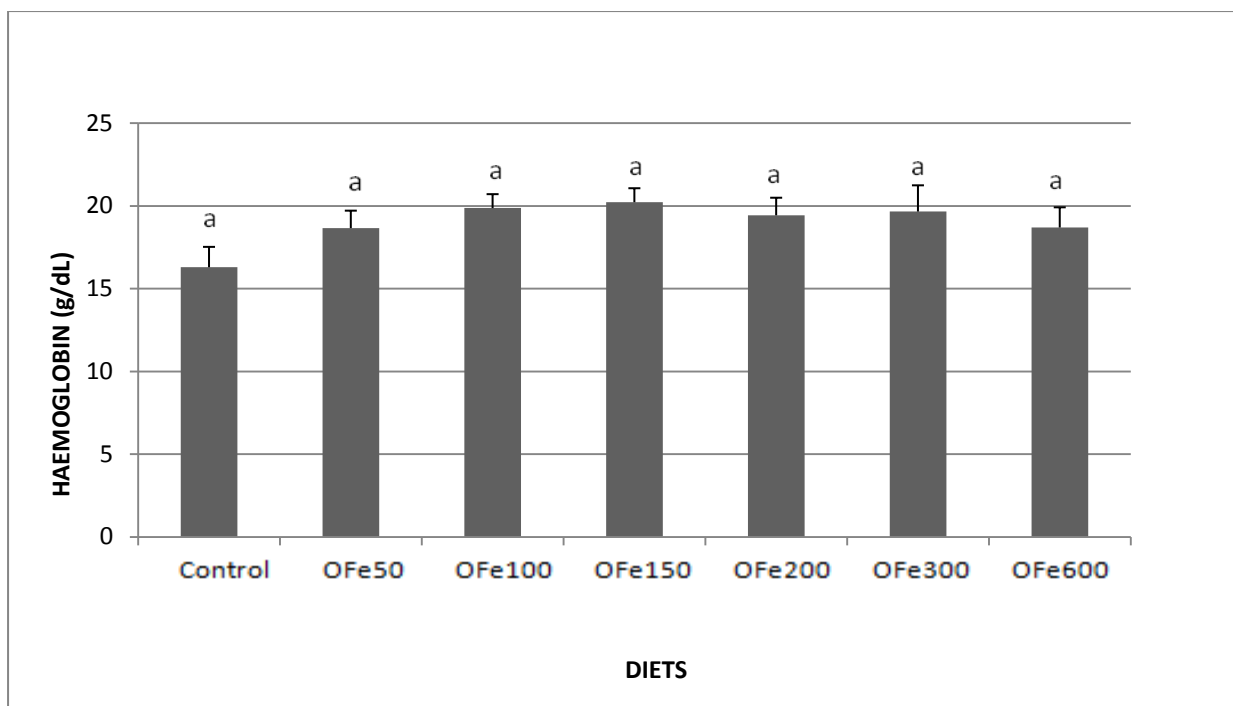
Fig. 3.8 The Hb on the gilthead sea bream fed the experimental diets for 12 weeks (g/dL). Values are the means (n = 18) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

3.3.4 Immunology

The immunological parameters examined were the respiratory burst activity and the antibacterial activity of serum. The immunological parameters for the gilthead sea bream groups fed on the seven experimental diets are presented graphically below (Fig. 3.9, 3.10). The analysis on the respiratory burst in the blood of the gilthead sea bream show no significant difference ($p>0.05$), with zymosan ($p=0.169$) and PMA ($p=0.653$). Although it is very important to mention that the OFe150 showed higher chemiluminessence levels compare to the other six experimental diets. On the contrary the antibacterial activity of serum shows, having a significant difference ($p=0.001$), that the difference levels of added organic iron have an effect of the immunological status of the gilthead sea bream, due to the fact

that p was <0.05. The fish fed on the OFe50 and OFe150 showed higher anti

bacterial activity in the serum of the fish compare to the fish fed the control, OFe200

and OFe300 diets. The diets OFe100 and OFe600 did not have a significant

difference with any other diet. Fig. 3.9 and 3.10 show the respiratory burst as well as

the statistically different antibacterial activity of serum on the fish groups fed the
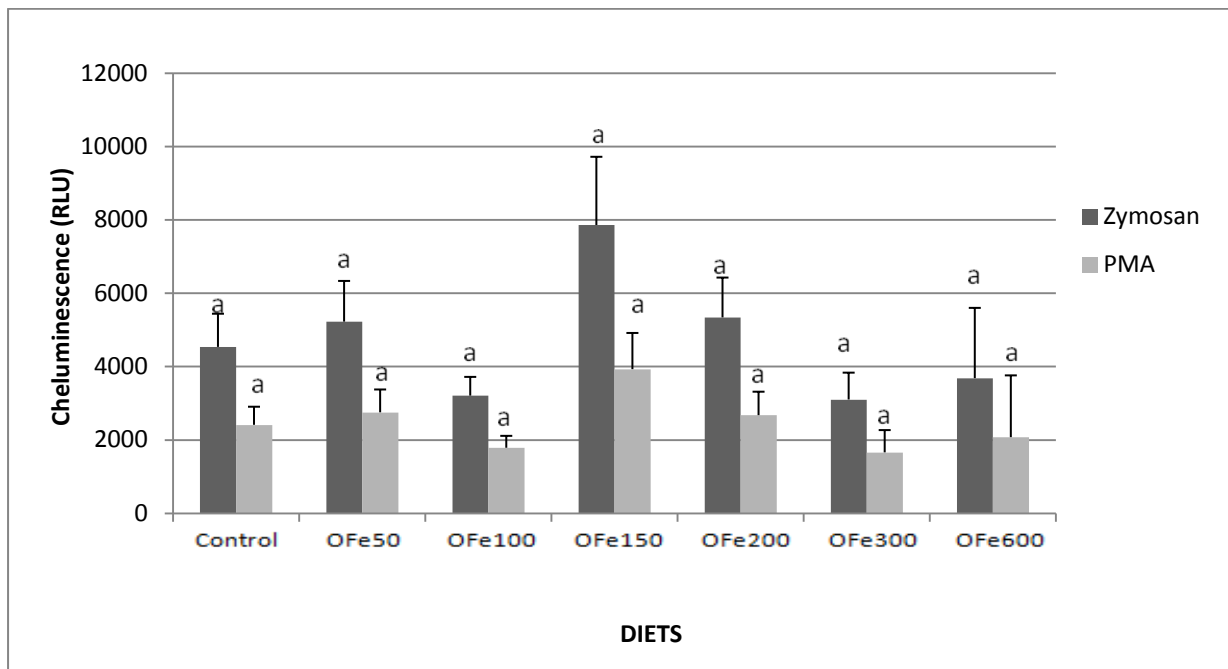
experimental diets.

Fig. 3.9 The respiratory burst activity in the blood of the gilthead sea bream fed the experimental diets for 12 weeks (RLU). Values are the means (n = 18) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common (between diets, not zymosan and PMA) letter are not significantly different at p<0.05.
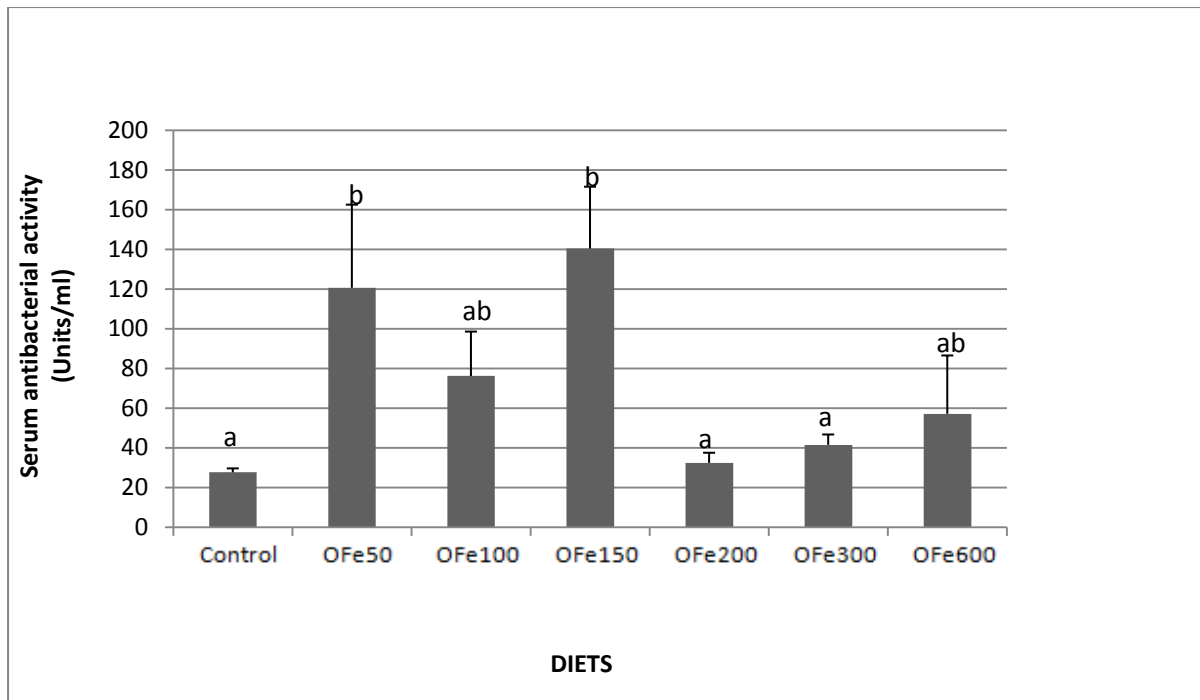
Fig. 3.10 The antibacterial activity of serum in the gilthead sea bream fed the experimental diets for 12 weeks (Units/mL). Values are the means (n = 18) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

3.4 Discussion

The present study was designed in order to evaluate the organic iron metabolism by the gilthead sea bream and eventually identify an optimum level that could be used by the aquaculture industry in order to prevent diseases and deficiencies and consequently improve the fish health. This study was one of the first investigations in iron supplementation in Mediterranean farmed species (Rigos *et al.,* 2010). An important obstacle that has to be overcome was the design of the experimental diets. The raw ingredients used for the diets formulation should have the lowest amount of iron possible, so the addition of organic iron in the premix should not interfere with the existing iron. On the other hand Rumsey & Ketola (1975) observed a relatively slow growth on Atlantic salmon species fed purified

diets. Another issue that had to be addressed was the formation of the diets which should be similar to the commercial type sea bream feeds. Before the purchase of the fish each individual ingredient is analysed for iron concentration, evaluated and the diets formulated with regard to the three criteria mentioned previously. A low level (5%) of mineral free casein from bovine milk was used to increase the protein levels while wheat gluten was the main ingredient. Fish meal and soybean meal included at levels of 15% due to the high iron content they had. The iron content in the final diets was quite high (62.17 mg/Kg), although the supplemented organic iron was the factor investigated in the current experiment and its effect of the gilthead sea bream's growth performance, iron tissue concentration, haematological and immunological parameters.

The organic iron supplementation did not show significant difference on the growth performance of the gilthead sea bream fed the seven experimental diets. The final weight of the fish as well as the specific growth rate (SGR) was not affected by the different levels added in the diets. Although there was no significant differences the fish groups fed the diets with no supplementation of organic iron and the lowers (50 mg/Kg) supplementation of organic iron, control and OFe50 respectively, show a higher SGR compare to the groups fed the rest of the diets. A possible reason for that could be the large standard deviations appearing in these groups. In addition there were no significant differences in the FCR (feed conversion ration) of the fish groups fed the experimental diets. The findings regarding the growth performance of the gilthead sea bream come in agreement with studies in Atlantic salmon that were fed iron supplemented feeds (Andersen *et al.,* 1996, 1997, 1998) where the growth of the fish were not improved significantly by the inclusion of iron in their diets. It also comes in agreement with studies in red sea bream showing that the growth of the

fish is not related with the supplementation of iron in the diets of the fish (Sakamoto & Yone, 1976). In contrast channel catfish fed purified diets without iron supplementation show a reduction in their growth (Gatlin & Wilson, 1986).

The statistical analysis showed no significant differences in tissue iron concentration among the fish fed the seven experimental diets. More specifically all three tissues (muscle, liver and spleen) analysed. Even there were no significant differences in the iron concentration in the specific tissues of the gilthead sea bream groups fed the seven experimental diets, there were numerically increased iron concentration values as the iron supplementation in the fish diets was increased. In all three tissues the fish fed the OFe150 and OFe200 diets showed the highest iron concentration levels, while the fish fed OFe300 and OFe600 show a stabilization in iron storage (either dropping or having similar levels). This phenomenon observed in previous experiments in Atlantic salmon and sea bream species where the higher iron supplementations in the diets of the fish show similar amounts of stored iron in the tissues (Andersen *et al.,* 1996; Rasmussen, 1994; Rigos *et al.,* 2010). The regulation mechanism for iron metabolism in the fish in not well studied and is still unknown, so this stabilization in the higher iron supplementation was expected but not easily explained. In addition, Andersen *et al.*(1998), in a study with Atlantic salmon parr and Fe levels from 10 up to 400 mg/Kg, demonstrated that liver had higher iron concentration for the diet containing 200 mg/Kg of iron, compared to the diet containing 400 mg/Kg iron, which has showed a reduction. In accordance with this experiment, come findings with previous studies in sea bream fed diets with supplemented organic iron from 22 mg/Kg up to 300 mg/Kg showing that there was no significant difference in the iron concentration in the liver and muscle of the fish.

The haematological parameters analyzed were not significantly affected by the iron supplementation in the diet of the fish. The haematocrit and haemoglobin were not significantly different and as a result the increasing levels of organic iron in the diet of the gilthead sea bream does not affect these specific blood parameters. It is worth mentioning that the in Hb the values measured reach the highest level in the fish group fed the OFe150 diets and then the values start dropping slightly as the organic iron supplementation was increased up to 600 mg/Kg. This comes in agreement with sea bream studies where the Hb and Htc of the sea bream fed five diets supplement with organic iron (from 22 mg/Kg up to 300 mg/Kg) showing no significant difference in the blood analysis of the fish (Rigos *et al.,* 2010). The RBC counts had no significant difference as well, although there was an increasing trend where the values increased along side with the iron supplementation in the diets. Rigos *et al.* (2010) observed a significant reduction in the RBC counts of sea bream species fed semi-purified (22 mg/Kg) and 50 mg/Kg organic iron supplemented diets compare to the fish fed diets with an addition of 100, 200 and 300 ppm of organic iron (Rigos *et al.,* 2010). This could be explained due to the high iron concentration in the raw ingredients used in the experiment as the pre existing iron in the control diet was 62.17 mg/Kg. Hence the iron content in the raw ingredients may cover the erythrocyte requirements of the fish. In Atlantic salmon the RBC count was not significantly affected from the addition of iron in the diet of the fish at levels from 10 mg/Kg up to 400 mg/Kg of supplemented iron (Andersen *et al.,* 1996). Later studies found that Hb and Htc had a significant difference in fish fed diets with less that 30 mg/Kg of supplemented iron (Andersen *et al.,* 1998).

The respiratory burst analysis show no significant differences in the blood of the fish fed the seven experimental diets. Although a clear trend was observed with

the values of both zymosan and PMA to increase alongside the organic iron supplementation in the diets, reaching the highest levels on the fish fed the OFe150 diet and the dropping as the organic iron supplementation increased up to 600 mg/Kg. The finding comes in agreement with previous studies in gilthead sea bream where the fish fed diets with organic iron supplementation from 22-300 mg/Kg and there was no significant difference and a similar trend was observed with values reaching the highest levels at the 150 mg/Kg added organic iron diets and the dropping (Rigos *et al.,* 2010). The immunological analysis in the humoral immune system showed clear differences in the antibacterial activity of serum among the fish groups fed the OFe50 and OFe150 diets compare to the fish groups fed the control, OFe200 and OFe300 diets. The standard deviation was large even though the statistical difference indicated that an addition of 150 mg/Kg of organic iron in the diet of the gilthead sea bream could positively influence the health of the fish increase the antibacterial activity in the serum of the blood. Significant difference in the antibacterial activity of serum of sea bream species also observed in previous experiments where fish fed an diet supplemented with 100 mg/Kg of organic iron show reduced levels compare to the ones fed diets with 300 mg/Kg supplementation of organic iron (Rigos *et al.,* 2010). Andersen *et al*. (1998) observe that Atlantic salmon species are not significantly influenced by dietary iron administration for 8 and 20 weeks, in the total antibody in the serum, specific haemolytic complement activity and spontaneous haemolytic activity and serum, head–kidney and spleen lysozyme activities. While, Sealey *et al.* (1997) show that the iron-deficiency had no effect on the specific antibody response in channel catfish but accelerated the onset of mortality when the same species exposed to *Edwardsiella ictaluri* (Lim & Klesius, 1997; Lim *et al,.* 2000), but decreased macrophage chemotaxis (Lim & Klesius 1997;

Sealey *et al.,* 1997). Dietary experiments with Atlantic salmon, show a significant association between high concentrations of serum iron and mortality of fish infected with *Vibrio anguillarum* (Sherman, 1992). In addition to that it was indicated that there is a delicate balance between the host defence mechanisms need of iron and the need for iron to sustain microbial growth (Ravndal *et al.,* 1994). Summarising the findings of the current experiment it can be suggested the supplementation of 150 mg/Kg of organic dietary Fe in the feeds of gilthead sea bream in intensive aquaculture conditions could lead to improved immunological parameters. Especially in periods where either the water oxygen levels are low or haematophagus monogeneans attacks are expected. Future immunological and disease challenge studies are required to investigate this speculative hypothesis.

Chapter 4 Effects of organic and inorganic forms of iron on availability and metabolism of gilthead sea bream.

4.1 Introduction

Iron (Fe) is considered to be the most abundant trace element in most vertebrates and is essential to all cells in all known organisms including fish (Reily, 2004; Vallee, 1986). It has an active role in oxidation/reduction reactions as well as electron transport associated with cellular respiration (Watanabe *et al.,* 1997). Therefore, it is considered a vital nutrient in the animal diet. Fish obtain Fe both from their diets and by direct water absorption. Fish are unable to obtain adequate Fe from the surrounding water, so they must rely on their diet in order to cover their Fe needs (Bury & Grosell, 2003; Lall, 1989; Spry *et al.*, 1988). Fe related deficiencies make fish become more susceptible to infectious agents and as a result Fe supplementation in their diets will benefit their health (Lall, 1989; Olivia-Teles, 2012). Furthermore, reduced growth haematological suspension and poor FCR and even microcytic anaemia can be caused in farmed fish due to Fe deficiency (Tacon, 1992; Andersen *et al.,* 1996). The research for mineral requirements for fish is very poor, mainly due to the difficulties involved in their study (Lall, 2002). It is suggested that the easiest way to avoid mineral deficiency problems in aquaculture is the dietary mineral supplementation (Hardy, 2001; Olivia-Teles, 2012). The nutritional requirement for Fe in the fish feeds have been determined for several species including, yellowtail (*Seriola quinqueradiata*), red sea bream, common carp (*Cyprinus carpio*), Japanese eel, Atlantic salmon and channel catfish and varies from

30-170 mg/ Kg (Sakamoto & Yone, 1976, 1978a, b; Lall & Hines, 1987; Nose & Arai, 1979; Ikeda *et al.,* 1973; Gatlin & Wilson, 1986). The gilthead sea bream Fe requirements was determined from two parties. Rigos *et al.* (2010) suggested that an iron concentration between 100-200 mg/Kg should be the recommended additive level in the commercial diets of the fish. While more detailed previous experiments in this study dirtily the 150 mg/Kg the optimum amount in order to cover the nutritional needs of gilthead sea bream. The availability and utilization of Fe by fish depends on a number of factors. These are the dietary source and level, the concentration in the water, the body stores, and the interactions with other nutrients (Tacon, 1992; Lall, 2002). Moreover Fe absorption can be influenced by the chemical form, the digestibility of the diet, the animal age and health status and more importantly the proportion of organic and inorganic dietary components of the diet (Andersen *et al.,* 1996; Lall, 2002). The organic forms of Fe can be absorbed better from the fish compare to the inorganic Fe sources (Watanabe *et al,.* 1997). Studies in red sea bream using different Fe salts showed that there are differences in absorption. As a result the organic forms of Fe were more highly available to the fish and more effective in the prevention of anaemia (Watanabe *et al.,* 1997; Sakamoto & Yone, 1979).

The aim of this experiment is to compare the optimum organic Fe level that was determined from previous experimentation, with various levels of two inorganic Fe forms that are widely used in the Greek aquaculture industry, mainly due to the low cost compared to the organic Fe. The parameters that will be studied in order to evaluate the comparison are the growth performance, the Fe concentration in three selected tissues of the fish as well as the effect on the haematology of the fish.

4.2 Materials and methods

4.2.1 Experimental conditions

Juvenile sea bream with an initial weight 17 gr ±1. were obtained from a fish farm in Poros Island in the Saronikos gulf (partner to H.C.M.R.). The fish were acclimatized for 4 weeks in an unsupplemented diet in order to reduce the body stores of Fe (Baker, 1986), in the H.C.M.R. facilities in Agios Kosmas, Athens. All fish within the system were graded to ensure uniformity of fish size at the start of the trial period. Then fish were distributed randomly into 21 experimental cylindrical fibre grass tanks of 150L capacity. Each tank contained 30 fish equally distributed. The duration of the experiment was 12 weeks, while triplicate groups of fish fed seven experimental diets three times per day. The amount the gilthead sea bream fed was close to satiation. The photoperiod started at 07:00 in the morning and lasted until 18:00 in the afternoon (11:13, light:dark).The water was supplied by a closed recirculation biological filter with a flow rate around 1.65 per min. Each individual tank was supplied with liquid oxygen at 6ppm. During the experiment the temperature was maintained at 20°C ±2 and the salinity of the water in the experimental tanks was 38‰.

4.2.2 Diet Formulation

Seven semi-purified diets were formulated in order to compare and determine the optimum level of organic iron (Appendix 1) and the two main forms of commercial inorganic iron (Ferrous Sulphate $FeSO_4$ and Ferrous Carbonate $FeCO_3$) in three levels, one equal to the control diet, one 50 mg/Kg for Fe lower and one 50 mg/Kg of

Fe higher. The control diet, 150ORGFe was using organic iron at 150 mg/Kg (which identified as optimum form the first experiment), diets 100INFeS, 150INFeS, 200INFeS was using 100 mg/Kg, 150 mg/Kg and 200 mg/Kg of Ferrous Sulphate Iron (Appendix 2) respectively and diets 100INFeC, 150INFeC, 200INFeC was using 100 mg/Kg, 150 mg/Kg and 200 mg/Kg of Ferrous Carbonate Iron (Appendix 3) in that order. Each diet was tested in triplication in all the gilthead sea bream groups. Before the beginning of the experiment the fish pellets were crumbled and sieved of the appropriate size required for the fish specific life stage (4mm). The experimental diets of the study were designed in order to compare the optimum organic Fe level (150 mg/Kg) to two different inorganic forms at the equal level as well as to 50 mg/Kg lower and higher respectively. The composition of the diets is presented in Table 4.1 with a detailed list of the percentage of the ingredients used. All the ingredients were provided by Biomar Hellas except casein and iron that were provided by Sigma Hellas and Alltech Hellas respectively. Mineral free casein for bovine milk was the mail protein used at a high level while due to the need to reduce as much as possible the Fe levels in the raw ingredients of the diets and as a result to add the tree different Fe forms in the premix so that the aim of the experiment could investigated. The main difference among the diets is the organic Fe and the two types of inorganic Fe forms varying in three different levels for each one. The Table 4.2 shows the Fe concentration in each of the seven experimental diets. Finally, the chemical composition of the seven experimental diets show that fish feeds used in the experiment had crude protein at 47.6±0.4%, crude fat at 16.7±0.3% and ash at 6.5±0.2%.

Table 4.1 The composition of the seven experimental diets (%).

	Control (150ORGFe)	100INFeS	150INFeS	200INFeS	100INFeC	150INFeC	200INFeC
Fish meal	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Wheat meal	37.0	37.0	37.0	37.0	37.0	37.0	37.0
Wheat gluten	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Fish Oil	16.0	16.0	16.0	16.0	16.0	16.0	16.0
Casein	35.0	35.0	35.0	35.0	35.0	35.0	35.0
Premix	0.3	0.3	0.3	0.3	0.3	0.3	0.3
cellulose	1.5	1.6	1.5	1.5	1.6	1.5	1.5
Organic Iron	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Iron Sulphate	0.0	0.1	0.2	0.2	0.0	0.0	0.0
Iron Carbonate	0.0	0.0	0.0	0.0	0.1	0.2	0.2
Total	100	100	100	100	100	100	100

Table 4.2 The Fe concentration in each of the seven experimental diets.

	Control (150ORGFe)	100INFeS	150INFeS	200INFeS	100INFeC	150INFeC	200INFeC
Fe concentration (mg/Kg)	168.3	117.2	170.8	224.3	122.7	162.2	219.1

4.2.3 Sampling

Prior to sampling there was a 24 h starvation period for the fish. At the end of the experiment the fish from each tank was anesthetised (phenoxyethanol at 50 uL/L for 5 minutes) so the sampling could take place. During the sampling all fish form the 21 tanks were individually weighted. The initial and final weights were measured and are demonstrated in Fig.4.1. A total number of eighteen fish from each dietary treatment (six fish from each of the triplicate tanks) were collected for hematological (red blood cell count, hematocrit, hemoglobin) where blood was drawn from the

caudal vein with 1 ml syringe. In addition a total number of forty five fish from each dietary treatment (fifteen fish from each of the triplicate tanks) were used for Fe concentration analysis where selective tissues [muscle (above the lateral line), liver and spleen] were collected. Lastly, the specific growth rate (SGR) of the fish was calculated by using the final and initial weight of the fish divided by the duration of the experiment using the formula presented in section 2.1.

4.2.4 Data analysis

The evaluation of the data collected from each measured parameter was statistically analysed using the software SPSS 11.5.1. The triplicate tanks for each experimental diet were pooled for each parameter analysed. Statistical analysis between dietary groups was performed by One-Way ANOVA followed by a Tukey's multiple range comparison test. In addition some secondary tests have been performed, such as normality tests and post hoc tests. The level of significance for all tests was set at $p < 0.05$. Finally the graphics prepared in order to present the findings were created using the Microsoft excel software.

4.3 Results

4.3.1 Growth

From the beginning (June) until the end (September) of the 12 week trial the fish initial and final weight measured for each tank separately and presented in details alongside with the specific growth rate (SGR) that was calculated according

to the previously mentioned formula. The final weight of the gilthead sea bream as well as the SGR did not show any significant differences. The final weight (p=0.301) of the fish as well as the SGR (p=0.665) were not significantly affected by dietary treatment. The FCR (p=0.115) as well as the mortality rate (p=0.646) show no significant differences among the fish groups fed the experimental diets Fig. 4.1 and 4.2 have the graphic presentation of the initial and final weight as well as the SGR and FCR for the gilthead sea bream fed the seven experimental diets, while Table 4.3 presents the mortality rates.
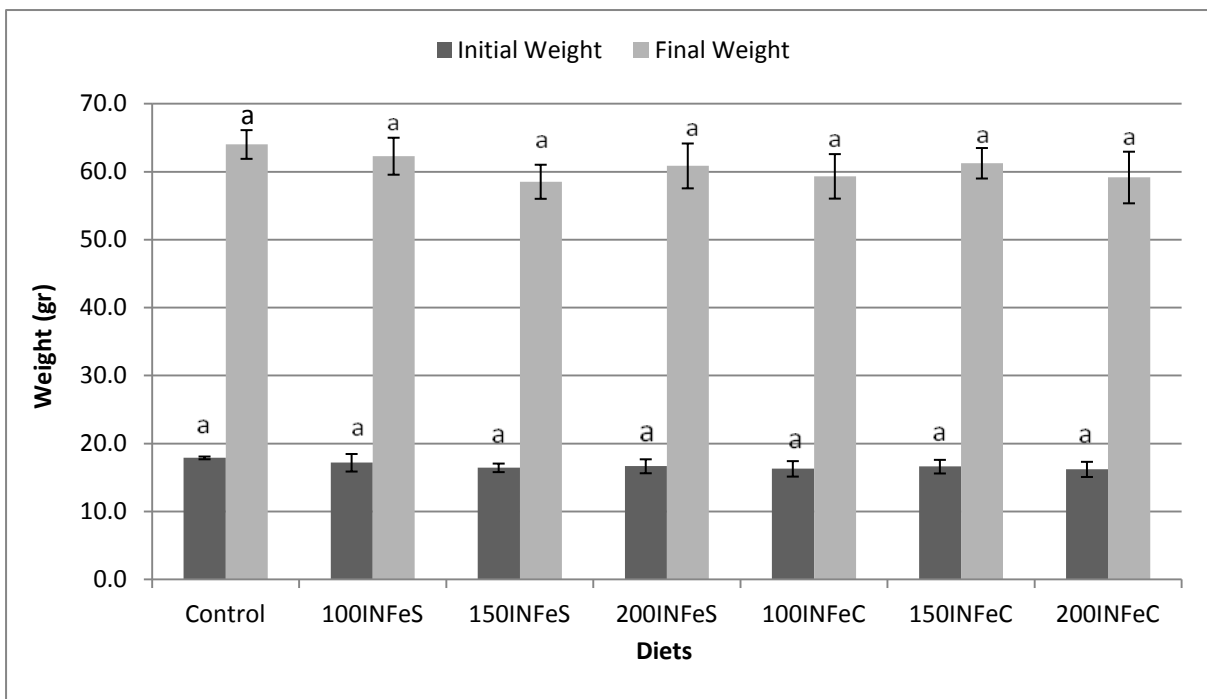
Fig. 4.1 The initial and final weight of the gilthead sea bream treated with the experimental diets for 12 weeks. Values are the means (n = 15) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.
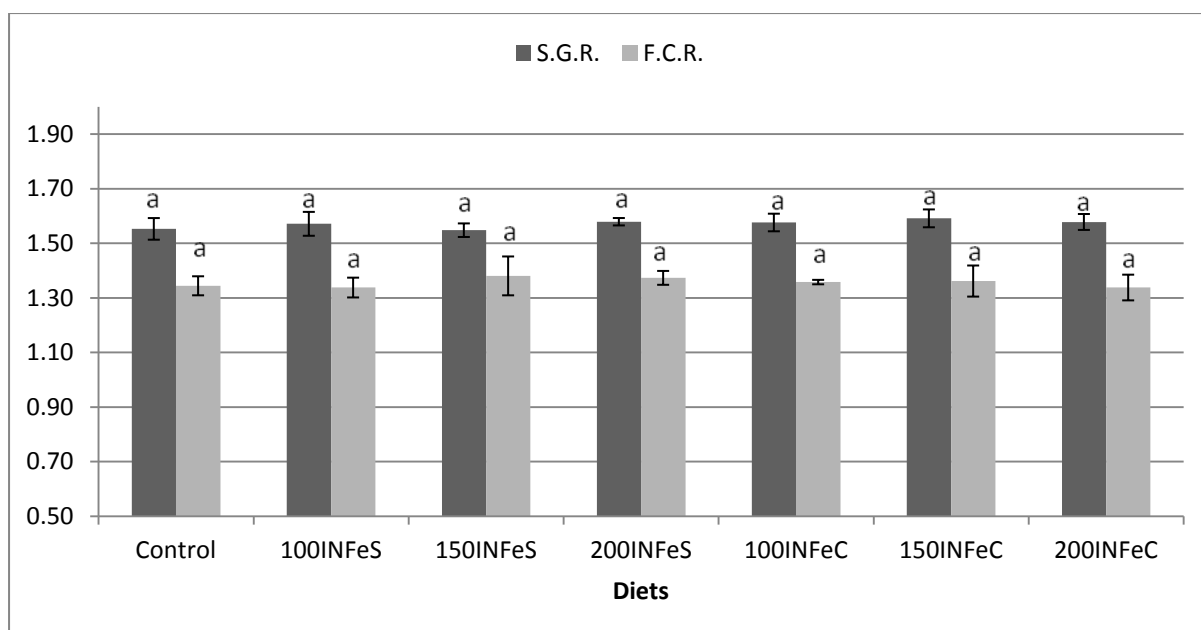
Fig. 4.2 The SGR and FCR of the gilthead sea bream treated with the experimental diets for 12 weeks. Values are the means (n = 15) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Table 4.3 The mortality rate (%) in each of the seven experimental groups.

Diets	Control	100INFeS	150INFeS	200INFeS	100INFeC	150INFeC	200INFeC
Mortality rate (%)	5.7 ±3.5[a]	8.7 ±3.1[a]	9.0 ±4.4[a]	5.7 ±3.1[a]	8.0 ±3.6[a]	11.0 ±3.5[a]	8.3 ±5.5[a]

4.3.2 Tissue concentration analysis

For the tissue concentration analysis the organs selected were the liver, spleen and muscle of the gilthead sea bream treated with the seven experimental diets for 12 weeks. The liver and muscle were analysed individually for each fish while spleen was pooled because it is a small organ and it didn't meet the minimum weight for the analysis needed several spleens. Fig. 4.3, 4.4 and 4.5 shows the Fe

concentration in the specific tissues (muscle, liver and spleen. The statistical analysis show that there were no significant differences in the Fe concentration in none of the fish groups fed the experimental diets. The Fe concentration in the muscle ($p = 0.071$) of the fish was not significantly affected by the different experimental diets. Equally the liver ($p = 0.119$) and spleen ($p = 0.283$) show no significant deference in the Fe concentration of the gilthead sea bream groups that were fed each of the seven experimental diets. Although there was no significant differences, it appears that the gilthead sea bream fed the Ferrous carbonate diets (INFeC) showed reduced levels of Fe concentration in all three tissues. In the same lines the Fe concentration in the spleen of the fish fed the control diets showed the higher levels even though the large standard deviation does allow any safe conclusions.

Fig. 4.3 The Fe tissue concentration in the muscle of gilthead sea bream fed the experimental diets for 12 weeks (mg/Kg). Values are the means (n = 15) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p < 0.05$.

Fig.4.4 The Fe tissue concentration in the liver of gilthead sea bream fed the experimental diets for 12 weeks (mg/Kg). Values are the means (n = 15) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig.4.5 The Fe tissue concentration in the spleen of gilthead sea bream fed the experimental diets for 12 weeks (mg/Kg). Values are the means (n = 15) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

4.3.3 Haematological analysis

The haematological analysis shows no significant difference for any of the parameters that were examined. Fig. 3.6, 3.7 and 3.8 shows each of the three haematological parameters analysed, the haematocrit (Htc), the haemoglobin (Hb) and finally the red blood cell (RBC) count. The Htc (p=0.340) of the gilthead sea bream groups fed the experimental diets was not affected. In addition the RBC count (p=0.692) and the Hb (p=0.565) did not have a significant difference at any of the seven experimental diets.

Fig. 4.6 The effects on the Htc of the seven experimental diets in the gilthead sea bream. Values are the means (n = 18) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 4.7 The effects on the RBC of the seven experimental diets in the gilthead sea bream (10^5mL^{-1}). Values are the means (n = 18) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

Fig. 4.8 The effects on the Hb of the seven experimental diets in the gilthead sea bream. Values are the means (n = 18) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

4.1 Discussion

In the present study the aim was to investigate the effect on gilthead sea bream fed with different Fe forms supplemented in their diets. A diet with the supplementation of an organic Fe form used as a control diet while two different types of inorganic Fe (ferrous sulphate, ferrous carbonate) in three levels (one 50 m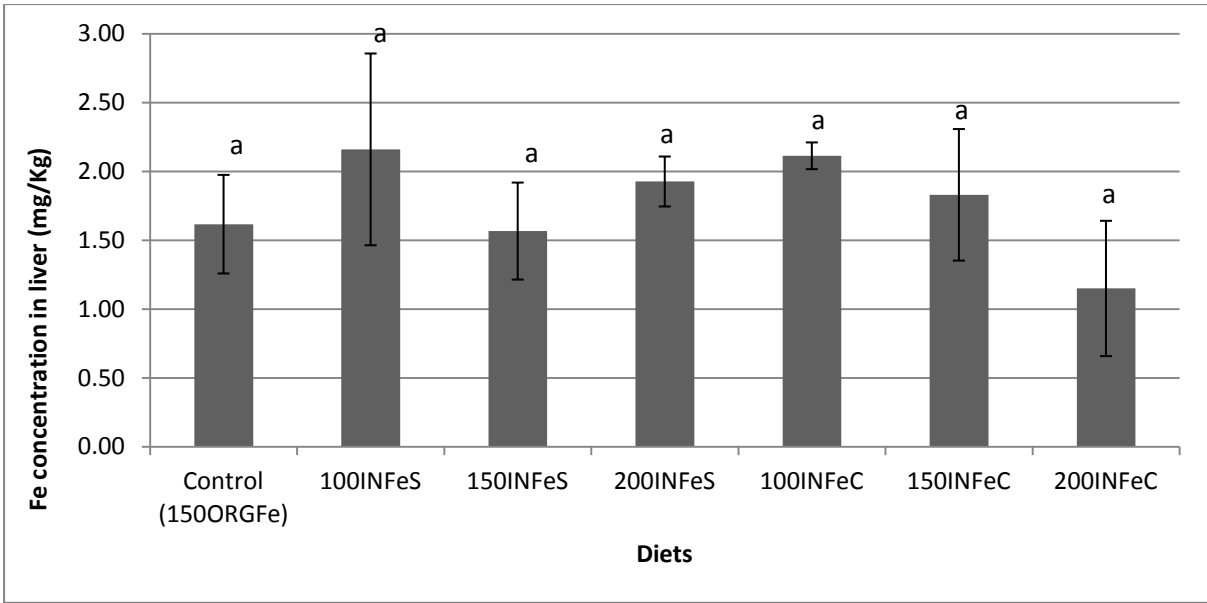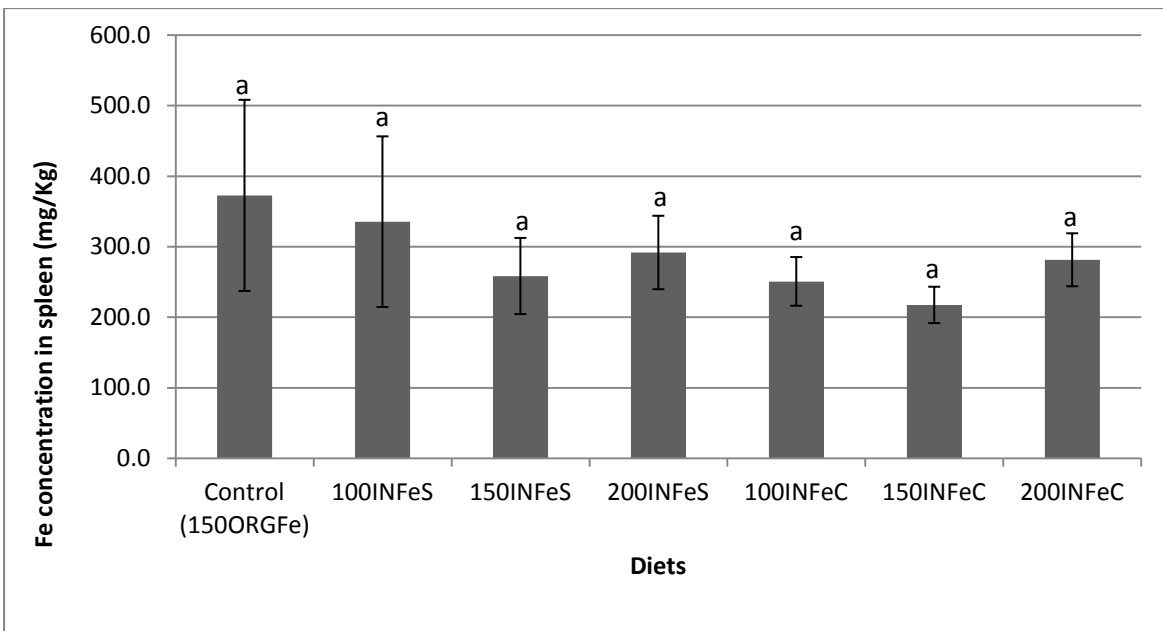g/Kg lower than the control, one equal, 150 mg/Kg, with the control and one 50 mg/Kg higher than the control) used in order to investigate potential differences in the growth, tissue concentration and haematology of the fish. In order to achieve that, a semi purified diet has to be designed so the Fe levels in the diets, before the addition of the addition of the three experimental Fe forms, could be as low as possible. Studies in Atlantic salmon show a relatively slow growth of fish fed on purified diets (Rumsey & Ketola, 1975). However Shearer *et al.* (1993) suggested the casein/gelatine can be a suitable protein source in order to conduct nutrient requirement studies in fish. As a result, the formulation of the diets in the current experiment used mineral free casein from bovine milk as the main protein at a levels of 35% of the diet, keeping the iron concentration in the raw ingredients at around 18.3 mg/Kg without compromising the protein levels and the palatability of the feeds (as pilot studies indicated, before the beginning of the experiment).

The seven experimental diets used for the experiment supported the fish with a satisfactory growth (with an initial weight of 16.7g ±1.2) and almost triple their weight during the 12 week trial. The SGR was equally satisfactory and it was around 1.5 for all the experimental groups. No significant differences were observed in the gilthead sea bream fed the experimental diets both for the actual growth of the fish and the specific growth rate. The final weight had a no statistical significant p value

while the p value for the SGR and FCR were higher than 0.05. The absence of significant retardation in the growth performance of the fish groups comes in agreement with previous studies in gilthead sea bream where different fish groups fed with a diets supplemented with organic and inorganic Fe in levels up to 200 mg/Kg, and there was no significant differences neither in the final weight of the fish nor in the specific growth rate (Rigos *et al.,* 2010). As a result the different Fe forms (organic, inorganic) do not have an effect on the growth of the fish. Baker (1986) observed that growth retardation in fish is a symptom quite common of many nutrient deficiencies, but the mineral deficient (Fe in this particular case) is not considered to be a factor. A number of studies with Atlantic salmon and red sea bream showed that no significant weight gained in the fish once the minimum Fe requirements were covered (Sakamoto & Yone, 1976; Andersen *et al.,* 1996, 1997, 1998). In this particular experiment the levels of Fe in the seven experimental diets was around the suggested level for sea bream and as a result they are meeting the nutritional requirement of the fish regardless of the Fe forms.

The selected tissues analysed in order to measure the Fe concentration were the liver, the spleen and the muscle since these organs are used to measure Fe concentration in the body (Bury *et al.,* 2012). The levels of Fe concentration in the selected tissues did not show any significant difference among the fish fed the experimental diets. The analysis in the muscle of the fish show that there was no significant difference, while in liver the p value was higher than 0.05 and as a result statistically significant. From both this tissues we can observe that the organic Fe in the diet of the fish did not have a significant higher effect on the Fe concentration in these organs. Although we can observe similar levels in the control and NFeS diets, the NFeC show lower values in the muscle. Andersen *et al.* (1996) stated that there

is an unknown regulation mechanism for iron metabolism and in order to understand it further research has to be done. The Fe concentration in the spleen of the gilthead sea bream show no significant differences. Although there was no significant difference the fish fed the control diet (organic Fe) had higher levels of Fe in the spleen than the high standard deviation could possibly mask any statistical difference. The results in the tissue concentration analysis are in accordance with previous experiments in gilthead sea bream where no significance was observed between various organic Fe levels (22-300 mg/Kg) compared to inorganic Fe form at 200 mg/Kg (Rigos *et al.,* 2010). Even though the organic iron is more bioavailabe (20% bioavailability) compared to inorganic froms (5% bioavailability), there is a limited ability to be absorbable (Oates & West, 2006). In conclusion the Fe concentration of organic form shows not significant differences to the inorganic forms although in the case of spleen there was higher availability of organic Fe compare to inorganic.

The haematological analysis in the gilthead sea bream reveal that there were no significant differences among the groups fed the experimental diets. There were three parameters used in the experiment, the haematocrit (Htc), the red blood cell counts (RBC) and the haemoglobin (Hb). The Htc didn't show any significant difference among the fish groups fed the different experimental diets. Same results observed in the RBC count and the Hb where the supplementation of organic and inorganic forms of Fe did not affect these parameters in the haematological analysis in the gilthead sea bream. It is worth mentioning that the higher Htc and RBC count levels appear to be presented in the fish groups fed the control diet, which had the supplementation of the organic Fe. Earlier experiments on sea bream fed various levels (50-300 mg/Kg) of organic Fe and inorganic Fe (200 mg/Kg ferrous sulphate)

show no significant differences among the inorganic Fe diet and the 100, 200 and 300 mg/Kg organic iron supplementation diets, neither at the haematological analysis (RBC counts, Htc, Hb) (Rigos *et al.,* 2010).

The present experiment indicates that the different iron forms (ferrous sulphate, ferrous carbonate) did not have an significant effect neither on the tissue concentration nor on the haematological parameters of the gilthead sea bream species fed the different experimental diets. Further studies with purified diets using more replicates and a wider range of both organic and inorganic Fe levels added in the diets, are required in order to identify the point where dietary organic Fe supplementation can have an significant effect on the growth and health of the fish.

Chapter 5 The effect of two specific oxygen deprivation stressors on the gilthead sea bream fed diets with different organic iron levels.

5.1 Introduction

Aquaculture is the faster growing industry in the food sector in the world according to FAO (2012). The Greek aquaculture industry is the leader in gilthead sea bream production, with a 55% of the total production in 2010 (Maniatis & Danchev, 2011; ICAP, 2009). Therefore it comes as no surprise the related problems and concerns to such intensive an increasing practice. A very important factor in fish performance under aquaculture conditions is stress (Pickering, 1992). Stress is an important consideration in successful aquaculture husbandry. It can affect either individual fish or populations at all levels, ranging from biochemical to behavioural changes (Iwama *et.al.,* 2012; Pickering, 1992). The biology of stress in fish as well as the physiological and behavioural responses to a wide variety of physical, chemical and biological stressors is well studied and there is an extensive literature with emphasis on the stressors seen in commercial aquaculture practices (Wendelaar-Bonga, 1997; Iwama *et al.,* 2012; Barton, 2000, 2002; Conte, 2004). In fish the primary stress response involves the release of catecholamines and the activation of the hypothalamic-pituitary-internal (HPI) axis. Originating from the hypothalamus, the corticotrophin releasing factor acts on the pituitary in order to synthesise and release corticotropin hormone, which in turn stimulates the synthesis as well as the mobilisation of glucocorticoid hormones (in teleost fish in cortisol) from the internal cells (Schreck, 1981; Wendelaar-Bonga, 1997). The stress response

evolved to enable fish to cope with hostile environments and as a result they play a very important role to preserve the individual. In modern aquaculture practices the conditions do not provide the stressed fish any possible escape and as a result the stress response may no longer be beneficial but in fact it can damage the health of the fish. Generally the effects of stress in fish depend on the type and severity, they can result in impaired growth, feeding, reproduction and can lead even to increased susceptibility to diseases and mortalities (Wendelaar-Bonga, 1997). Fish reared under commercial aquaculture conditions are associated with a number of husbandry related stressors which can have a negative effect in the health of the fish some of these can be the increased fish density and poor water quality (i.e., low dissolved oxygen levels, undesirable temperature, high pH, increased levels of carbon dioxide, high ammonia levels, nitrite, hydrogen sulfide, organic matter in the water), injuries during handling and anoxic conditions (i.e., netting, capture, sorting, shipping), inadequate nutrition and finally poor sanitation (Rottmann *et al.,* 1992; Iwama *et al.,* 2012). According to the fish farmers two of the most common stressors in the Greek aquaculture industry are the overcrowding and the netting of the fish for various reasons (Nengas, personal communication). Both these stressors are associated with low oxygen levels to anoxic conditions. Irons (Fe) active role in oxygen transport and cellular respirations (Andersen *et al.,* 1998) could very well prevent to a certain extent the negative effect of these specific stressors to the health of the fish, by providing the fish organism with the necessary Fe in their diets.

The aim of the present study was to compare the effect on gilthead sea bream fed three experimental diets with different organic Fe levels (50, 150 and 250 mg/Kg), for a total number of two common stressors in aquaculture practises. The parameters that will be examined are the growth of the fish, the tissue concentration

in lever spleen and muscle, as well as the haematological and the immunological effects of these stressors to the fish fed the experimental diets.

5.2 Materials and methods

5.2.1 Experimental conditions

The aquaculture laboratories in the H.C.M.R., in Athens, Greece, host the experiment. Gilthead sea bream with an average initial weight 35 gr., from a fish farm near Larisa in the Malian gulf (partner to H.C.M.R.) distributed into nine experimental cylindroconical fire glass tanks of 1000 L capacity. The experiment was designed to contain two stages where after the completion of the first stage (acute stressor), the experiment was continued with the rest of the fish until the end of the second stage. As a result, the gilthead sea bream had exactly the same conditions (for both stages) in order to serve the needs of each experimental stressor subjection (chronic and acute). Each tank contained 60 fish equally distributed randomly. Before the beginning of the experiment the fish were acclimatized for 4 weeks in an unsupplemented diet in order to reduce the body stores of Fe (Baker, 1986). The duration of the experiment was 12 weeks (August until November), where triplicate groups of fish fed the three experimental diets four times per day. The photoperiod started at 08:00 in the morning and lasted until 19:00 in the afternoon (11:13, light:dark). The amount of feed was close to satiation. The water was supplied by a pump from the sea and filtered by a multilayer filter before end in the tanks and the flow rate was around 2L/min. Each individual tank was supplied

with liquid oxygen at 7ppm. During the experiment the temperature was maintained at 23°C ±2 and the salinity of the water in the experimental tanks was 38‰.

5.2.2 Diet Formulation

Three experimental diets were fed to fish for this trial. The main ingredients were mineral free casein from bovine milk and wheat meal in order to minimise the non additive Fe levels without compromising the protein levels as well as the palatability of the feeds. All the raw ingredients were obtained by Biomar Hellas while casein was provided from Sigma Hellas. The difference between the experimental diets was the supplementation of organic Fe (Appendix 1) in various levels explained below. The control diets was containing 150 mg/Kg of organic Fe (the defined suggested level from previous experiments), while other diets has a ±100 mg/Kg of Fe inclusion. OFe50 had a 50 mg/Kg organic Fe inclusion and OFe250 has 250 mg/Kg of organic Fe added to the diet. Each diet was given to fish in triplicate groups randomly distributed for all experimental diets. Before the beginning of the experiment the fish pellets were crumbled and sieved in order to have the appropriate diameter which was 4mm for the specific size of the gilthead sea bream used in the experiment. The crumbled and sieved of the feed continued during the experiment as the fish were growing and they needed a larger diameter pellet.

The composition of the diets in the current experiment was designed in order to minimize the iron content in the ingredients as much as possible and be as close as possible to the commercial type diets that used in the Greek aquaculture industry. Mineral free casein from bovine milk was used to replace the fish meal in order to reduce the Fe concentration in the raw ingredients. In the same line wheat meal was

used to replace soybean meal in the diets due to the lower Fe level that it was containing. As a result the gilthead sea bream in the current experiment were fed semi-purified diets with the inclusion of organic Fe in the premix at three different levels. The chemical composition of the three experimental diets show that the percentage of crude protein was at 47.2±0.4%, of crude fat at 16.6±0.3% and ash at 6.8±0.3% in the fish feeds used in the current experiment. Table 5.1 presents the percentage (%) of each individual ingredient used in the formulation of the three experimental diets. Table 5.2 show the Fe concentration in each experimental diet. The gilthead sea bream exposed to both chronic and acute stressors fed the same diets since the beginning of the experiment.

Table 5.1 The composition of the three experimental diets were gilthead sea bream fed for 12 weeks (%).

	Control (OFe150)	OFe50	OFe250
Fish meal	5.0	5.0	5.0
Wheat meal	37.0	37.0	37.0
Casein	35.0	35.0	35.0
Fish oil	16.0	16.0	16.0
Wheat gluten	5.0	5.0	5.0
Premix	0.3	0.3	0.3
Cellulose	1.6	1.7	1.5
Organic Fe	0.1	0.0	0.2
Total	100	100	100

Table 5.2 The Fe concentrations in each of the three experimental diets were gilthead sea bream fed for 12 weeks.

Diets	Control (OFe150)	OFe50	OFe250
Fe concentration (mg/Kg)	162.1	76.4	268.8

5.2.3 Sampling and oxygen deprivation stressors exposure methodology

The design of the experiment consists of a two stages oxygen deprivation stressors, after the 12 week feeding period. The first oxygen deprivation stressor would be an acute one, while the second one would be a chronic. The chronological order stages of the oxygen deprivation stressor experiments decided, so there will be no interference between the two stages. All the fish used for the first stage of the experiment were used for sampling (and then terminated) and second stage of the experiment continued with the rest of the fish. The acute oxygen deprivation stressor that the fish were subjected was the netting. The following stage was a long term oxygen deprivation stressor where fish were exposed to overstocking. The specific methodology used is explained in detail below.

By the completion of the 12 week feeding period the first stage of the experiment took place. The fish exposed to the acute oxygen deprivation stressor collected from the tank with a 2 meter long net placed immediately and as gently as possible to a 50L bucket for 1 minute. Then the fish collected with a 6'' depth nylon

mesh net and lifted steadily outside of the buckets water and kept for 30 seconds. After the completion of the 30 second oxygen deprivation stressor blood was drawn (from the caudal vein, using 1 ml syringe) immediately from the first 8 fish from each tank for the haematological (haematocrit, haemoglobin and red blood cell count) and immunological analysis (respiratory burst activity and serum antibacterial activity). After that the fish were killed instantly by an incision in the brain, in order to be weighted. The final stage of the sampling was the collection of the samples for the Fe concentration analysis where a total number of 15 fish (including the 8 used for immunological and haematological samples) for each tank. The procedure described was followed for each fish separately, for all experimental tanks.

After the completion of the sampling of first stage of the experiment, a 7 day (1 week) period started where the remaining fish exposed to the chronic oxygen deprivation stressor (second stage of the experiment) by elevated stocking density (overcrowding). The elevated stocking density stressor experiment designed to be achieved by exposing the fish to a state where they will be overcrowded in the tank. The commercial practice in sea bream production in the Greek aquaculture industry describes the recommended stocking density at 10 Kg per m^3. At the current experiment the stocking density of the gilthead sea bream were 25 Kg per m^3. The adjustment in the 1000L fibre glass tanks applied by weighting the fish after the sampling of the first stage of the experiment and lowering the water level in the tank to the level where the fish will be 2.5 times more crowded that the maximum allowed by the regulations. By the completion of the period (1 week) where the fish subjected to the overcrowding condition the sampling took place. The fish were not anesthetised, each individual fish was collected with the net and immediately placed so as to take blood samples (from the caudal vein, using 1 ml syringe(for the

immunological (respiratory burst activity and serum antibacterial activity) and haematological analysis (haematocrit, haemoglobin and red blood cell count). The total number collected for blood samples were eight fish from each tank. The next step was to kill the fish by an incision in the brain, placed in the electronic scale and record the weight of the fish. Followed by that a total number of 15 fish (including the ones where blood samples taken from) used for the Fe concentration analysis where the selected tissues (muscle from above the lateral line, liver and spleen) were collected.

Lastly the specific growth rate (SGR) of the fish was calculated (using the formula presented in section 2.1) by deducting the final from the initial weight of the fish divided by the duration of the each experimental stage.

5.2.4 Data analysis

The evaluation of the data collected from each measured parameter was statistically analysed using the software SPSS 17. The triplicate tanks for each experimental diet were pooled for each parameter analysed. Statistical analysis between dietary groups was performed by One-Way ANOVA followed by a Tukey's multiple range comparison test. In addition tests have been performed such as normality tests and post hoc tests. The level of significance for all tests was set at $p < 0.05$. Finally the graphics prepared in order to present the findings were created using the Microsoft excel software.

5.3 Results

5.3.1 Growth

For the growth performance of the gilthead sea bream, the initial and final weights were measured at the beginning and at the end of each trial respectively. Moreover the specific growth rate (SGR) was calculated both for the fish exposed to acute stressor (netting) and chronic stressor (overstocking). For both trials neither the final weights of the fish nor the SGR were statistically different. The supplemented organic Fe did not have an effect on the final weight ($p=0.261$) and the SGR ($p=0.649$) on the fish exposed to the acute stressor. Similar results occur to the gilthead sea bream exposed to the chronic stressor, where the three levels of organic Fe did not have an effect neither on the final weight ($p=0.102$) nor the SGR ($p=0.609$). The FCR show no significant differences neither in the acute stressor trial ($p=0.575$), nor at the chronic stressor trial ($p=0.457$). No mortalities occurred in any of the treated groups in the current experiment. The initial and final weights for both trials presented separately in Fig. 5.1, while Fig. 5.2 presents the SGR for both stressor exposure trials together, while Fig. 5.3 shows the FCR.

Fig. 5.1 The initial and final weight for both A.S. and C.S. exposed gilthead sea bream trials (gr.). Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 5.2 The SGR for both A.S. and C.S. exposed gilthead sea bream trials. Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.
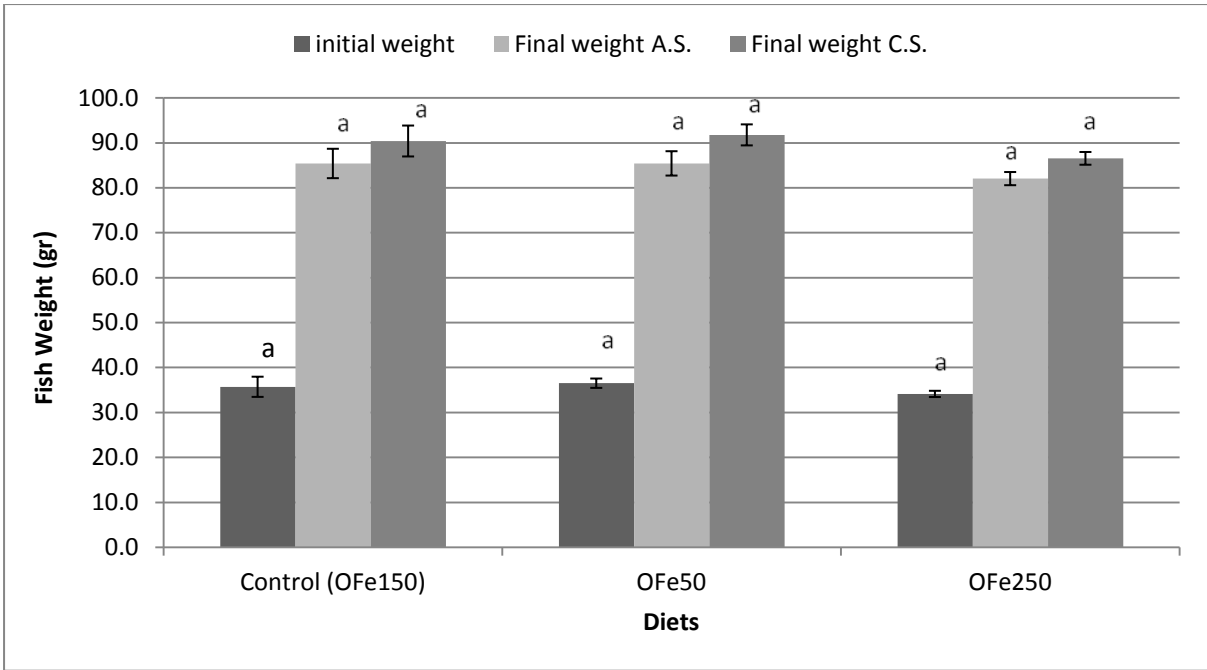
Fig. 5.3 The FCR for both A.S. and C.S. exposed gilthead sea bream trials. Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

5.3.2 Tissue concentration

The tissue concentration analysis shows the Fe concentration in the selective tissues of muscle, liver and spleen. Fig. 5.4, 5.5 and 5.6 show graphically the Fe concentration in the three selected tissues of the fish exposed to the acute stressor while Figures 5.7, 5.8 and 5.9 show the same values but for the gilthead sea bream that were exposed to the chronic stressor. All tissue concentration analysis did not show any significant differences. The exposure to the acute stressor did not have a significant effect on the fish fed the three Fe supplemented diets in the levels of 50, 150 and 250 mg/Kg show no significant difference in spleen (p=0.103), muscle (p=0.42) and liver (p=0.411). It is worth mentioning that both in liver and spleen the Fe concentration had higher concentration in the previously mentioned tissues as the Fe levels in the experimental diets was increased. There was no significant

difference in the spleen (p=0.877), muscle (p=0.589) and liver (p=0.801) of the gilthead sea bream fed the experimental diets and exposed to the chronic stressor. A similar trend was observed here as well, as the Fe concentration in specific tissues increased in the fish groups as they feed in higher Fe concentration experimental diets. Even though there was no statistical significance a noticeable exception occurs in the Fe absorption in the liver of the fish fed the control diet where it seems to have the higher values that both the other fish groups fed the rest diets.

Fig. 5.4 The Fe concentration in the spleen of the gilthead sea bream fed the three experimental diets and exposed to the acute stressor (A.S.) (mg/Kg). Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

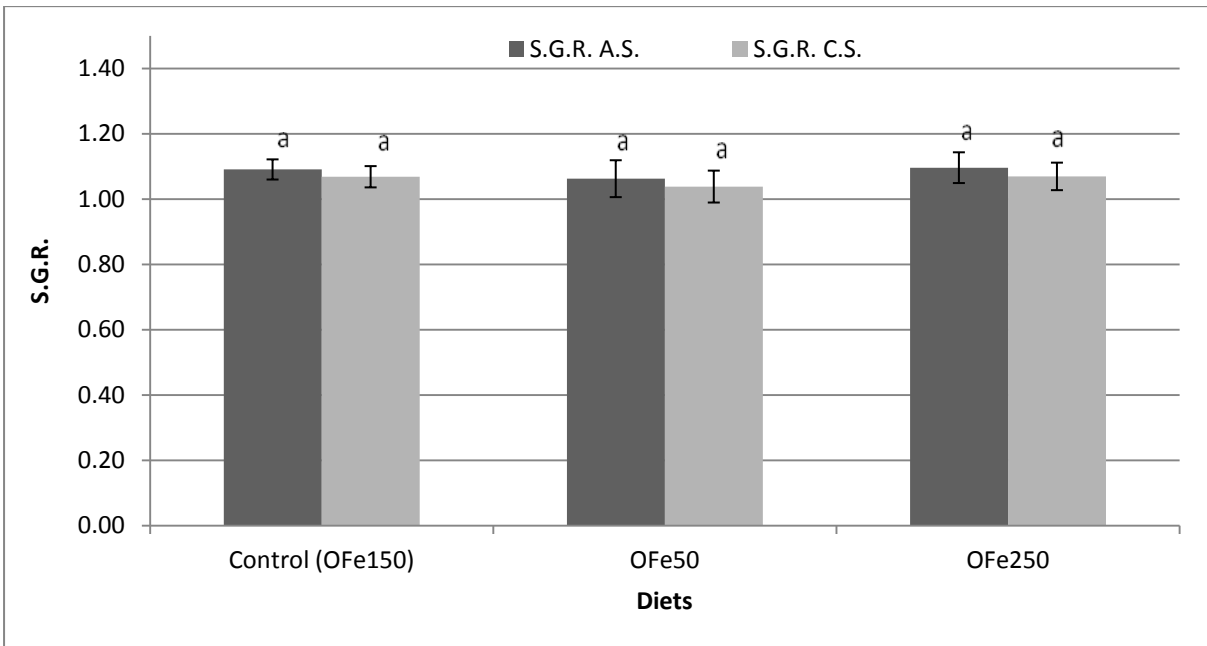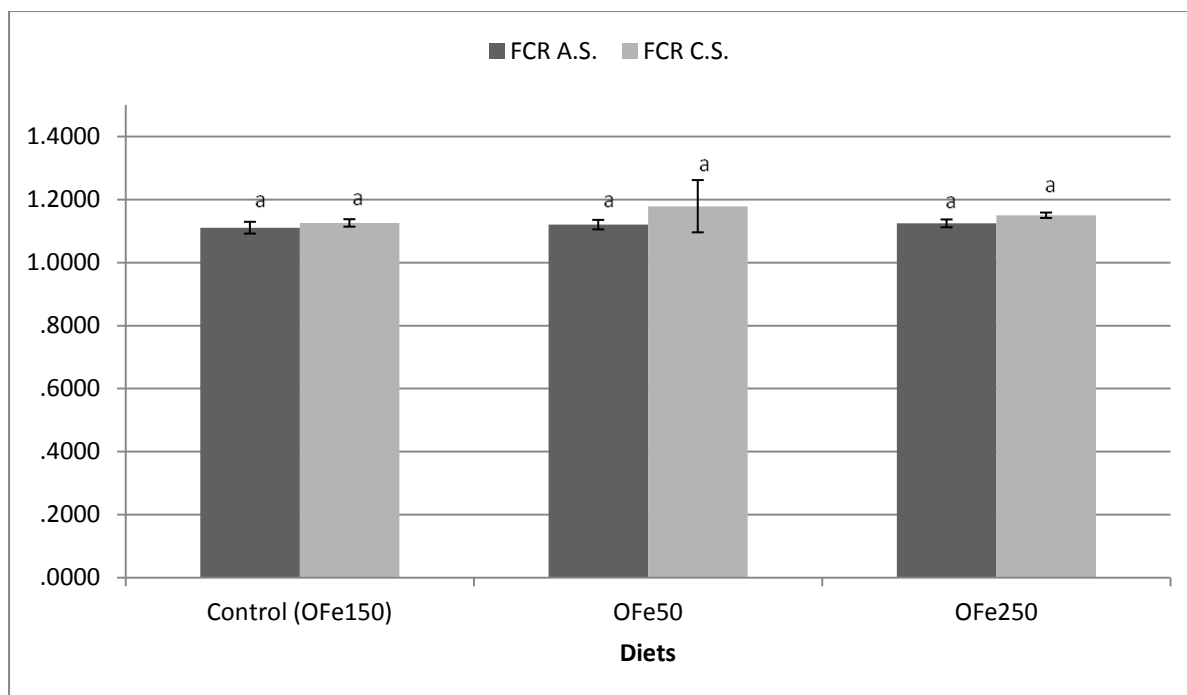Fig. 5.5 The Fe concentration in the muscle of the gilthead sea bream fed the three experimental diets and exposed to the acute stressor (A.S.) (mg/Kg). Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

Fig. 5.6 The Fe concentration in the liver of the gilthead sea bream fed the three experimental diets and exposed to the acute stressor (A.S.) (mg/Kg). Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

Fig. 5.7 The Fe concentration in the spleen of the gilthead sea bream fed the three experimental diets and exposed to the chronic stressor (C.S.) (mg/Kg). Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 5.8 The Fe concentration in the muscle of the gilthead sea bream fed the three experimental diets and exposed to the chronic stressor (C.S.) (mg/Kg). Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.
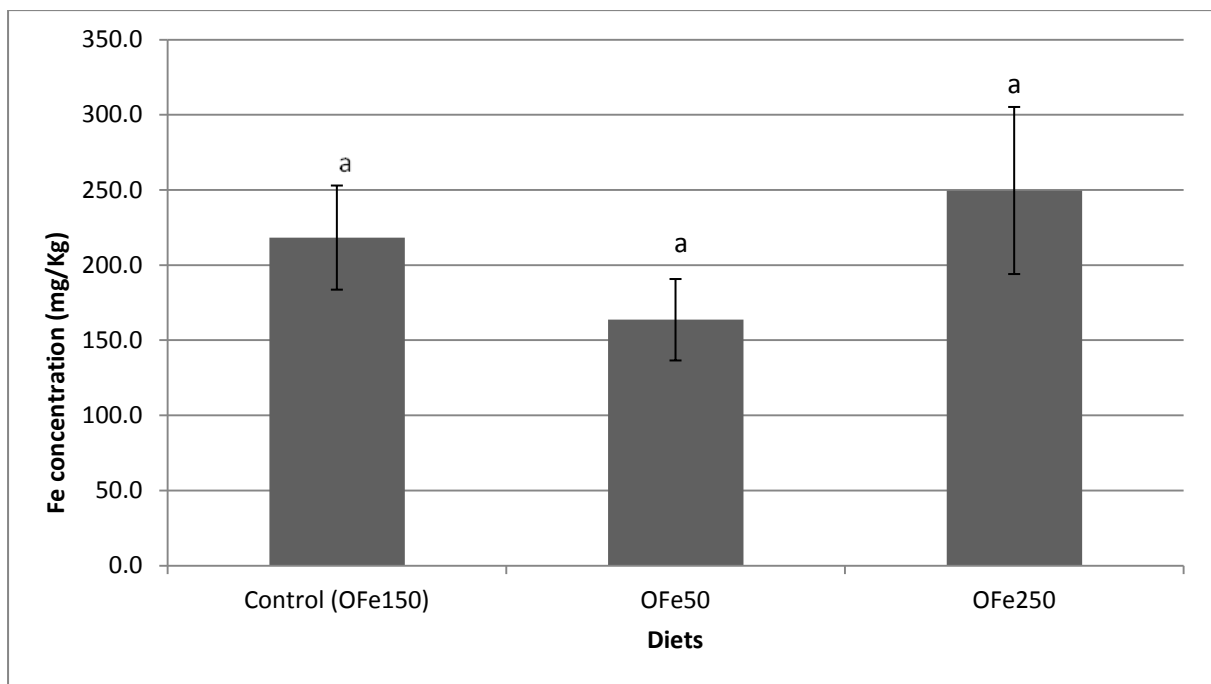
Fig. 5.9 The Fe concentration in the liver of the gilthead sea bream fed the three experimental diets and exposed to the chronic stressor (C.S.) (mg/Kg). Values are the means (n = 23) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.
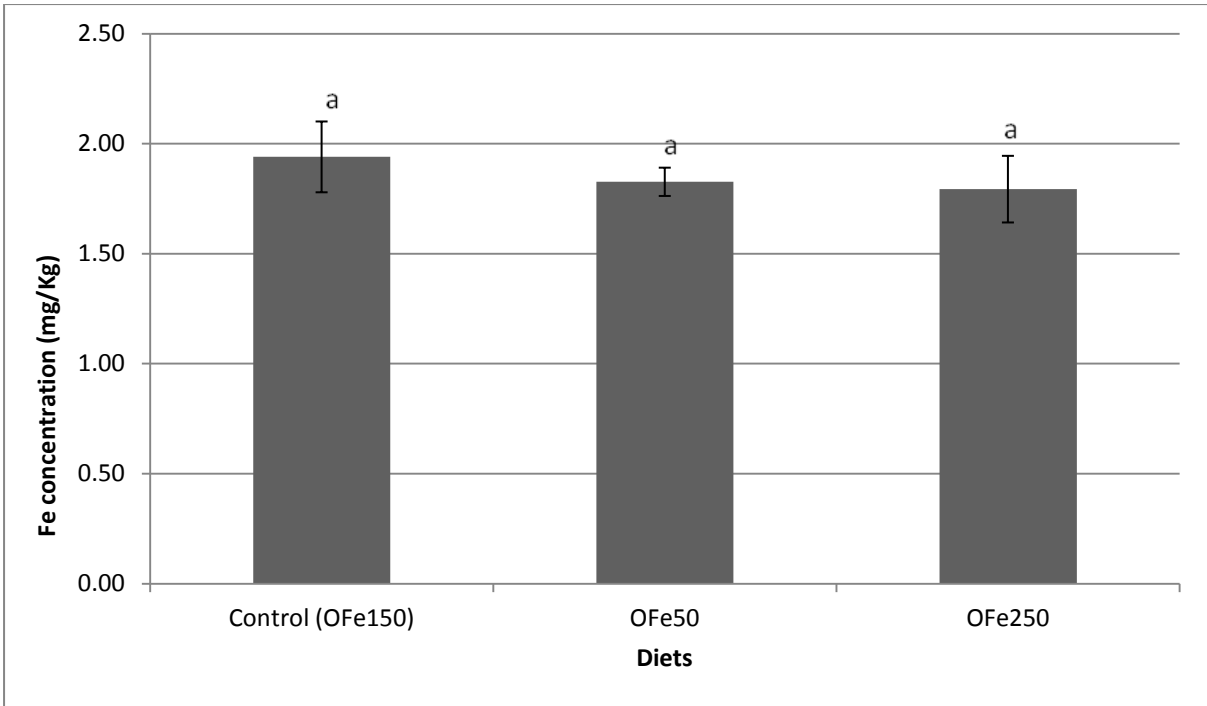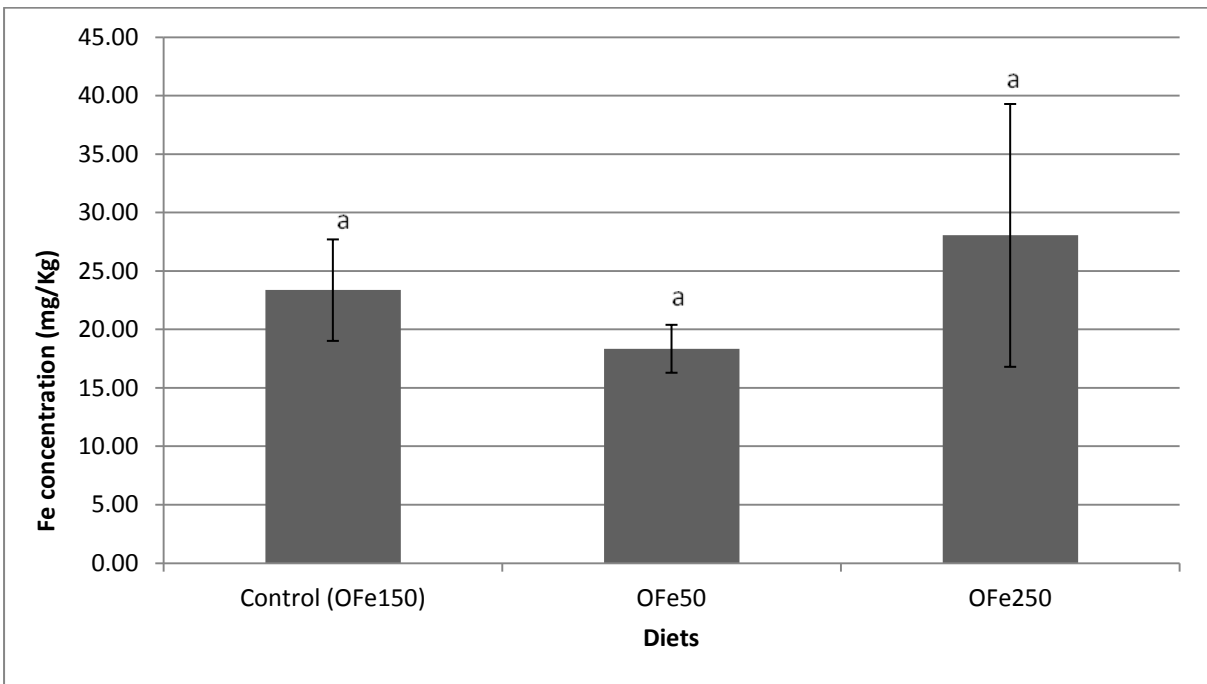
5.3.3 Haematological analysis

The haematological values that were analysed were the haematocrit (Htc), the red blood cell (RBC) counts and the haemoglobin (Hb). The gilthead sea bream fed the experimental diets did not have a significant effect on the haematological parameter when exposed to the acute stressor. No significant differences were observed on Htc ($p=0.067$), RBC counts ($p=0.564$) and Hb ($p=0.704$) between the experimental groups. The fish exposed to the chronic stressor show no significant difference in the Htc ($p=0.707$) and RBC counts ($p=0.682$) of the fish fed the experimental diets. On the other hand the haematological analysis for the fish exposed to the chronic stressor, show that there was significant lower Hb ($p=0.018$) levels on the fish group fed the OFe50 diet compare to the ones fed the OFe250 diets. A general observation in both stressor exposure trials, regardless the statistical

significance show higher values for the control and OFe250 diets compare to the OFe50 diet. Fig. 5.10, 5.11 and 5.12 present the three haematological values (Htc, RBC and Hb) for the gilthead sea bream fed the experimental diets and finally exposed to the acute stressor, while Fig. 5.13, 5.14 and 5.15 present graphically the same haematological values for the fish exposed to the chronic stressor.

Fig. 5.10 The Htc of the gilthead sea bream fed the three experimental diets and exposed to the acute stressor (A.S.) (%). Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

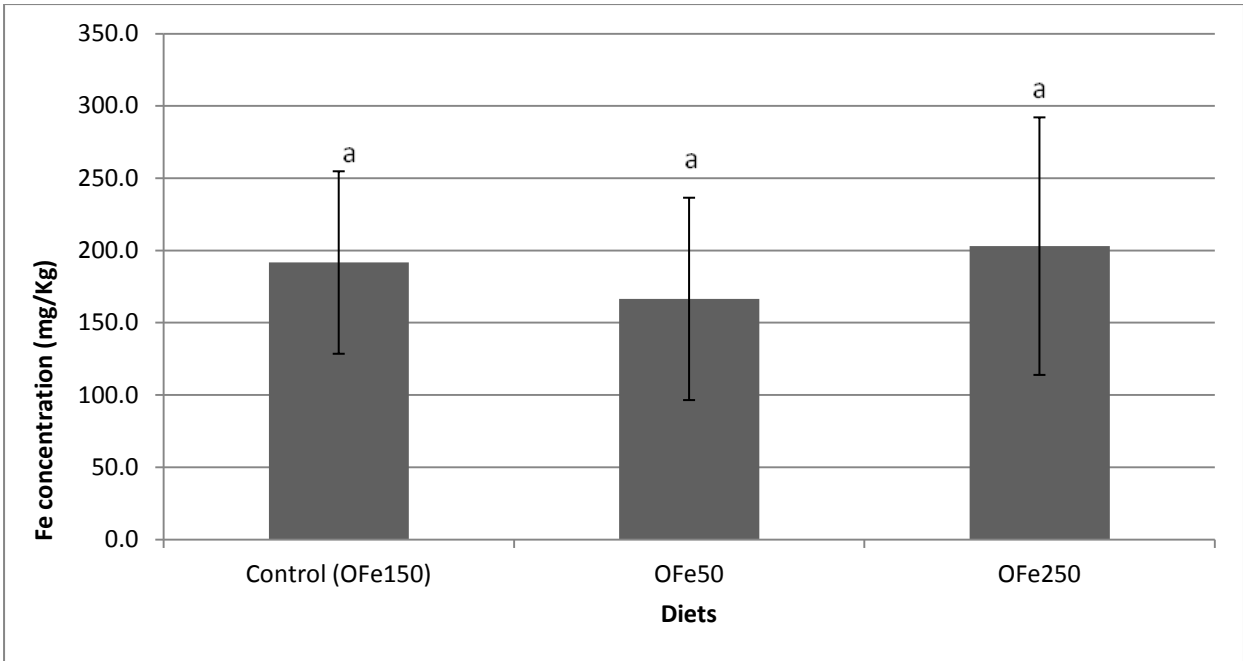Fig. 5.11 The RBC counts of the gilthead sea bream fed the three experimental diets and exposed to the acute stressor (A.S.) (10^5mL^{-1}). Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.
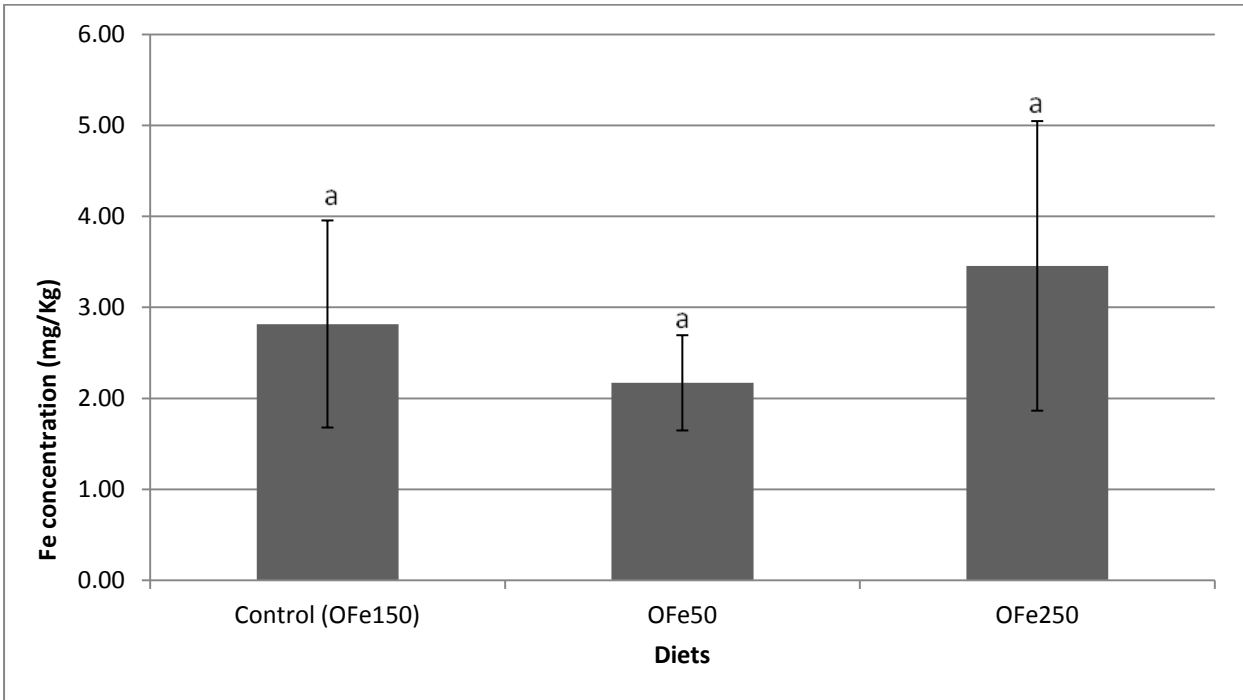

Fig. 5.12 The Hb of the gilthead sea bream fed the three experimental diets and exposed to the acute stressor (A.S.) (g/dL). Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

Fig. 5.13 The Htc of the gilthead sea bream fed the three experimental diets and exposed to the chronic stressor (C.S.) (%).Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.
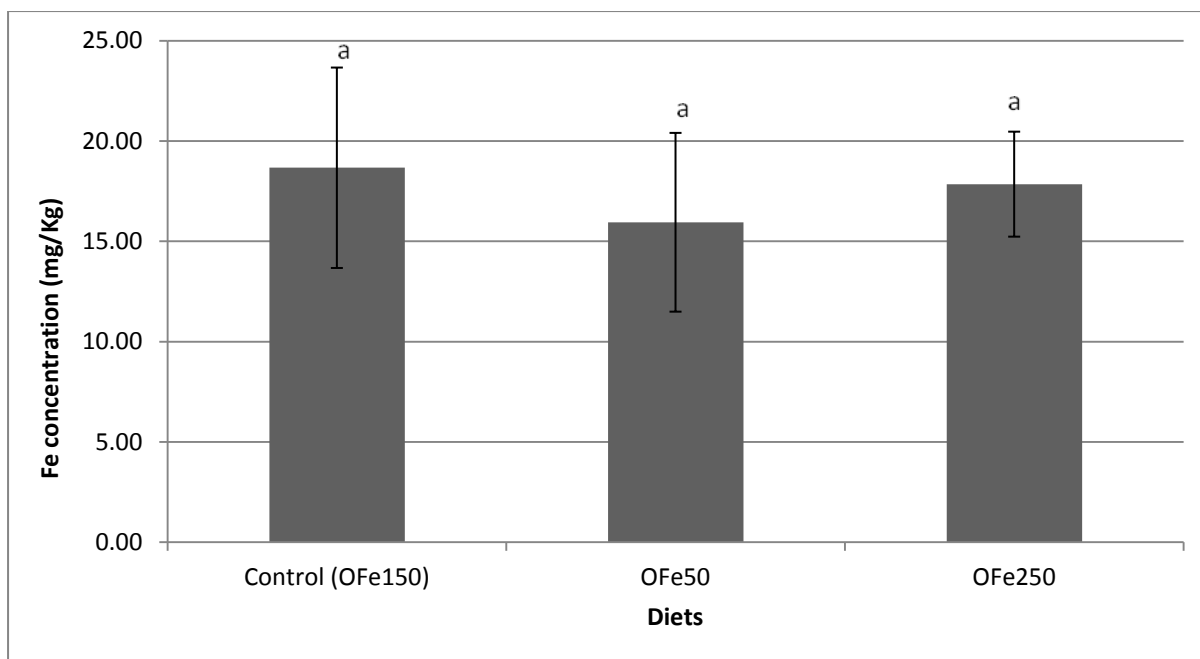

Fig. 5.14 The RBC counts of the gilthead sea bream fed the three experimental diets and exposed to the chronic stressor (C.S.) ($10^5 mL^{-1}$). Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.
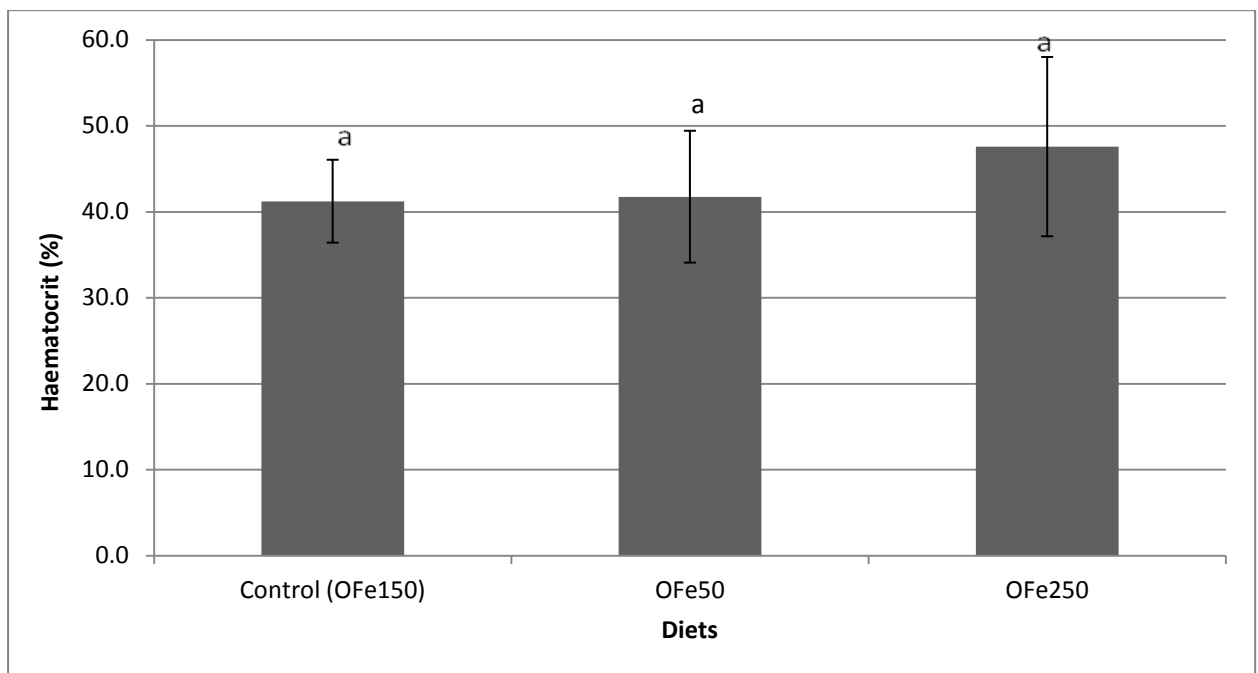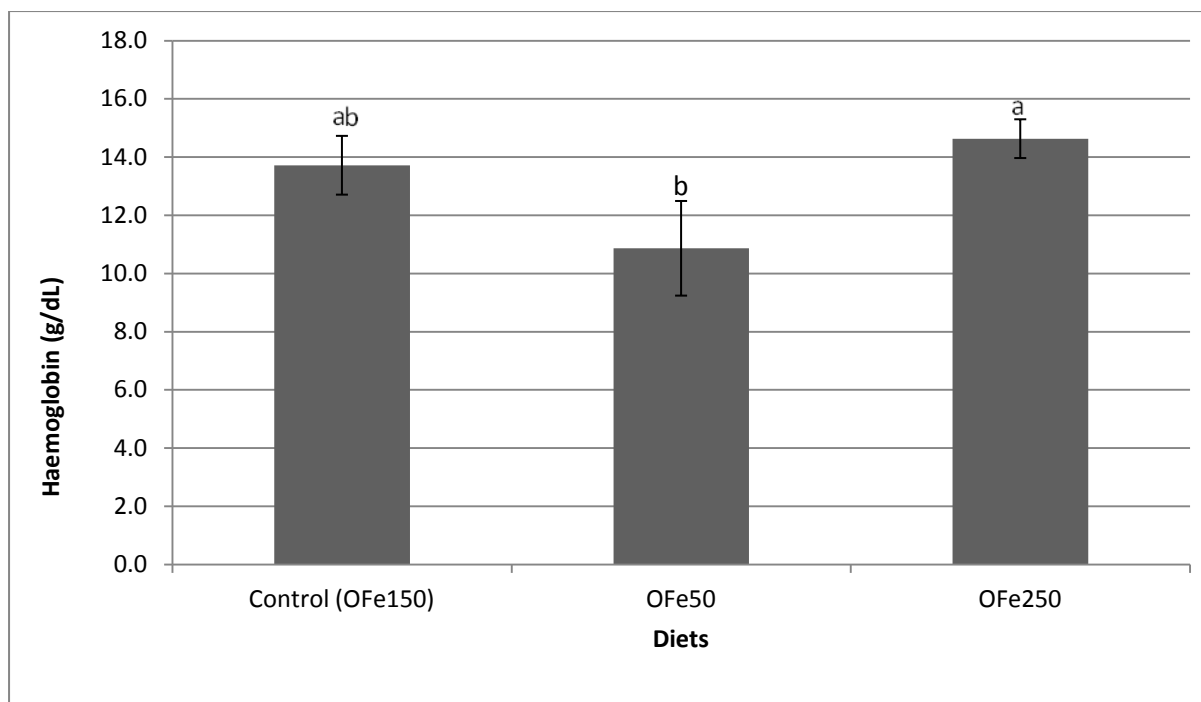
Fig. 5.15 The Hb of the gilthead sea bream fed the three experimental diets and exposed to the chronic stressor (C.S.) (g/dL). Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

5.3.4 Immunological analysis

The immunological parameters analysed consist of two types. The first examines the humoral immune system of the fish and the second the cellular immune system, the antibacterial activity of serum and the respiratory burst respectively. These immunological parameters were measures for both trials where the fish exposed to the chronic and acute stressors. The fish groups fed the experimental diets and exposed to the acute stressor did not show a significant effect on the antibacterial activity in the serum (p=0.416). Absence of significant difference were revealed in the respiratory burst measurements of zymosan (p=0.341) and PMA (p=0.300). However, it is worth mentioning that in both parameters the control diet show higher values. Fig. 5.16 and 5.17 present the immunological values and the graphical presentation of the findings. Absences of

significant difference also occur in the gilthead sea bream exposed to the chronic stressor. The antibacterial activity of serum (p=0.373) was not affected by the different organic iron levels the fish groups fed upon. The zymosan (p=0.078) and PMA (p=0.325) show that the respiratory burst activity in the fish fed the experimental diets and exposed to the chronic stressor was no affected by the different organic iron levels in the treatments. The large standard deviation could be the reason that the OFe250 diet appear to be the lowest one in the antibacterial activity and zymosan analyses. Fig. 5.18 and 5.19 show the immunological values of the fish exposed to the chronic stressor.
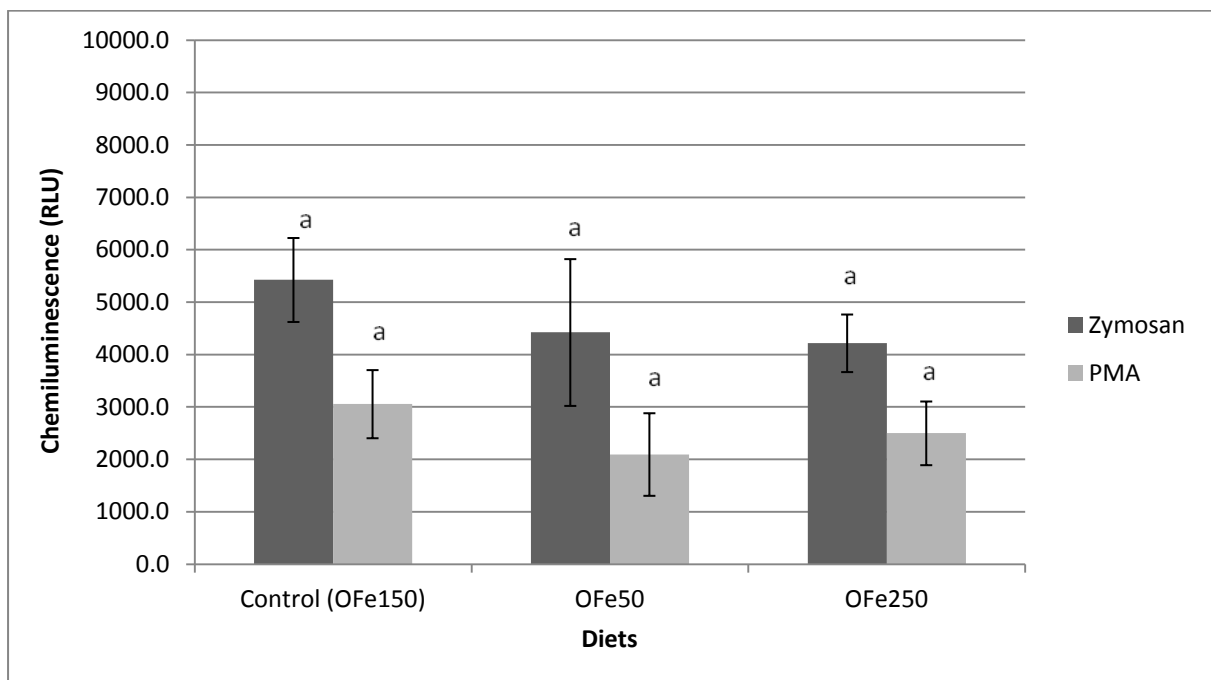
Fig. 5.16 The chemiluminescence of the gilthead sea bream fed the three experimental diets and exposed to the acute stressor (A.S.) (RLU). Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.
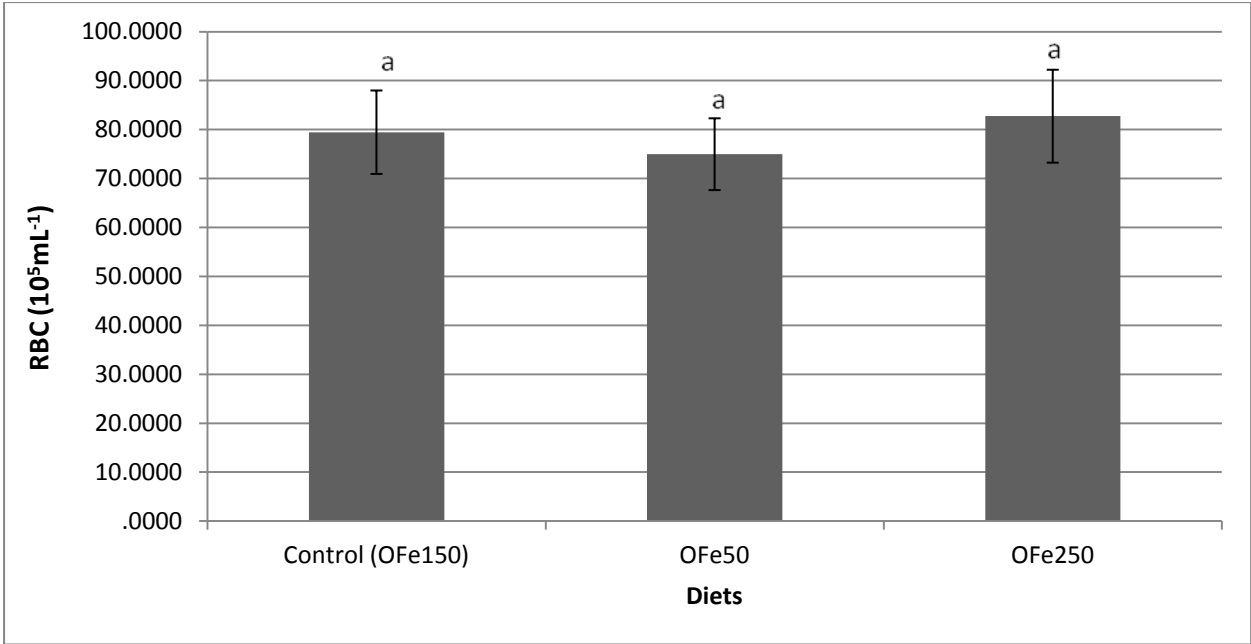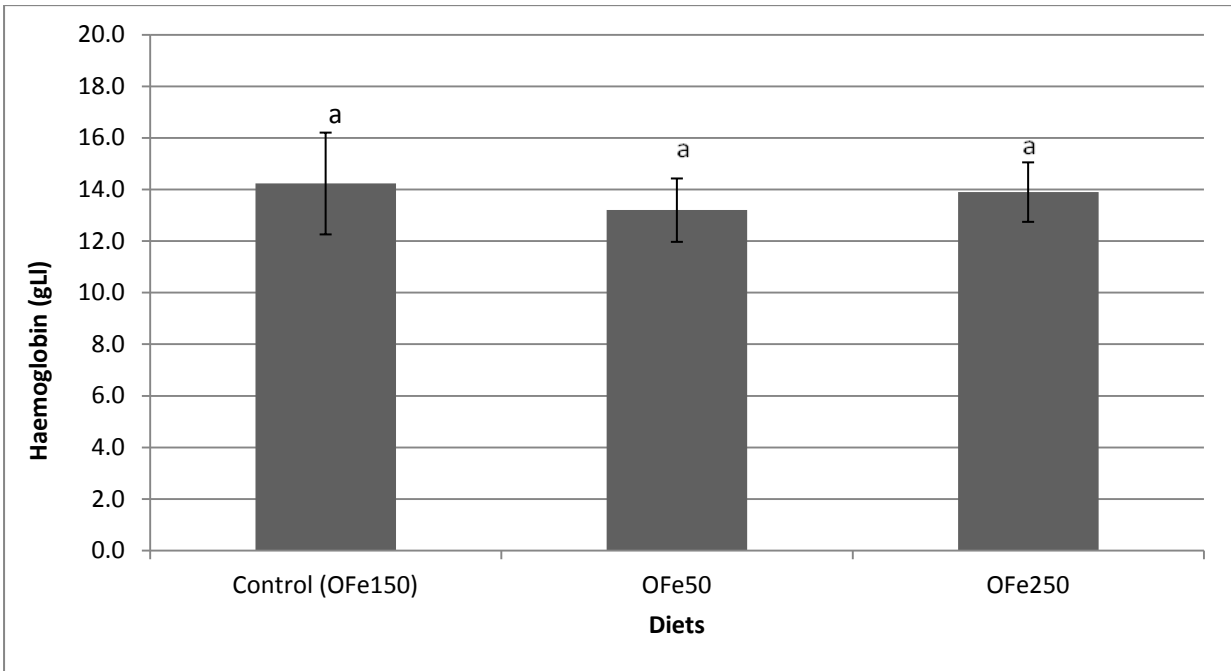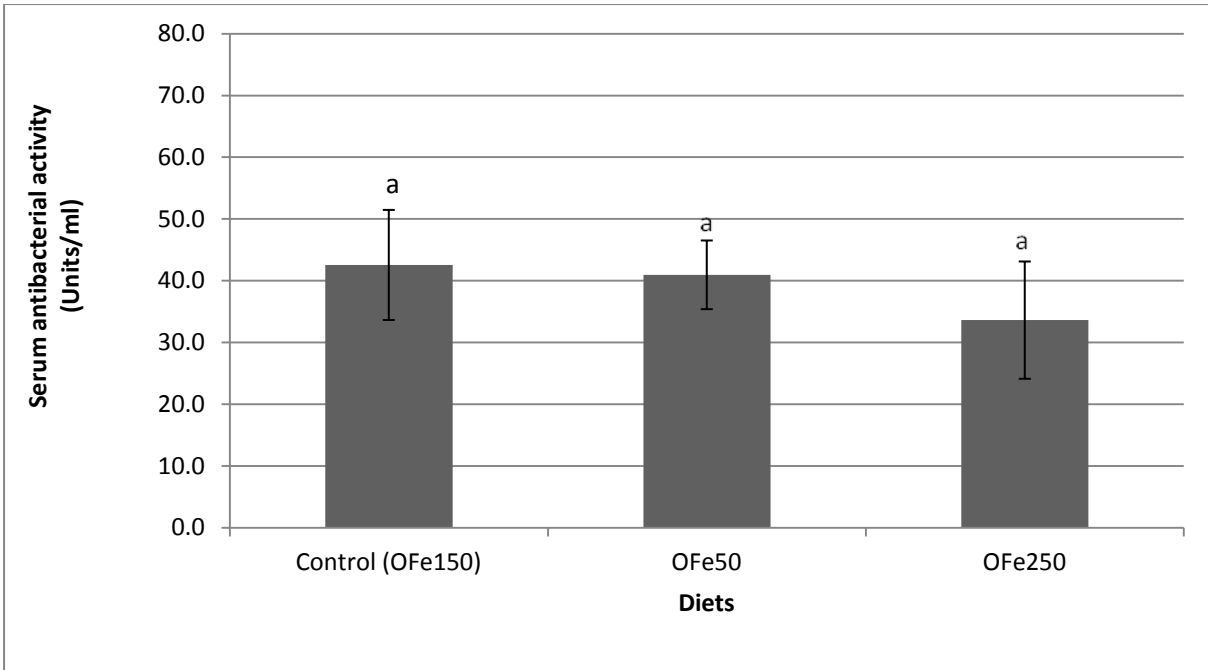
Fig. 5.17 The antibacterial activity of serum of the gilthead sea bream fed the three experimental diets and exposed to the acute stressor (A.S.) (Units/mL). Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.
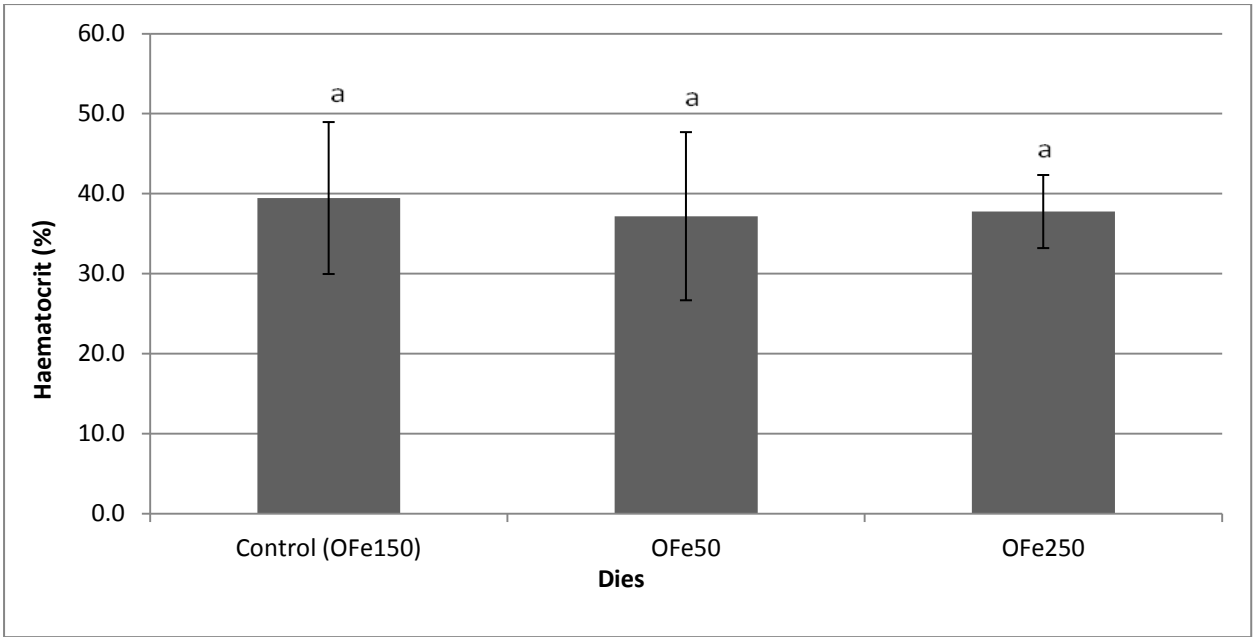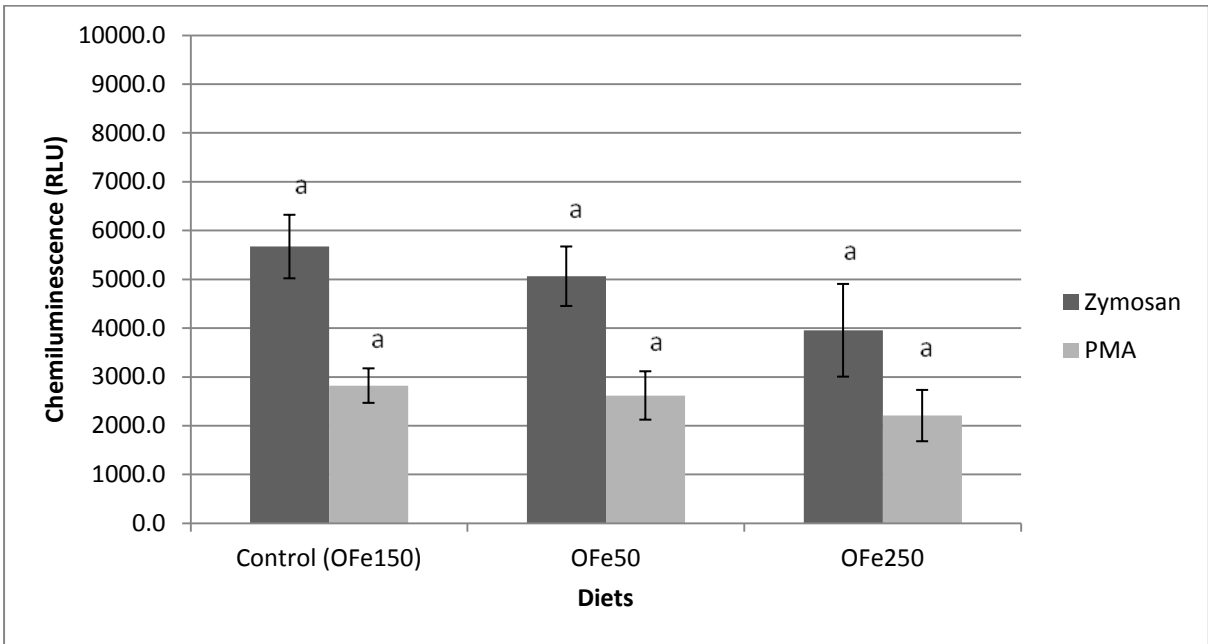

Fig. 5.18 The chemiluminescence of the gilthead sea bream fed the three experimental diets and exposed to the chronic stressor (C.S.) (RLU). Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.
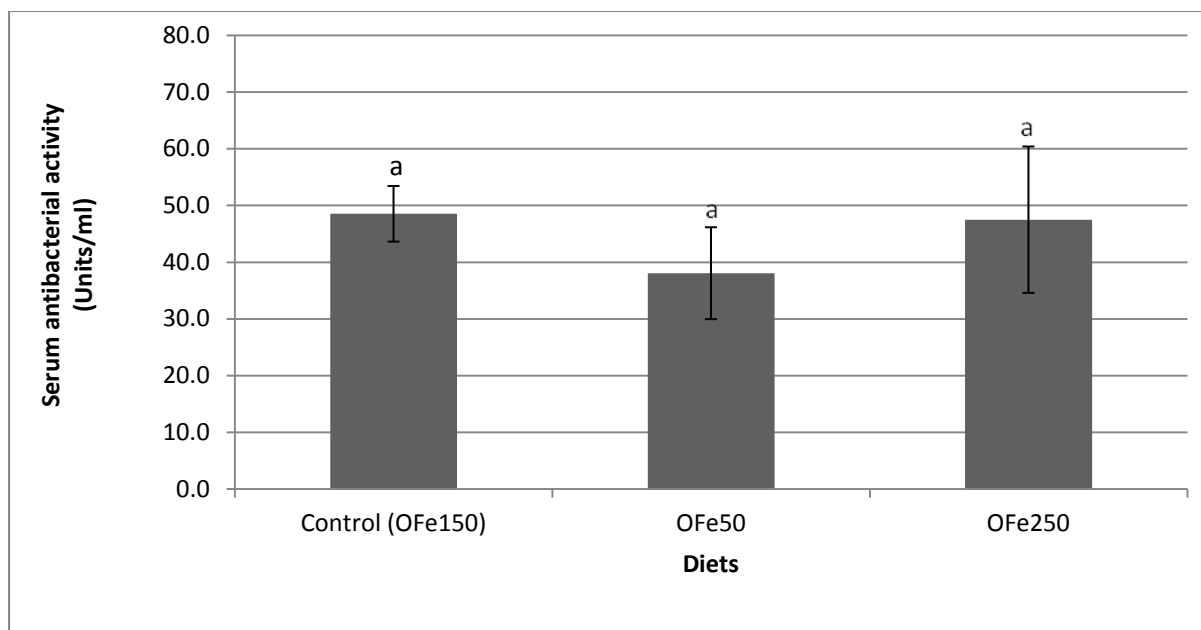
Fig. 5.19 The antibacterial activity of serum of the gilthead sea bream fed the three experimental diets and exposed to the chronic stressor (C.S.) (Units/mL). Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p < 0.05$.
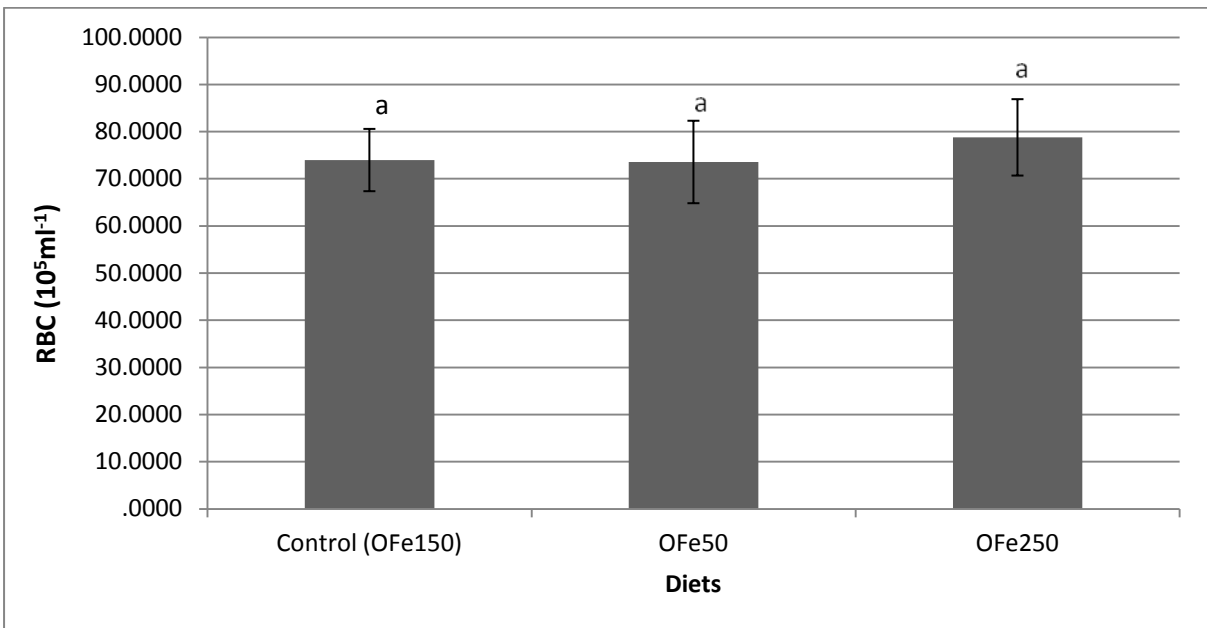
5.4 Discussion

In some cases commercial aquaculture is often closely associated to poor fish husbandry. Thus, stressors are almost unavoidable in intensive fish farming conditions. Some common stressors related to aquaculture are handling, sorting, grading, transporting, crowding and poor water quality. These stressors can result from massive mortalities to suppressed disease resistance and growth rate, which in all cases lead to suboptimal production (Tort *et al.,* 2011; Schreck, 1982; Fagerlun, 1995; Barton & Iwama, 1991). According to the fish farmers, two of the most common stressors in the Greek aquaculture industry are the overcrowding and the netting of the fish (Nengas, personal communication). These stressors found in commercial aquaculture practices are associated with oxygen deprivation. The hypothesis is that the supplementation of organic Fe in the diets of gilthead sea

bream could improve fish health and resistance, to the effects of hypoxic and anoxic conditions, due to Fe active role in oxygen transport and cellular respiration (Lall, 1989). The experimental diets contained as main ingredients mineral free casein from bovine milk and wheat meal, and the only difference among the three formulations was the levels of organic Fe supplementation ranging from 50, 150 and 250 mg/Kg.

The growth performance of the fish was measured and analysed for both stages. First the acute oxygen deprivation stressor by netting the fish and second a chronic stressor by elevating stocking density in the tank (overcrowding). The acute oxygen deprivation stressor did not show any significant difference on the fish growth performance as expected, due to the acute exposure to the stressor at the end of the experiment. The fish exposed to the elevated stocking density (chronic oxygen deprivation stressor) did not show any growth reduction, neither in the final weight nor in the SGR and FCR. As observed in the first experiment and the related literature, when the fish Fe requirements are met (Table 5.2) the growth of the fish is not affected (Andersen *et al.,* 1996, 1997, 1998; Rigos *et al.,* 2010) not even through the duration of the second stage that they were exposed to the overcrowding stressor for one week. In sea bream, both acute and chronic stress causes a clear reduction in growth hormone (GH) levels (Rotllant *et al.,* 2000a, b, 2001), though the absence of growth reduction can indicate that the stressors were not significant to affect the fish growth performance. Studies have shown that high stocking density produce, in several cultured sparids, poor feed utilization, poor growth, chronic stress, alterations in metabolism and even mortality (Rotllant *et al.,* 1997; Montero *et al.,* 1999; Sangiao-Alvarellos *et al.,* 2003). The limited number of studies with low fish stocking density manipulation under controlled water quality is an unfortunate

event (Pavlidis & Mylonas, 2011). Though, recent studies have shown that the overstocking effect on intensively reared aquaculture fish is mediated through the available space for the needs of the cultured species, the water quality and social interactions (Ellis *et al.,* 2002; Fanouraki *et al.,* 2007; Turnbull *et al.,* 2008). Fanouraki *et al.* (2007) observed that, when group and tank sizes were reduced, even at a low stocking density, at 5 Kg m^{-3}, the maintenance cost of red porgy (*Pagrus pagrus*) is higher than the one at high stocking density of 25 Kg m^{-3}.

The Fe concentration analysis in the liver, muscle and spleen of the gilthead sea bream fed the experimental diets were not significant different. The 30 second exposure to the acute stressor did not present any effect of the Fe concentrated in the selected tissues. Hence, the fish fed the three experimental diets did not have a significant effect in the spleen, muscle and liver of the fish groups analysed while exposed to the acute oxygen deprivation stressor. The gilthead sea bream fed the three organic Fe supplemented diets and exposed to the elevated stocking density (chronic oxygen deprivation stressor) show no significant difference in the spleen, muscle and liver that were analysed. A similar trend in the Fe concentration observed in both acute and chronic stages as well as Fe concentration in the tissues increased as the supplementation of organic Fe increased in the experimental diets. This is in agreement with studies where the higher iron supplementations in the diets of Atlantic salmon and sea bream species show no significant differences in the amounts of stored iron in the tissues (Andersen *et al.,* 1996; Rasmussen, 1994; Rigos *et al.,* 2010). The exposure of the gilthead sea bream to the acute and chronic oxygen deprivation stressors for 30 second and 1 week respectively, probably wasn't enough time as they were previously fed with the Fe supplemented diets for 12

weeks. It should be highlighted that as Baker (1986) observed fish need no less than 4 week in order to reduce the body stores of Fe.

The haematological and immunological parameter were analysed through blood samples from the gilthead sea bream, after the completion of the fish exposure to both acute and chronic oxygen deprivation stressors. The gilthead sea bream groups fed with the experimental diets and exposed to the acute oxygen deprivation stressor did not show any significant difference in any of the haematological parameters measured (Htc, RBC counts and Hb). On the contrary, the fish groups fed with the OFe50 diet and exposed to the elevated stocking density stressor for a week show a significant reduction on the haemoglobin levels compared to the ones fed with the OFe250 diets. The Haematocrit and red blood cell counts did not show a significant difference among the fish fed with the three experimental diets and exposed to the elevated stocking density stressor for 1 week. Furthermore the immunological analysis did not show a significant effect on the parameters analysed at either of the two stages of the experiment. The fish groups exposed to the acute oxygen deprivation stressor were not affected by the different organic Fe supplementation levels in the diet, neither in the humoral nor in the cellular immune system analyses, where antibacterial activity in the serum, the respiratory burst measurements for zymosan and for PMA p values were lower than 0.05. The gilthead sea bream groups which were overcrowded for 1 week time also did not show any significant difference in the antibacterial activity of serum or the respiratory burst activity, while zymosan and PMA. Literature suggests that husbandry stressors related to aquaculture can have an effect on the haematological profile and the immunological functions of sparidae (Pavlidis & Mylonas, 2011). However in the present study the absence of cortisol level measurements could neither rule out nor

support the possible effect of stress in the findings. The only significant reduction observed in the haemoglobin levels of the fish fed the OFe50 diet, which indicates that the organic Fe supplementation should be higher than 50 mg/Kg in order to meet the gilthead sea bream haematological requirements. The acute oxygen deprivation stressor to the fish groups probably was too short to cause any significant effects on the immunological and haematological parameters. Studies in acute stressor exposure (background and netting) of red porgy species lasted 2 hours in order to show effects (Van Der Salm *et al.*, 2006),while air exposure and confinement in sea bream lasted for 30 minutes (Arends *et al,.* 1999). On the other hand the absence of significant difference in the immunology, Htc and RBC counts in the fish exposed to the elevated stocking density stressor, literature indicates that fish may adapt to hypoxic conditions considering their evolutionary history and that enables them to cope with the unavoidable aquaculture stressors in a better way and have better chances of survival (De Almeida-Val *et al.,* 2006). Generally, sparids seem to tolerate husbandry related stressors better than other aquaculture species. In sparidae species, the response to the human presence is considered to be of lower avoidance reaction. For instance, fish farmers may perform grading exercises in the gilthead sea bream species with minor disturbance and losses. One of the reasons that scientist explain such a difference may be either genetic or because of a faster process of domestication (Pavlidis & Mylonas, 2011).

To conclude the only significant difference observed in the current experiment was the increased haemoglobin levels of the fish fed the OFe250 diet compare to the fish fed the OFe50 while exposed to the overstocking stressor for a week. All the other parameters analysed show no significant differences. An dietary organic Fe suplementation in the diet of sea bream appears to have an positive effect on the

fish while coping with oxygen deprivation stressors related to poor husbandry. Although future experiments could apply stressors in sea cages more realistic to an aquaculture environment. In addition a more in depth study in the interactions between different trace minerals and the effect on the fish could give a better understanding of the role of dietary organic Fe in the gilthead sea bream.

Chapter 6 Organic iron in the diet of gilthead sea bream and the interaction with various levels of zinc and copper.

6.1 Introduction

Fish require minerals in their normal life processes. These can be derived from ambient water although feeds considered being the major source of iron in fish (Watanabe *et.,* 1997; Lim *et al.,* 2000). It has been established that trace minerals are essential and have beneficial effects in animal nutrition and especially fish farming. Trace elements like Fe, Zn and Cu are essential for fish and serve important functions in their living cells (Lall & Bishop, 1977; House, 1998; Lorentzen & Maage, 1999). Studies in human and animal science confirm that there are several dietary mineral–mineral interactions, presumably through competition for absorption binding sites (Thomson & Valberg, 1972; Sandström *et al.,* 1985; Davis & Mertz, 1987; Hurley & Keen, 1987; Morris, 1987). Iron is considered to be one of the most important micronutrients due to its effect on immune system functions and host defence against infections (Beisel, 1982; Bhaskaram, 1988). It has an important role in oxygen transport and cellular respiration, so it is essential for the functioning of organs and tissues of fish (Lim *et al.,* 2000). Equally important elements for farmed fish are Zn and Cu. Zn is an important trace element, as it is presented in all organs, tissues and fluids and acts as a stabilizer of membranes and cellular components, although there is no specific storage in the body of the fish (Chvapil, 1973; Vangen & Hemre, 2002). While Cu, is essential nutrient for fish because it is involved in the activity of enzymes, it also has metabolic roles, moreover it is important for iron

metabolism as part of antioxidant and electron transport enzymes (Lorentzen *et al.,* 1998; Watanabe *et al.*, 1997). It plays a crucial role in Fe metabolism as it is needed in animals in order to utilize Fe. Studies in swine and rats with Cu deficiency have shown that they develop anaemia similar to those of a Fe deficiency status. So it is suggested that there is a direct connection between the morphological and biochemical similarities, between iron deficiency anemia and copper deficiency anemia, so that was due to a defect in iron metabolism. As a result there is a relation in the role copper plays in anemia, due to the mobilization of the tissue iron, the formation of the mitochondrial heme, and the reduction of erythrocytes' half-lives (Ramirez-Cardenas *et al.*, 2005; Smith & Medlicott, 1944; Lahey *et al.*, 1952; Saari *et al.*, 1995). In fish the whole-body Cu and Fe status are known to be negatively influenced by high levels of Zn in the diet (Knox *et al.,* 1984; Eid & Ghonim, 1994), but the mechanism involved remains unclear (Bury *et al.,* 2011). In human nutrition, high Zn levels can adversely affect the hematological parameters which are related to the inhibition of Fe and Cu uptake in the gut (Maret & Sandstead, 2006; Stefanidou *et al.,* 2006). Ingestion of excess Zn in chicks has been shown to reduce tissue Fe and Cu concentrations as well as reduce hematocrin and hemogloibn values leading to anaemia (Pimentel *et al.,* 1992). Due to the limited study and knowledge of trace mineral function it is generally assumed that they are similar to those in other vertebrates (Lall, 1989).

The aim of the this study is to identify and evaluate how the high and low levels of dietary Cu and Zn could affect the sea bream species that fed diets with suggested organic Fe (150 mg/Kg). The parameters that will be examined are the growth of the fish, the selective mineral concentrations in the liver spleen and muscle of the fish, as well as the haematological and immunological factors.

6.2 Materials and Methods

6.2.1 Experimental conditions

Juvenile sea bream, with an initial average weight of 51±3 gr., were obtained from a fish farm near Larisa in the Malian gulf and transported to the H.C.M.R. facilities in Agios Kosmas, Athens. The fish were acclimatized for 4 weeks in an unsupplemented diet in order to reduce the body stores of Fe (Baker, 1986). Then the fish were distributed into 15 experimental cylindroconical fibre glass tanks of 250 L capacity. Each tank contained 26 fish randomly distributed. The total duration of the experiment was 12 weeks from August until November during which triplicate groups of fish fed the five experimental diets four times per day. The photoperiod started at 08:00 in the morning and lasted until 19:00 in the afternoon (11:13, light:dark). The amount of feeds fed to gilthead sea bream in each daily feeding schedule was close to satiation. The water was supplied by a pump from the sea and filtered by a multilayer filter before ending in the tanks. The flow rate was approximately 1.9 L/min and the liquid oxygen that was supplied at each individual tank was 7 ppm. During the experiment the temperature was maintained between 22°C ±2 and the salinity of the water in the experimental tanks was 38‰.

6.2.2 Diet Formulation

The experimental diets had a total number of five semi-purified diets; these were the Control, OFeCuMin, OFeCuMax, OFeZnMin and OFeZnMax. The dominant ingredients were the mineral free casein from bovine milk and the wheat meal in order to minimise the non additive Fe levels without compromising the protein levels

and the consistency of the feeds. The composition of the experimental diets as well as the Fe concentration in each diet is given in Tables 6.1. By using an electrothermal atomic absorption spectrometry we measure the Fe, Zn and Cu levels in each out of the five experimental diets (Table 6.2). The formulation of the diets (especially for Zn and Cu) was based on three factors, the legal limits of mineral addition in the diet of the fish, the lowest levels of minerals that could be reached in the raw materials used without compromising the diets and finally the common practices and knowledge from the sea bream farms in the Greek aquaculture industry. The control diet has a total amount of 150 mg/Kg organic iron as well as 5 mg/Kg organic Cu and 200 mg/Kg organic Zn. OFeCuMin and OFeCuMax had the same amount of organic Fe (150 mg/Kg) as the control while Cu levels was the lowest possible in the first one and the highest allowed by legislation for the second one. The diets OFeZnMin and OFeZnMax had the same amount of organic Fe (150 mg/Kg) and the amount of Zn was the lowest possible for OFeZnMin and the highest allowed by legislation (modified to the meet the Greek aquaculture practices) for OFeZnMax. Each diet was tested in triplication in all experimental diets. The five experimental diets were identical and as a result the chemical composition (%) was the same for all seven of them. The diets used in the current experiment had levels of crude protein at 47.1±0.6%, crude fat at 17.1±0.2% and ash at 6.9±0.2%. The initial and final weights were measured and are demonstrated in Fig. 6.1. Before the beginning of the experiment the fish pellets were crumbled and sieved at 4mm diameter.

Table 6.1 Composition (%) of the five experimental diets.

Diets / Ingredients	Control	OFeCuMin	OFeCuMax	OFeZnMin	OFeZnMax
Fish meal	5	5	5	5	5
Casein	35	35	35	35	35
Wheat meal	27	27	27	27	27
Fish oil	16	16	16	16	16
Corn meal	5	5	5	5	5
Starch	10	10	10	10	10
Premix	0,3	0,3	0,3	0,3	0,3
Cellulose	1,3	1.5	1.5	1.4	1.1
Fe	0,2	0,2	0,2	0,2	0,2
Zn	0,2	0	0	0,1	0,4
Cu	0,005	0	0,01	0	0
Total	100	100	100	100	100

Table 6.2 The levels of Fe, Zn and Cu in each of the five experimental diets.

	Control	OFeCuMin	OFeCuMax	OFeZnMin	OFeZnMax
Fe (mg/Kg)	161.00	164.07	164.03	168.02	163.02
Cu (mg/Kg)	5.01	2.29	8.08	2.27	2.38
Zn (mg/Kg)	208.07	21.25	21.51	50.09	407.01

6.2.3 Sampling

Prior to sampling there was a 24 h starvation period for the fish. At the end of the experiment the fish from each tank was anesthetised in phenoxyethanol at 50 uL/L for 5 minutes so the sampling could take place. During the sampling all fish form the 15 tanks were individually weighed. From a total number of 24 fish from each dietary treatment (8 fish from each of the triplicate tanks) blood was collected (with 1 ml syringe from the caudal vein) for hematological (RBC count, Htc, Hb) and immunological analysis (antibacterial activity of serum, respiratory burst (RB) activity in blood). In addition a total number of 45 fish from each dietary treatment (15 fish from each of the triplicate tanks) were used for Fe concentration analysis in selective tissues (muscle from above the lateral line, liver, spleen). Finally the specific growth rate (SGR) of the fish was evaluated by calculating the final and initial weight of the fish divided by the duration of the experiment using the formula shown in section 2.1.

6.2.4 Data analysis

The evaluation of the data collected from each measured parameter was statistically analysed using the software SPSS 17. The triplicate tanks for each experimental diet were pooled for each parameter analysed. Statistical analysis between dietary groups was performed by One-Way ANOVA followed by a Tukey's multiple range comparison test. In addition tests have been performed such as normality tests and post hoc tests. The level of significance for all tests was set at $p < 0.05$. Finally the graphics prepared in order to present the findings were created using the Microsoft excel software.

6.3 Results

6.3.1 Growth

The results on the growth performance of the sea bream fed the experimental diets are presented below. For each individual diet there are values for the initial weight of the fish during the beginning of the experiment as well as the final weight of the fish at the end of the trial. In addition the SGR and FCR of the fish are calculated and presented as well. The statistical analysis shows that there were no significant differences in the growth of the fish regarding either the initial weight (p=0.634) and final weight (p=0.357) or the SGR (p=0.481) and FCR (0.251). Fig. 6.1 and 6.2 present graphically the initial and final weight of the fish and both the SGR and FCR between the experimental groups, respectively. The mortality rate (p=0.566) of the fish groups fed the experimental diets is presented in Table 6.3.

Table 6.3 The mortality rate (%) in each of the seven experimental groups.

	Control	OFeCuMin	OFeCuMax	OFeZnMin	OFeZnMax
Mortality rate (%)	3.7 ± 2.1^a	3.0 ± 1^a	4.7 ± 2.5^a	6.0 ± 2^a	5.7 ± 1.2^a

Fig. 6.1 Initial and final weights of gilthead sea bream fed the five experimental diets for 12 weeks. Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.
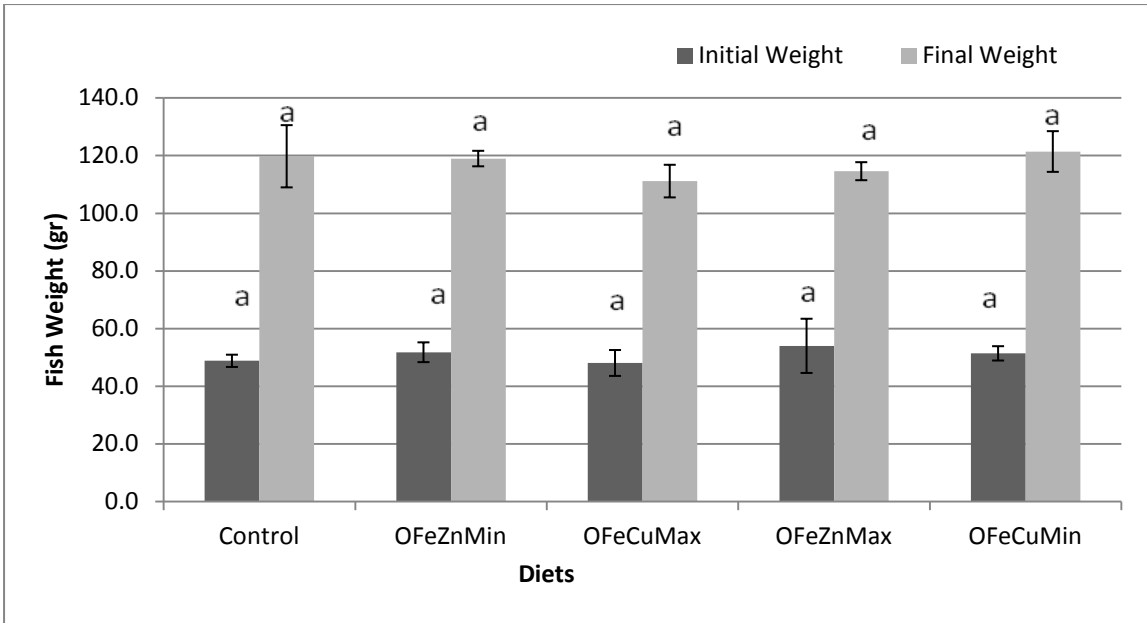

Fig. 6.2 The SGR and FCR of the gilthead sea bream fed the five experimental diets for 12 weeks. Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

6.3.2 Tissue concentration analysis

The specific organs that were selected for the tissue concentration analysis were in the liver, the spleen and the muscle of the sea bream. The liver and muscle was analysed individually for each fish while spleen was pooled due to the small size of the organ. Below there is a detailed presentation of the examined trace minerals Fe, Zn and Cu in each of the selected organs of the sea bream fed upon the experimental diets for 12 weeks. There were no significant difference between the experimental diet groups in none of the concentrations of each trace element. The Fe concentrations in the fish fed the tested diets was not significantly different in muscle ($p=0.199$), liver ($p=0.644$) and spleen ($p=0.239$). Likewise, the Cu concentrations in muscle ($p=0.301$), liver ($p=0.339$) and spleen ($p=0.074$) did not have any significant difference between the experimental diets, however, the OFeCuMax diets displayed the highest values. In the same line, there was no significant difference in Zn concentrations of the selected tissues, muscle ($p=0.261$), liver ($p=0.056$) and spleen ($p=0.092$). The graphic presentation of the tissue Fe, Zn and Cu concentration analysis in spleen, liver and muscle of gilthead sea bream fed the experimental diets for 12 weeks are presented below. Fig. 6.3, 6.4 and 6.5 shows the Fe concentration in each individual tissue. Fig. 6.6, 6.7 and 6.8 shows the Cu concentration in each individual tissue and finally the Zn concentrations in spleen, muscle and liver are shown in Fig. 6.9, 6.10 and 6.11.

Fig. 6.3 Fe concentration in the spleen of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

Fig. 6.4 Fe concentration in the muscle of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.
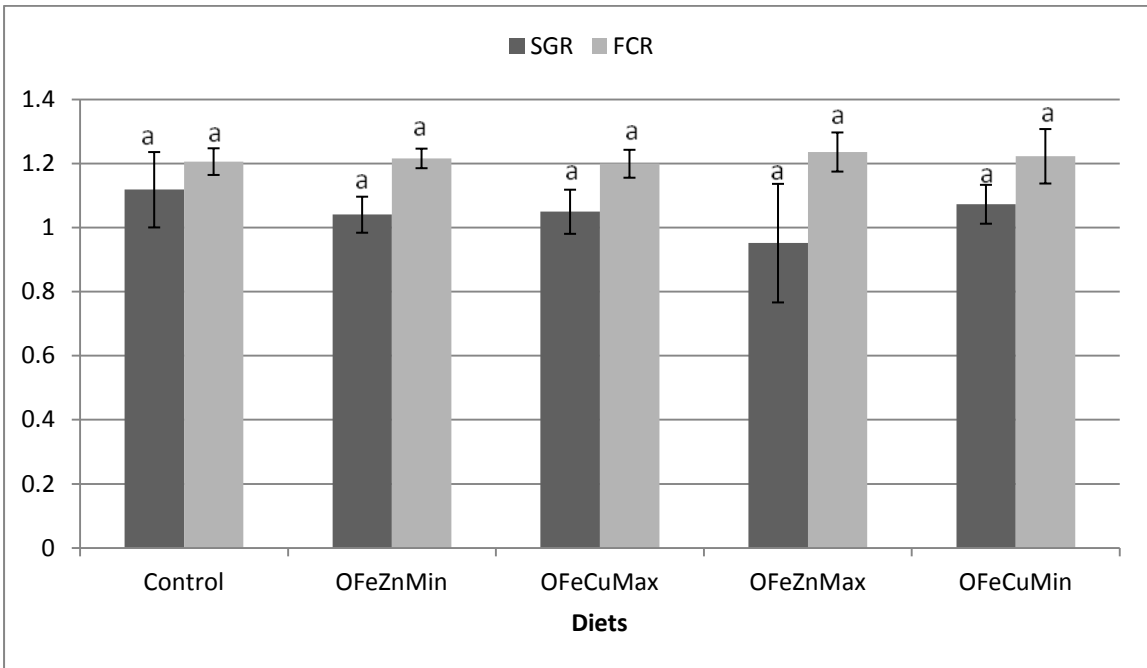
Fig. 6.5 Fe concentration in the liver of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p < 0.05$.

Fig. 6.6 Cu concentration in the spleen of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p < 0.05$.

Fig. 6.7 Cu concentration in the muscle of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

Fig. 6.8 Cu concentration in the liver of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.

Fig. 6.9 Zn concentration in the spleen of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

Fig. 6.10 Zn concentration in the muscle of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.
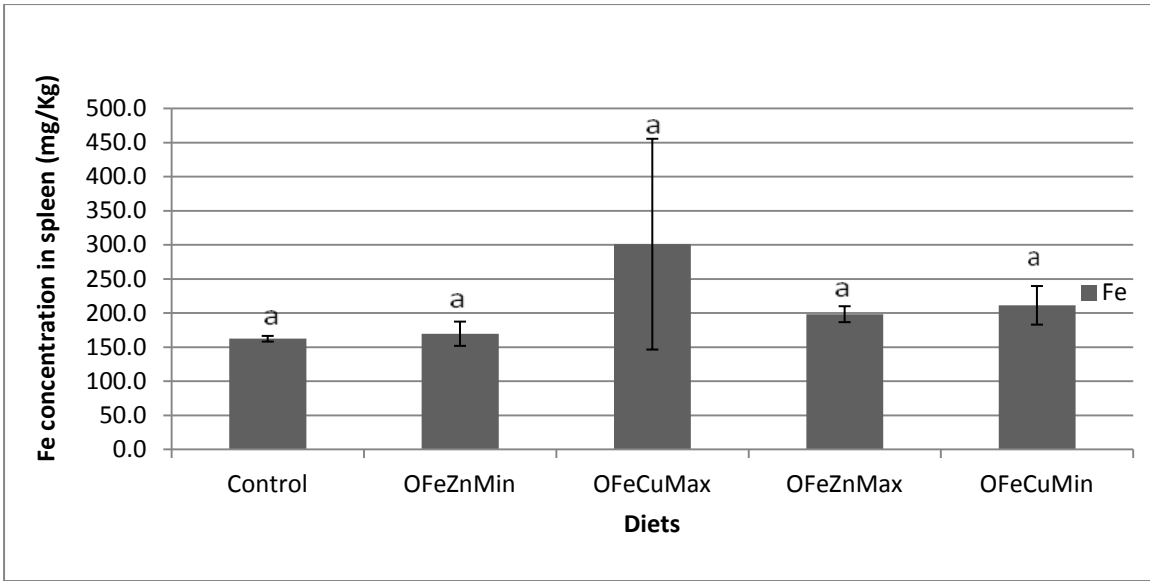
Fig. 6.11 Zn concentration in the liver of gilthead sea bream fed the experimental diets for 12 week (mg/Kg). Values are the means (n = 45) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.
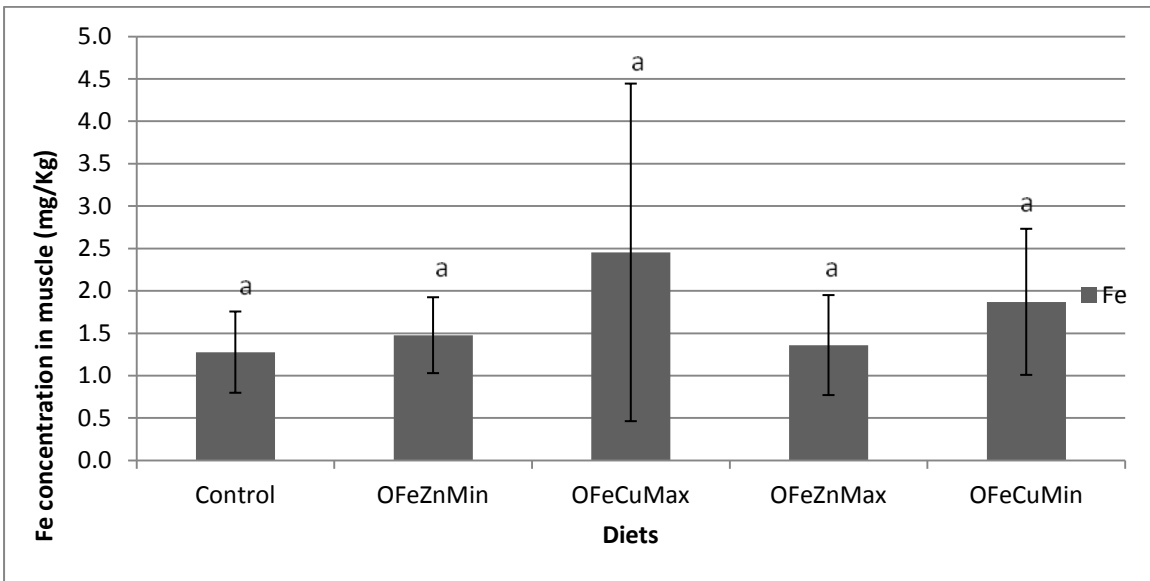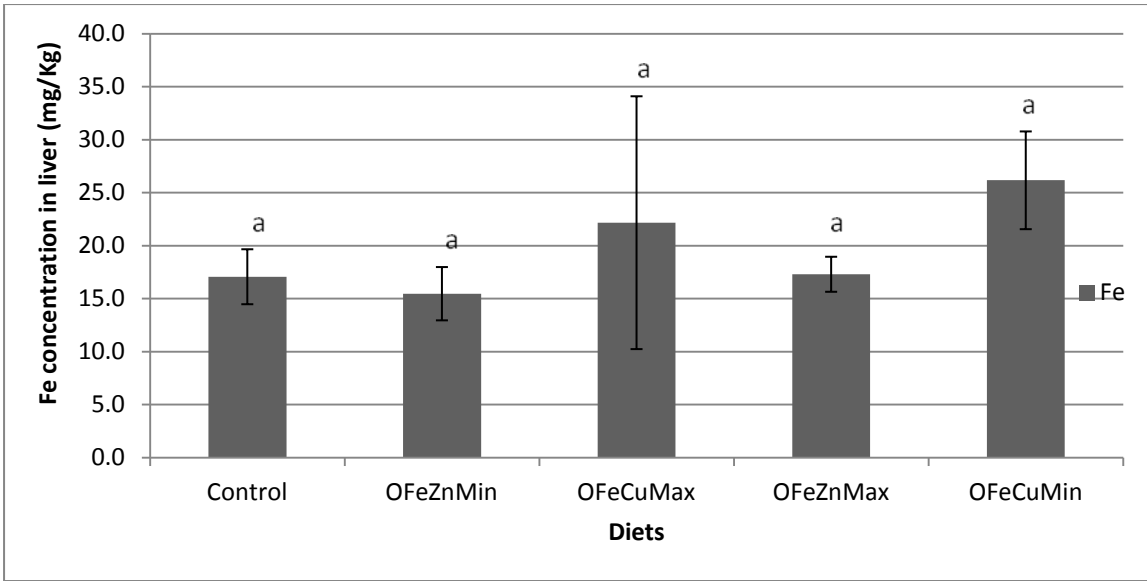
6.3.3 Haematological analysis

The haematological analysis includes the analysis of red blood cell (RBC) count, the haematocrit (Hct) and haemoglobin (Hb). The figures below present the values for each haematological parameter. The RBC in fish were not significantly different (p= 0.317) between the experimental groups. The Htc values shows that there was a significant difference (p= 0.001) in fish fed OFeZnMin, OFeCuMin, OFeZnMax diets comparing to the fish receiving the OFeCuMax feed. The control diet wasn't significantly different with any other experimental diet. Significant differences were also observed in the Hb (p= 001) as the gilthead sea bream fed OFeCuMin and OFeZnMax had higher Hb values compare to the ones treated with the Control and OFeCuMax diets. In addition the fish fed with OFeCuMax had also significantly lower Hb levels compared to the fish that received the OFeZnMin diet. Fig. 6.12, 6.13 and 6.14 demonstrate graphically the above findings.

Fig.6.12 Effects on the red blood cell count in gilthead sea bream fed the experimental diets for 12 week (10^5mL^{-1}). Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

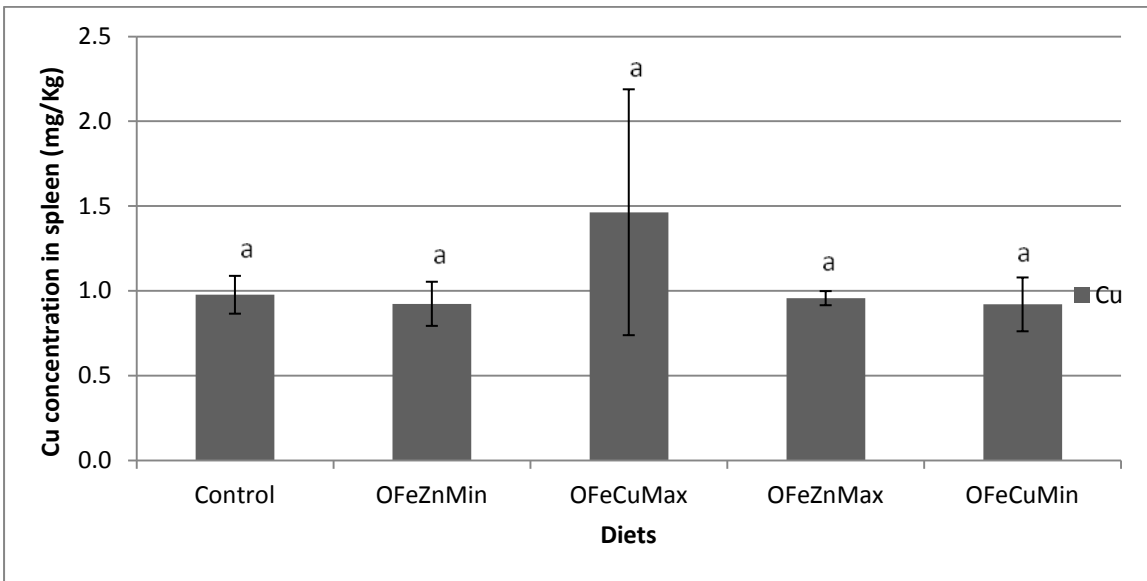Fig.6.13 Effects on the haematocrit in gilthead sea bream fed the experimental diets for 12 week. Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.

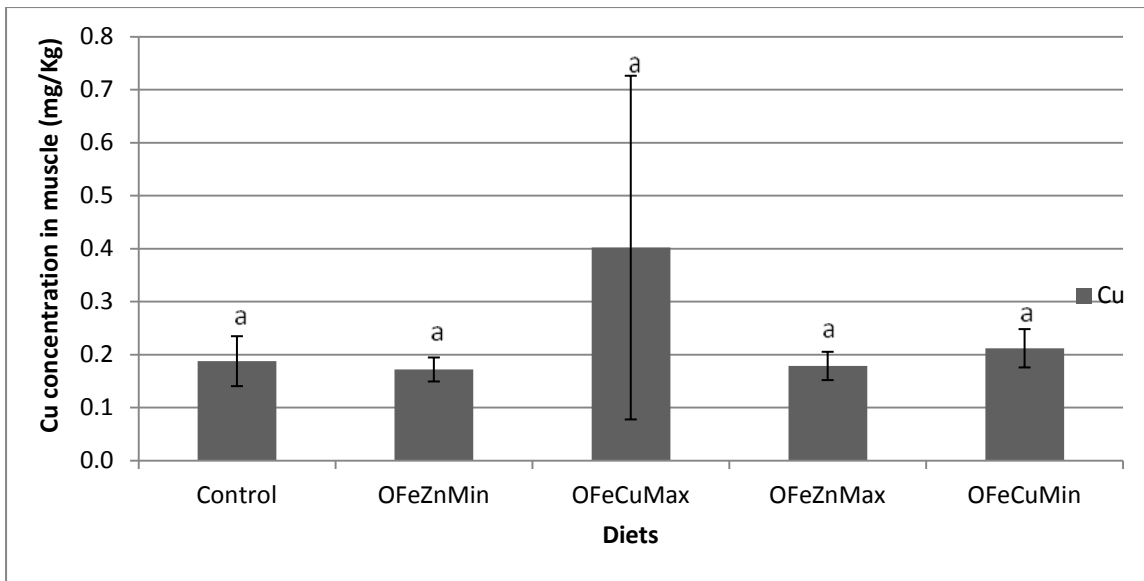Fig. 6.14 Effects on the haemoglobin in gilthead sea bream fed the experimental diets for 12 week. Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p < 0.05$.
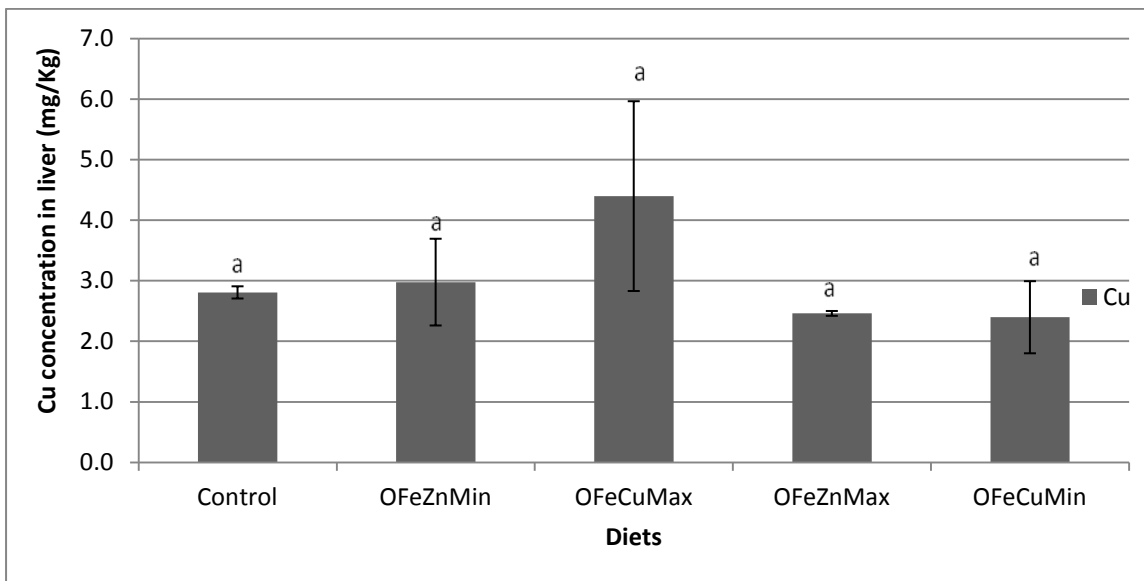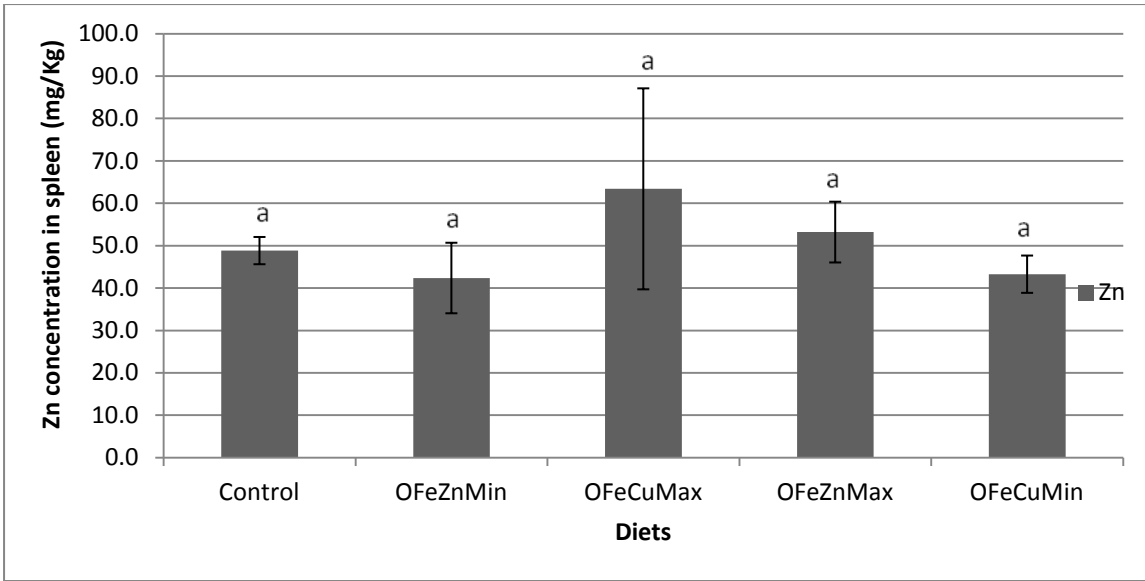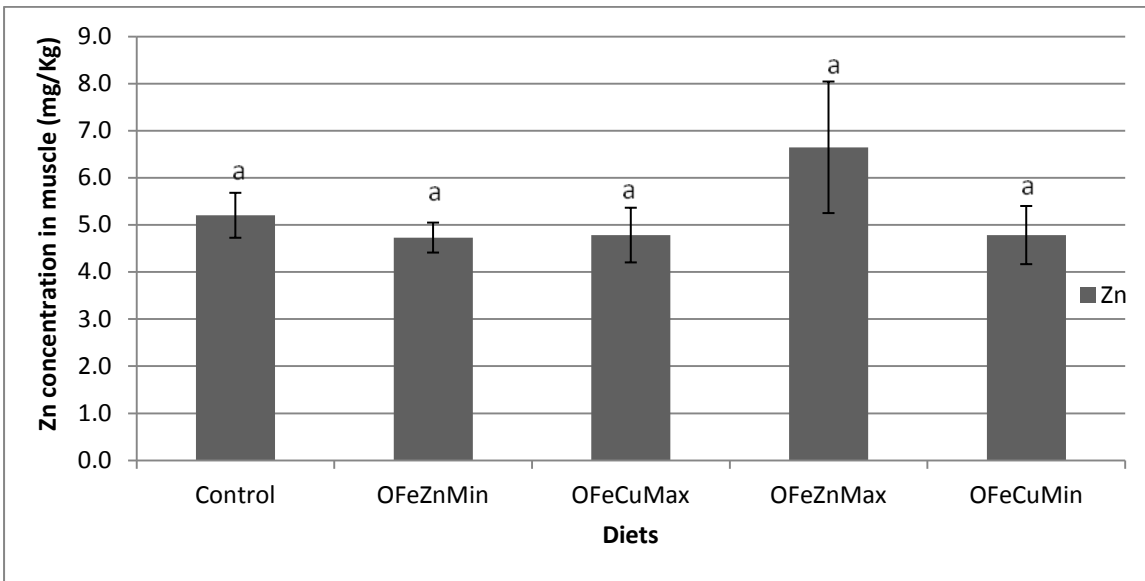
6.3.4 Immunological parameters

The immunological analyses in the current experiment reveal that the gilthead sea bream groups were not affected by the mineral interaction in their diets. The respiratory burst did not have a significant effect in the cellular immune system in neither the zymosan ($p=0.112$) nor in PMA ($p=0.320$). Even there is no significant difference a reduction is observed in the fish groups fed the OFeCuMin, compared to the fish groups fed the other diets. However, the large standard deviation could be a reason either for the reduction or the absence of statistical difference. The immunological analysis on the humoral immune system showed that there was not an effect on the fish fed the experimental diets. The measurements show no statistical difference on the antibacterial activity ($p=0.494$) on serum of the gilthead sea bream fed the five experimental diets with the various mineral interactions. Despite the absence of statistical difference the control diet showed higher levels

than the other diets and the large standard deviations could prevent a possible significant difference with OFeCuMin diet which was almost 25% lower. Fig. 6.15 and 6.16 present graphically the chemiluminescence and the antibacterial activity of serum in the gilthead sea bream fed the five experimental diets, respectively.
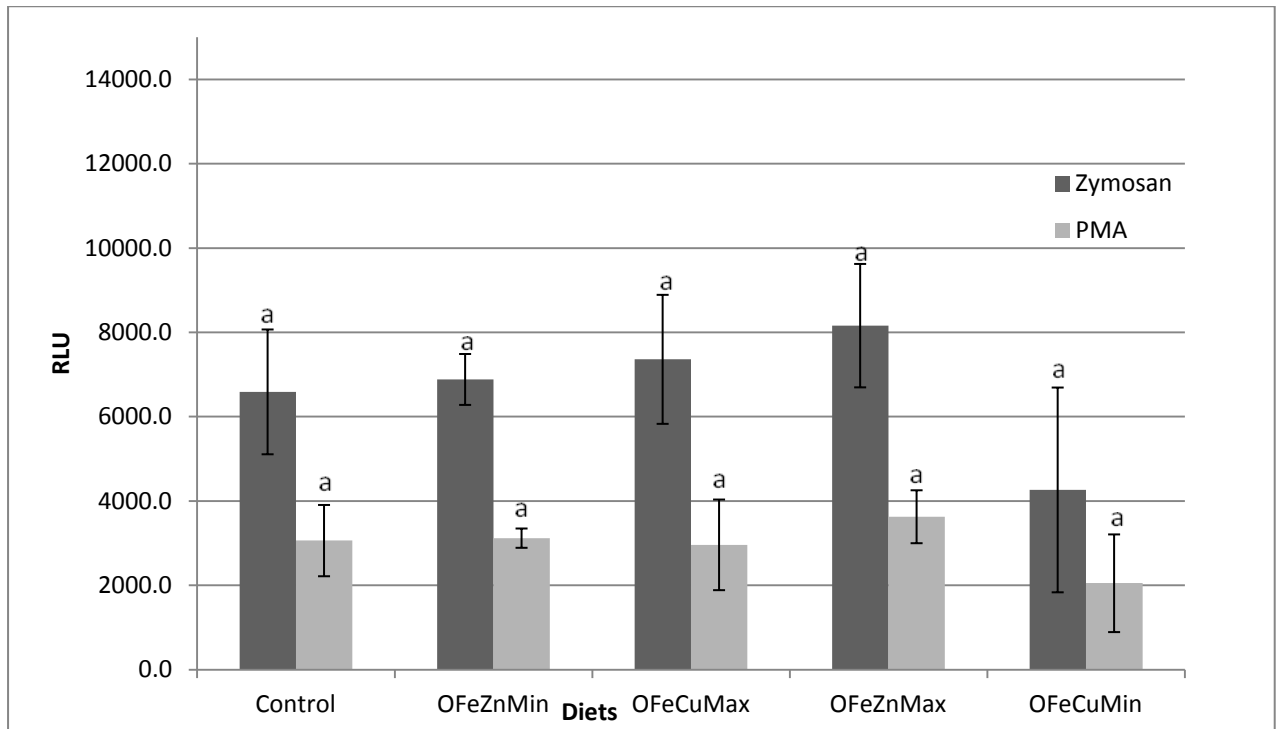
Fig.6.15 The chemiluminescence in the blood of gilthead sea bream fed the experimental diets for 12 week. Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at $p<0.05$.
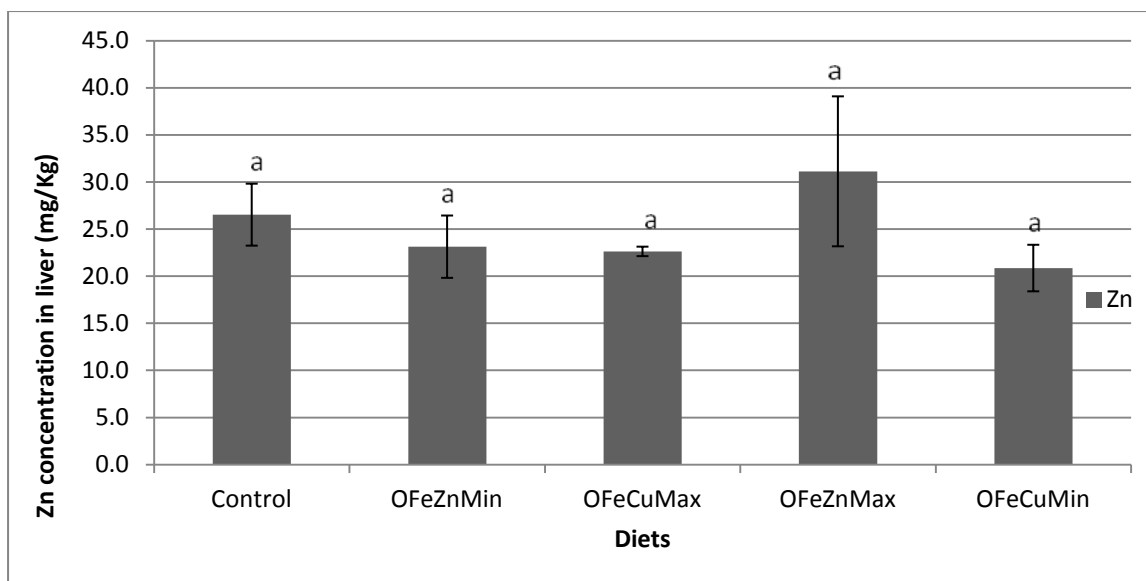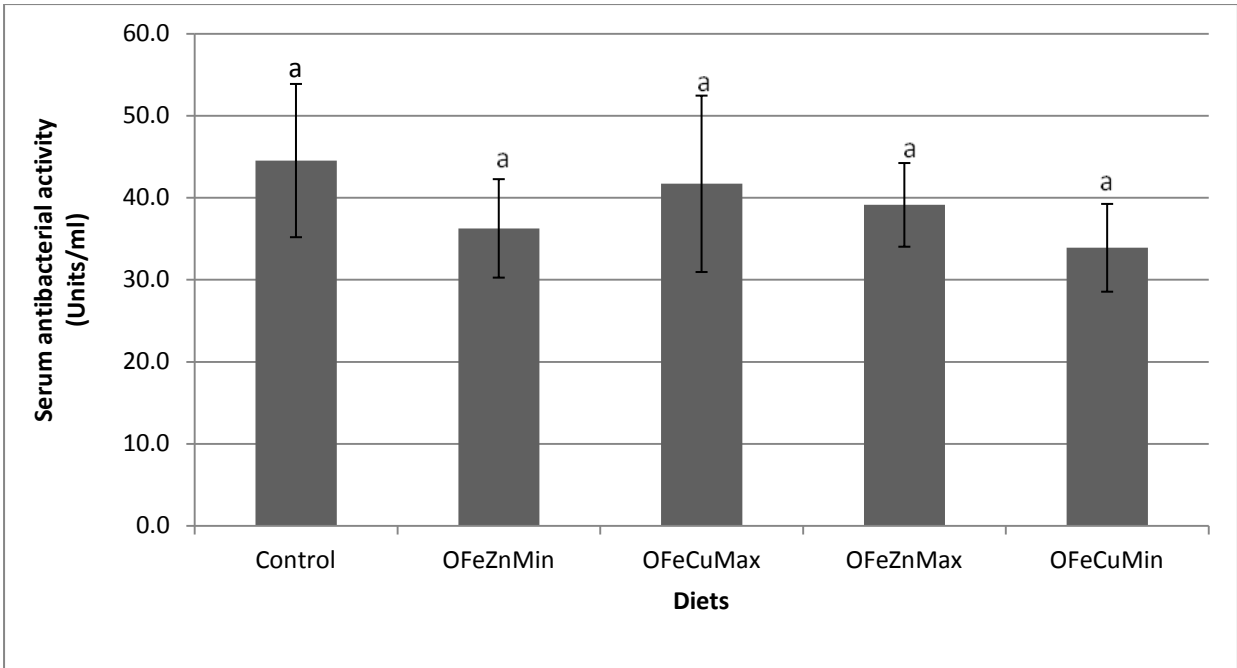
Fig. 6.16 The antibacterial activity of serum in gilthead sea bream fed the experimental diets for 12 week. Values are the means (n = 24) of three replicate tanks expressed with the standard deviation between tanks. Means sharing a common letter are not significantly different at p<0.05.
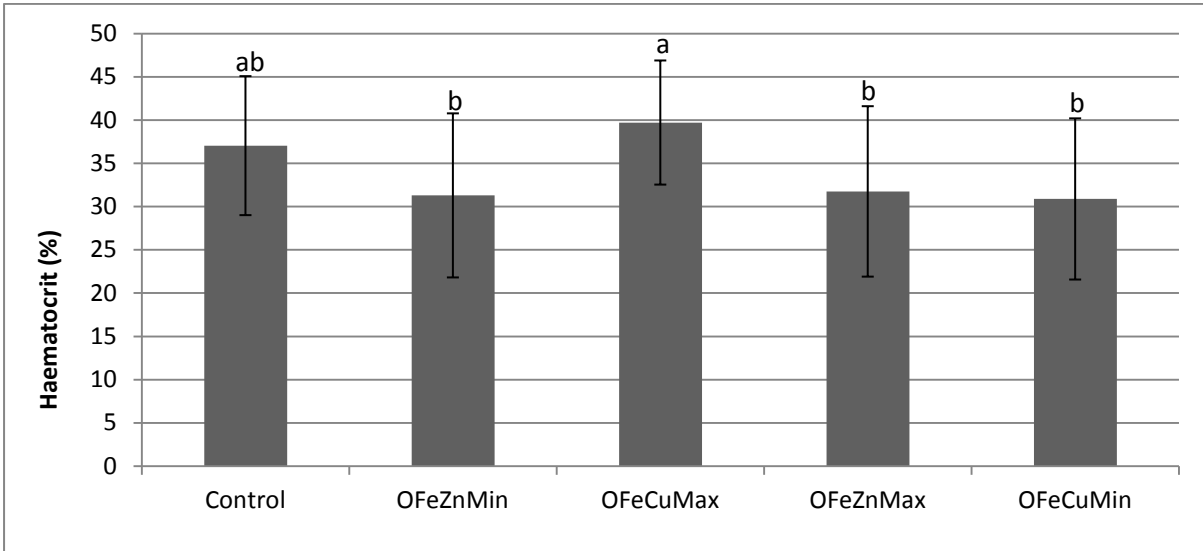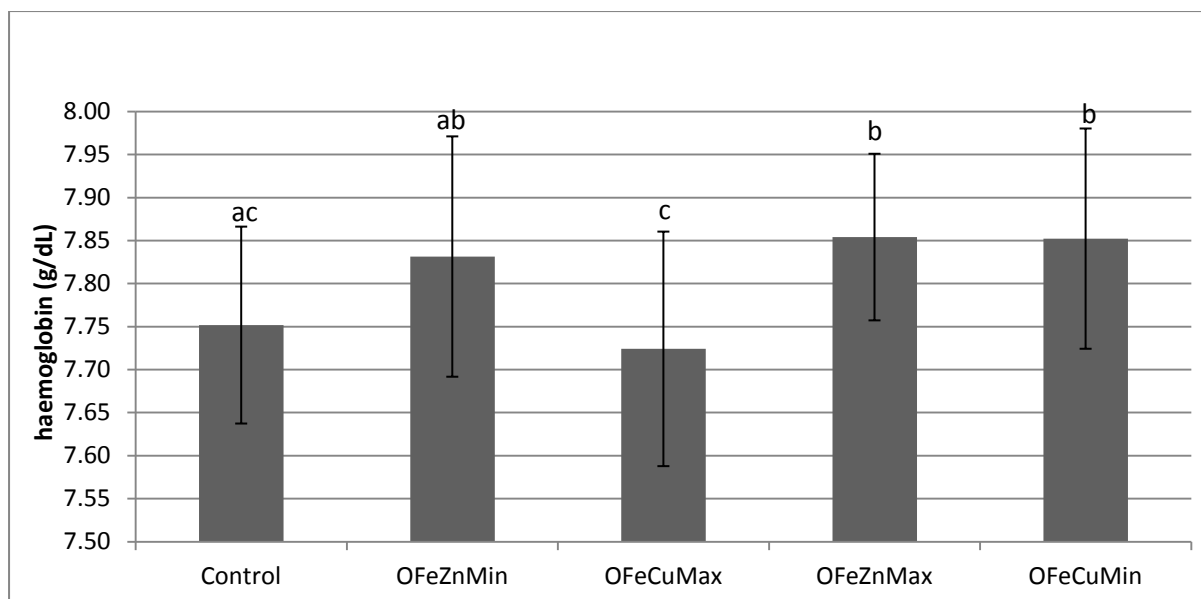
6.4 Discussion

It is well documented that dietary Fe, Zn and Cu are essential trace minerals for fish nutrition although there are cases that they can interact, having either synergetic or antagonistic effects (Reily, 2004; Halver & Hardy, 2002). The present study was conducted in order to evaluate the effects on gilthead sea bream fed on the suggested (by the first experiment of the current study) organic Fe level (150 mg/Kg) that was defined from previous experiments and the interactions with different dietary minerals which are used in commercial aquaculture diets. Dietary organic Zn and organic Cu were the selected trace minerals that were added in the experimental diets in order to investigate the interactions with organic dietary Fe and the effects on growth, tissue concentration, haematological and immunological parameters. The specific levels that were added to the diets were extreme, having high and low (based on three factors described in materials and methods and are the legal limits, the diets palatability and the Greek aquaculture sector common practices) values for both Zn and Cu. The control diet had the Cu and Zn levels which are widely used in sea bream feeds, according to the Greek fish feed manufacturing companies. The extreme values were designed according the EU legislation limits set for fish with some modifications. During the 12 week experiment the gilthead sea bream didn't show any significant differences among the fish fed the experimental diets. The final weight of the fish as well as the specific growth rate was not found to improve any of the factors mentioned. This comes in agreement with a number of studies that highlight that the inclusion on trace minerals didn't affect the growth of the organisms. Previous experiments with the inclusion of Fe and Zn in the diets of Atlantic salmon did not improve the growth performance (Maage &

Julshamn, 1993; Andersen *et al.,* 1996, 1998) neither in cases of interaction of Fe, Zn and Cu (Lorentzen & Magge, 1999). In addition there was no significant reduction in the growth of the fish. Growth reduction observed in rainbow trout, channel catfish, tilapia (*Oreochromis niloticus*) and carp in cases of fish fed diets with no supplementation of trace elements (Lim & Klesius, 1997; Lim *et al,.* 2000; Gatlin & Wilson, 1986; Shiau & Su, 2003; Satoh *et al.,* 1983a, b). In the current study all diets had an inclusion of trace elements (Fe, Zn and Cu). The findings suggest that the interaction of the coexistance of Fe, Zn and Cu in this form, as well as the various high and low levels, do not affect the growth of gilthead sea bream.

The tissue concentration among the experimental groups revealed no significant difference in fish liver, spleen and muscle. This analysis was aimed to give an understanding in the effects that can cause iron concentration in the selected tissues of the fish, the inclusion of extreme levels of Zn and Cu in their diets. Furthermore in order to present a broader picture that could improve the current knowledge in the subject, as well as to give a basis for further studies. There were measured the levels of Cu and Zn even though the selected tissues (with the exception of liver) do not work as storage nor usually measure their concentration in these specific organs (Grosell, 2011; Hogstrand, 2011). The knowledge in Cu distribution to the body of the fish is limited, although it is known to be carried to the liver and kidney (Reily, 2004) while Zn does not seem to exist in any specialized storage organ as well, but it can be found mainly in liver and in smaller amounts in gills as reported in rainbow trout, squirrelfish (*Holocentrus rufus*) and soldierfish (*Myripristis murdjan*) (Hogstrand *et al.,* 1995; Hogstrand & Haux, 1996; Thompson *et al.,* 2002, 2003). The interaction of high and low Zn and Cu with the 150 mg/Kg organic Fe in the five different treatments, didn't affect the Fe concentration in

spleen, muscle and liver. This was expected due to previous studies showing iron storage levels to be stabilised in levels higher than 100 mg/Kg (Rigos *et al.,* 2010; Andersen *et al.,* 1996). Nutritional trials in rainbow trout and tilapia have shown that high Zn levels in the diet have a negative effect on Fe and Cu uptake in fish (Knox *et al.,* 1982, 1984; Eid & Ghonim, 1994) although various species (like rainbow trout and Atlantic salmon) respond differently to dietary Cu due to their regulatory mechanisms in the liver (Lorentzen *et al.,* 1998). The absence of significance showed in the present work than the Fe concentration in the spleen, liver and muscle of gilthead sea bream is not affected from the specific levels of Zn and Cu used in the experimental treatments.

The haematological analyses in gilthead sea bream groups fed the experimental treatments show no significant differences in the erythrocyte (RBC) count. In contrast the haematocrit and haemoglobin values show a clear significance on the fish groups. The sea bream treated with the OFeCuMax diets show higher levels of Htc in the blood compared to the fish fed OFeZnMin, OFeZnMax and OFeCuMin. There was no significance between control and OFeCuMax. The final haematological parameter show a significant reduction in the Hb levels of fish fed the control diet compared to the fish fed the OFeZnMax and OFeCuMin diets, in addition even lower Hb values were observed among the fish fed the OFeCuMax diets compare to the fish fed the OFeZnMin, OFeZnMax and OFeCuMin diet. There was no significance between control and OFeCuMax of the fish fed these diets. It is worth mentioning that the RBC count analysis show an obvious but not significant incensement in the erythrocytes of fish fed control and OFeCuMax compared to the ones fed upon the rest diets, similar to the Htc results. This could very well be due to the large standard deviation between fish fed OFeZnMin, OFeZnMax and OFeCuMin

which could mask any significant difference. The Hb analyses show a significantly lower value in control and OFeCuMax while the Htc values showed significantly higher values for OFeCuMax and a similar trend for the control diet compared to the fish fed the other diets. This could be due to the small sized erythrocytes (explained by the comparison of RBC count and Htc results) that were carrying a lower amount of Hb as presented in the results (McPhee & Ganong, 2005; Smith *et al.,* 2004; Vander *et al.,* 1997). Previous studies in Atlantic salmon show that Fe supplementation does not affect the RBC count significantly (Andersen *et al.,* 1996) although other parameters such as Htc and Hb can be affected by dietary Fe levels addition in the diet. In human nutrition, the most sensitive known functions to be adversely affected by high dietary Zn include hematological parameters which are related to inhibition of Fe and Cu uptake (Maret & Sandstead, 2006; Stefanidou *et al.,* 2006). Fisher *et al.* (1981) reported that the excess of dietary Zn can induce the Cu bounded thionein and ultimately induce a relatively Cu deficiency while studies in chicks show that extra dietary Cu does not have an antagonistic effect with Zn (Pimentel *et al.,* 1992). In related studies with Atlantic salmon species haemoglobin reduction observer in fish fed diets with Fe lower than 30 mg/Kg (Andersen *et al.,* 1996). The findings in the current study may suggest that there is a relationship between optimum organic Fe levels and the addition of organic Cu (higher than 5 mg/Kg) leading to higher Htc values although further and more extended research is needed in order to identify what is the reason causing an effect on the size of the erythrocytes leading to lower Hb values. Further experimentation would be necessary in order to support this.

The immunological parameters did not show any significant difference among the fish groups fed either of the experimental diets. The immunological parameters

analysed in the fish show no significant differences probably due to the supplementation of the suggested organic Fe levels (150 mg/Kg) in all of the diets. Berger, (1996) observed that the fish immune system could be compromised either due to deficiency or excess of Fe. The fish of all treatments were able to sustain microbial growth due to Fe levels that covered the organisms needed for their defence mechanisms (Ravndal *et al.,* 1994). The immunological results both for respiratory burst and complement activity indicate that the interaction with various levels of Zn and Cu does not affect the dietary Fe ability to compromise or improve the fish immune system. The fish groups fed the experimental diets did not show any significant differences in the respiratory burst analysis neither of the zymosan (p=0.112) nor in PMA (p=0.320). The antibacterial activity of serum (p=0.494) was also not affected by the high and low levels of Zn and Cu coexisting in the experimental diets with the suggested level of organic Fe. Studies in rats show that Cu affects basic properties of the immune system, while Cu deficiencies could impair the neutrophil functions (Babu & Failla, 1990) and even cause anaemia to mammals and compromise the immune system (O'Dell, 1982; Davis & Mertz, 1987). The Cu concentration was at least 2.27 mg/kg, and it could be a reason for the absence of significant differences in the results.

The present study demonstrates that the incorporation of organic Fe and Cu levels around 150 mg/Kg and between 5-8 mg/Kg respectively can increase the haematocrit. In addition the results of haemoglobin and RBC count indicate that the previously described Fe and Cu levels interaction could result smaller erythrocytes in the blood of the fish.

Chapter 7 General Discussion

The experiments carried out in the current thesis were designed in order to address and extend the knowledge on nutritional issues and challenges related with the production of gilthead sea bream in the Greek aquaculture industry. At the end of the previous decade many fish farms in the Greek aquaculture sector suffered mortalities and deficiencies associated with a gill parasite (*Microcotyle* spp.). As a result the gilthead sea bream suffered from anaemia and sub lethal losses while they were cultured in the sea cages. In order to improve the fish health and provide resistance to the effects of the parasitic infection, several fish farms in Greece incorporated in the diets of the fish organic iron (Athanassopoulou *et al.,* 2005; Nengas personal communication, 2007). Fe has an active role in oxygen transportation associated with cellular respiration and oxidation/reduction reactions (Watanabe *et al.,* 1997; Lim *et al.,* 2000). Moreover Fe is considered to be one of the most important micronutrients due to its effect on immune system functions and host defence against infections (Beisel, 1982; Bhaskaram, 1988). Despite the nutritional importance of Fe in the diet of gilthead sea bream and the need of guidance from the commercial practices, the studies in dietary Fe in Mediterranean species are lacking. The information about Fe requirements in fish, and more specifically in absorption and metabolism, are limited and as far as Mediterranean fish are concerned studies are almost nonexistent (Watanabe *et al.,* 1997; Rigos *et al.,* 2010). Therefore, a series of experiments were designed with the collaboration of the University of Plymouth and the Hellenic Centre of Marine Aquaculture, in order to understand the effects of organic Fe in the diets of the gilthead sea bream species, in relation to a number of conditions and parameters.

The first experiment (Chapter 3) was designed as a direction indicator for the subsequent trials. Seven experimental diets from no added organic Fe and supplementation of up to 600 mg/Kg organic Fe were fed to gilthead sea bream in order to understand the effects of growth and haematology of each level to the fish and therefore determine the optimum levels that could be suggested as inclusion levels in the aquaculture industry. The overall findings of the experiment show that the growth of the fish was not affected by the inclusion of the organic Fe in the diet, which was shown both in the final weights of the fish groups as well as the specific growth rate of the fish. That was in acordace to the initial hypothesis as studies in Atlantic salmon and sea bream fed diets with the supplementation of Fe didn't show any improvement in the growth (Andersen *et al.,* 1996, 1997, 1998; Rigos *et al.,* 2010). The addition of organic Fe did not affect significantly neither the haematological parameters (haematocrit, haemoglobin, red blood cell count), nor the Fe concentration in the liver, spleen and muscle, of the sea bream groups fed the experimental diets. The only significant difference observed was the antibacterial activity of serum, showing higher levels in the fish groups fed the with 50 and 150 mg/Kg supplementation of organic iron in the diets. The respiratory burst showed no significant difference (probably due to the large standard deviation propably caused by few replicates), although the diet with the 150 mg/Kg organic Fe supplementation had the higher levels (as observed in Fe concentration in muscle, Hb and Htc analyses). An issue that may mask possible significant differences was the high Fe concentration in the raw materials, as shown in the control diets (62.17 mg/Kg) which had no additional Fe. This level should be a lot lower because many of the requirements could be covered from this level alone. More extended research and analysis in the ingredients types and levels as well as palatability test resulted to

address that issue in the next experiments and lower the Fe concentration in the raw materials. Furthermore future experiments could extend the organic Fe supplementation levels from purified diets to higher that 1000 mg/Kg supplementation in order to have a better understanding. In the current experiment, the need to keep diets similar to the commercial ones as well as the EU limitation in Fe supplementation which had to be similar to the ones in the commercial practise, prevented us from having a wider and more informative picture. Overall, it can be suggested that an organic Fe supplementation of 150 mg/Kg (regarding the cost of the product) diet should be the recommended additive level in the feeding schedules of the gilthead sea bream (it has to be clear that is not a absolute suggestion, due to the fact that the basal level will change depending on the ingredients) during the whole production cycle, and especially before and during the periods which favour the increase of haematophagus monogeneans attacks that can highly compromise the haematological status of gilthead sea bream.

The following experiment (Chapter 4) used the suggested, as optimum organic Fe supplementation level (150 mg/Kg) as control and compare it with two different inorganic iron forms that are widely used in Greek aquaculture, mainly due to the lower cost compared to organic Fe. These inorganic iron forms were the ferrous sulphate and ferrous carbonate and were supplemented in the experimental diets at levels of 100, 150 and 200 mg/Kg. The literature suggests that organic Fe can be absorbed better compared to the inorganic forms, and as a result be more highly available to the fish and more effective in the prevention of anaemia (Watanabe *et al.,* 1997; Sakamoto & Yone, 1979). The diet formulation in the experiment were improved and the Fe concentration dropped to less than 20 mg/Kg, while the mineral free casein from bovine milk and wheat meal were the dominant

ingredients. No significant differences were observed in the growth performance factors of the fish. The Fe concentration analysis in the selective tissues show that the fish were not affected by the different types of Fe, while the concentration of Fe in the spleen was higher for the fish fed the control diet. Along the same line were the haematological parameters analysed where no significant difference was observed in the fish treated with the seven experimental diets. However, the fish fed the control showed higher number of erythrocytes in their blood as well as higher Hb level. The findings suggest that there is no statistical effect on the fish fed the organic or the inorganic Fe forms. However the higher levels shown in the haematological analysis (could be very important in commercial scale practices) indicate that there is a trend maintaining the initial hypothesis that needs to be investigated further. Some factors that may affect the significant difference and have to be under consideration in future research are the extended analysis of the diets, as the diet could have vitamin C and this could improve the absorbance of inorganic iron (Reilly, 2004; Mann & Turswell, 2007). The small size of the fish could have an effect on the quality of the tissues and blood sample collected and analysed. Future research, in order to have bolder differences among the diets with the inclusion of organic and inorganic forms of Fe, could have an equal levels of diets supplemented with organic and inorganic Fe, as well as a higher range of supplementation.

Aquaculture practices are often closely associated with husbandry related stressors. Two of the most common ones are the overcrowding and the transport between units, usually with nets. These oxygen deprivation related stressors could be prevented or minimized alongside with the harmful effects for the fish health, if Fe was supplemented in fish diets due to the role of trace mineral's active role in oxygen transportation and cellular respiration (Rottmann *et al.,* 1992; Lall, 2002; Iwama *et*

al., 2012; Pavlidis & Mylonas, 2011). The aim of the next experiment (Chapter 5) was to evaluate the effects of three levels of organic Fe (50, 150 and 250 mg/Kg) in sea bream species exposed to two different types of oxygen deprivation conditions, one acute (netting) and one chronic (overcrowding). The experimental results show no significant differences in the final weigh and specific growth rate of the fish fed the experimental diets and exposed to both oxygen deprivation conditions. The Fe concentration analysis in the liver, spleen and muscle also lack of significant differences, probably due to the short period of the fish exposed to the oxygen deprivation stressors (Baker, 1986). In addition, the immunological parameters analysed did not show any significant difference at the analysis of the two stages of the experiment. The only significant reduction observed in the haemoglobin level of the fish fed the OFe50 diet that exposed to the overcrowding stressor for a week. The short range of the iron supplementation levels as well as the not extended exposure of the fish to the oxygen deprivation stressors could be the reason for the lack of significant differences, since it was established from the first experiment that the iron requirements of gilthead sea bream are covered in the levels supplemented in the current experiment. However it will be suggested that a minimum addition of organic Fe in the diet of sea bream can improve the fish health and resistance to oxygen deprivation conditions in commercial aquaculture. Further experimentation could include cortisol measurement to the fish, in order to evaluate better the exposure conditions as well as to make safer assumptions. An investigation less oriented to the commercial aquaculture practices could stretch the supplementation levels from purified diets up to 1000 mg/kg, in order to have a better understanding. In addition a fish group without any exposure to either acute or chronic oxygen

deprivation stressors could be incorporated in the experimental design, in order to have a more distinguish comparison.

The final experiment (Chapter 6) took place in order to investigate the interactions between the suggested level of organic Fe and high and low levels of organic Zn and Cu, coexisting in the feeds of gilthead sea bream. The suggested level of organic Fe was determined from the first experiment. The levels of dietary Zn and Cu in the experimental diets were determined by three factors, the EU maximum limits, the lowest availability in the raw ingredients, and more importantly, the common practices and observations in the Greek aquaculture sector. The growth performance of the fish didn't show any significant differences among the fish fed the five experimental diets. The tissue concentration analysis covered all three trace elements levels in all the selected tissues of the fish. The main reason that Cu and Zn concentration (in the tissues) analysed and presented, was to be as available knowledge or even as blueprint for future research on the subject. Thus, the Fe concentration in the specific tissues show that at the specific levels examined, the mineral interaction had no significant effect in the fish Fe absorption. In the spleen and muscle the fish fed the OFeCuMax diet had the higher levels, although the extremely large standard deviation cannot lead to any safe assumptions. Along those lines, the gilthead sea bream fed the five experimental diets did not show any significant difference in the immunological parameters of the fish. Significant differences occurred in the haematological parameters analysed of the fish fed the experimental diets. The fish fed the OFeCuMax diet show higher Htc values compared to the fish fed OFeZnMin, OFeCuMin, OFeZnMax diets. While the gilthead sea bream fed Control and OFeCuMax had significantly lower Hb values compared to the fish fed with the OFeCuMin and OFeZnMax diets. In addition the fish fed with

OFeCuMax had also significantly lower Hb levels compared to the fish that received the OFeZnMin diet. Summarizing the findings of the experiment it could be said that the diets with the higher Cu levels (control, OFeCuMax) cause a negative effect on the in the Hb of the fish. In the majority of the analysis the standard deviations were quite large, and as a result, the findings should be approached with caution. The fact that the design of the experimental diets (Cu and Zn levels) was dictated by the commercial practices and requirements could very well narrow the rage of the results.

Concluding the whole thesis, consisting of the four experiments, it was an applied research in order to solve problems and address issue of the intensive culture of gilthead sea bream facing the Greek aquaculture sector. The whole research was designed to evaluate the effects of organic Fe supplementation in the diets of gilthead sea bream, regarding four pre-determined parameters, the growth performance, the Fe concentration in three Fe related organs (muscle, liver and spleen), the haematology and the immunology of the fish. The limited studies and knowledge in many areas of this area made in some cases the evaluation and approach of the findings quite challenging. Evaluating the whole research, the main issue under consideration for future research is the fact that the experiments serving the purpose of addressing specific issues in the aquaculture industry should be carried out in a aquaculture environment (for example sea cages) where they could be more applied and correct. In addition the sample would be higher for the analysis and the standard deviations drop down, leaving no room for any possible masking of statistical differences. On the other hand, experiments carried out in the lab, where the conditions are more controlled, should enable researchers to investigate the hypothesis with more freedom in the dosage ranges and the approach of the

experiments. To conclude the finding of the entire research programme, it can be suggested that gilthead sea bream recommended levels of organic Fe supplementation in the fish feeds could be at 150 mg/Kg and the Cu levels in the diets should be less than 5 mg/Kg. A 150 mg/Kg supplementation of organic Fe should improve the fish haematological and immunological parameters, as well as fish health in relation to resistance of haematophagus monogeneans attacks and aquaculture husbandry related oxygen deprivation stressors.

The work covered in the thesis could lead to a number of interesting and important future avenues of research. First and foremost it would be important to repeat the experiments from the current study in an environment similar to the ones that aquaculture practices are applied. Sea cages could be used in different farms across the country in order to test the finding in conditions identical to the ones that the industry has. In addition the current experiments could be tested in different life stages of gilthead sea bream species and investigate more vigorously the dietary organic Fe requirements as well as the effects on health and growth in this species in age groups. Moreover future research on Fe dependent enzymes could unravel a great amount of knowledge on more sensitive parameters that could be measured. Another area that could be more than enlightening regarding the health and resistance to pathogens of gilthead sea bream species could be a series of challenge experiments with other pathogens as well as fish exposed to the *Microcotyle* spp. parasite while fed diets with the suggested organic Fe level.

Appendices

Appendix 1. The data sheet of the organic Fe used as supplement in the

experimental diets

Appendix 2. The date sheet of the inorganic Fe form (ferrous sulphate) used as supplement in the experimental diets

Appendix 3. The date sheet of the inorganic Fe form (ferrous carbonate) used as supplement in the experimental diets

Chapter 7 List of References

- Adams, S.M. (1990a) Biological indicators of stress in fish. *American Fisheries Society Symposium Series*, **8**, 1-191.

- Adams S.M. (1990b) Status and use of biological indicators for evaluating the effects of stress in fish. *American Fisheries Society Symposium Series*, **8**, 1-8.

- Andersen, F., Maage, A. & Julshamn, K. (1996) An estimation of dietary iron requirement of Atlantic salmon *Salmo salar* L. parr. *Aquaculture Nutrition*, **2**, 41–47.

- Andersen, F., Lorentzen ,M., Waagbo, R. & Maage, A. (1997) Bioavailability and interactions with other micronutrients of three dietary iron sources in Atlantic salmon, *Salmo salar*, smolts. *Aquaculture Nutrition*, **3**, 239-246.

- Andersen, F., Lygren, B., Maage, A. & Waagbo, R. (1998) Interaction between two dietary levels of iron and two forms of ascorbic acid and the effect on growth, antioxidant status and some non-specific immune parameters in Atlantic salmon (*Salmo salar*) smolts. *Aquaculture,* **161**, 437-451.

- Arends, R.J., Mancera, J.M., Munoz, J.L., Wendelaar-Bonga, S.E. & Flik, G. (1999) The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. *Journal of Endocrinology*, **63**, 149–157.

- Athanassopoulou ,F., Ragias, V., Vagianou, S., Di Cave, D., Rigos, G., Papathanasiou, G. & Georgoulakis, J. (2005) Report of *Sparicotyle* (Microcotyle*) chrysophrii* Van Beneden and Hesse 1863, *Atrispinum seminalis* Euzet and Maillard 1973 and *Polylabris tubicirrus* Paperna and Kohn 1964

(Monogenea) on captive sea bream (*Sparus aurata*) and sharp snout sea bream (*Diplodus puntazzo*) in coastal Greece and Italy. *Bulletin of the European Association of Fish Pathologists*, **25**, 256–262.

- Babu, U. & Failla, M., (1990) Copper status and function of neutrophils are reversibly depressed in marginally and severely copper deficient rats. *The Journal of Nutrition*, **120**, 1700–1709.

- Baker, D.H. (1986) Problems and pitfalls in animal experiments designed to establish dietary requirements for essential nutrients. *The Journal of Nutrition*, **116**, 2239-2249.

- Barnable, G. (1986) *Aquaculture,* Volume 1 & 2. Lavoisier, Paris.

- Barton, B.A. (2000) Salmonid fishes differ in their cortisol and glucose responses to handling and transport stress*. North American Journal of Aquaculture*, **62**, 12–18.

- Barton, B.A. (2002) Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative & Comparative Biology*, **42**, 517–525.

- Barton, B.A. & Iwama, G.K. (1991) Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases*, **1**, 3-26.

- Bauchot, M.L., Hureau, J.C. & Miquel, J.C. (1981) *Sparidae.* Canada Funds-in-Trust. Department of Fisheries and Oceans Canada, by arrangement with the Food and Agriculture Organization of the United Nations, Ottawa.

- Beard, J.L., Dawson, H. & Pinero, D.J. (1996) Iron metabolism: a comprehensive review. *Nutrition Reviews*, **54**, 295-317.

- Beisel, W.R. (1982) Single nutrient and immunity. *The American Journal of Clinical Nutrition*, **35**, 417–468.

- Berger, L.L. (1996) Trace minerals: key to immunity. *Salt Trace Minerals,* **28**, 1–4.

- Bhaskaram, P. (1988) Immunology of iron-deficient subjects. In: Chandra, R.K. Ed., *Nutrition and Immunology*, pp. 149–168. Alan R. Liss, New York.

- Blaxhall, P.C. & Daisley, K.W. (1973) Routine haematological methods for use with Fish blood. *Journal of Fish Biology,* **5**, 771-781.

- Bodiga, S. & Krishnapillai, M.N. (2007) Concurrent repletion of iron and zinc reduces intestinal oxidative damage in iron- and zinc-deficient rats. *World Journal of Gastroenterology,* **13**, 5707-5717.

- Boobis, S. (1999) *Review of Copper. Expert Group on Vitamins and Minerals*. Ministry of Agriculture, Fisheries and Food, London.

- Brett, J.R. (1958) Implications and assessment of environmental stress. In: *The Investigation of Fish-PowerProblems* (ed. Larkin, P.A.). Institute of Fisheries, University of British Columbia, Vancouver.

- British Nutrition Foundation (1995) *Iron: Nutritional and Physiological Significance*. Chapman & Hall, London.

- Bury, N.R., Boyle, D.& Cooper, C.A. (2011) Iron. In: *Fish Physiology: Homeostasis and Toxicology of Essential Metals* (Farrell, A., Brauner, C.J. & Wood, C.M. eds), Volume 31A, pp. 201-251. Academic Press, UK.

- Bury, N. & Grosell, M. (2003) Iron acquisition by teleost fish. *Comparative Biochemistry and Physiology Part C*, **135**, 97–105.

- Buttriss, J. (2001) The mysteries of iron metabolism: magnetic attractions. *Nutrition Bulletin*, **26**, 271-2.

- Chvapil, M. (1973) New aspects in the biological role of zinc: a stabilizer of marcomolecules and biological membranes. *Life Sciences*, **13**, 1041-1049.

- Chesters, J.K. (1991) Trace element-gene interactions with particular reference to zinc. *Proceedings of the Nutrition Society*, **50**, 123- 129.

- Conte, F.S. (2004) Stress and the welfare of cultured fish. *Applied Animal Behaviour Sciences*, **86**, 205–223.

- Davis, K.D. & Mertz, W. (1987) Copper. In: *Trace Elements in Human and Animal Nutrition* (Mertz, W. ed.), 5th edition, Vol. 1, pp. 301–364. Academic Press Inc., New York.

- De Almeida-Val, V.M.F., Gomes, A.R.C. & Lopes, N.P. (2006) Metabolic and physiological adjustments to low oxygen and high temperature in fishes of the Amazon. *The Physiology of Tropical Fishes*, **21**, 443-500.

- Eid, A.E. & Ghonim, S.I. (1994) Dietary zinc requirement of fingerling *Oreochromis niloticus. Aquaculture*, **119**, 259–264.

- Ellis, T., North, B., Scott, A.P., Bromage, N.R., Porter, M. & Gadd, D. (2002) The relationships between stocking density and the welfare of farmed rainbow trout. *Journal of Fish Biology*, **61**, 493–531.

- Ewing, W.N. & Charlton, S.J. (2007) *The Minerals Directory, 2nd edition, pp.90-130.* Context Publication, Packington, England.

- Fagerlund, U.H.M, MvBride, J.R. & Williams, I.V. (1995) Stress and rolerance. In: *Physiological Ecology of Pacific Salmon* (Groot, C., Margolis, L. & Clarke, W.C. eds.), pp.461-503. University of British Columbia Press, Vancouver.

- Fanouraki, E., Divanach, P. & Pavlidis,M. (2007) Baseline values for acute and chronic stress indicators in sexually immature red porgy (*Pagrus pagrus*). *Aquaculture*, **256**, 294–304.

- FAO-Food and Agriculture Organization of the United Nations (2010) Cultured Aquatic Species Information Programme. *Sparus aurata*. In: FAO Fisheries and Aquaculture Department [online] (eds. F. Colloca & S. Cerasi). Rome. Available at: http://www.fao.org/fishery/culturedspecies/Sparus aurata/en.

- FAO. 2012. Aquaculture topics and activities. State of world aquaculture. Text by Rohana Subasinghe. In: *FAO Fisheries and Aquaculture Department* [online]. Rome.

- Fisher, P.W.F., Giroux, A. & L'Abbe, M.R.L. (1981) The effect of dietary zinc on intestinal copper absorption. *The American Journal of Clinical Nutrition*, **34**, 1670-1675.

- Gatlin, D.M. & Wilson, R.P. (1986) Characterisation of iron deficiency and the dietary iron requirement of fingerling channel catfish. *Aquaculture*, **52**, 191–198.

- Grosell, M. (2011) Copper. In: *Fish Physiology: Homeostasis and Toxicology of Essential Metals* (Farrell, A., Brauner, C.J. & Wood, C.M. eds), Volume 31A, pp. 53-81. Academic Press, UK.

- Halver, J.E. & Hardy, R.W. (2002) Fish Nutrition. In: *Fish Nutrition* (Halver, J.E. & Hardy, R.W. eds.). Academic Press, Elsevier rd Science, San Diego, USA.

- Hardy R.W. (2001) Nutritional deficiencies in commercial aquaculture: likelihood, onset, and identification. In: Nutrition and Fish Health (ed. by C. Lim & C.D. Webster), pp. 131–147. Haworth Press, Inc, London.

- Harte, E.B., Steenbock, H., Waddell, J. & Elcenhjem, C.A. (1928) Iron nutrition VII. Copper is a supplement to iron for haemoglobin building in the rat. *Journal of Biological Chemistry*, **77**, 797-812.

- Haschke, F., Ziegler, E.E., Edwards, B.B. & Foman, S.J. (1986) Effect of iron fortification of infant formula on trace mineral absorption. *Journal of Pediatric Gastroenterology and Nutrition*, **5**, 768-73.

- Hogstrand, C. (2011) Zinc. In: *Fish Physiology: Homeostasis and Toxicology of Essential Metals* (Farrell, A., Brauner, C.J. & Wood, C.M. eds), Volume 31A, pp. 135-199. Academic Press, UK.

- Hogstrand, C. & Haux, C. (1996) Naturally high levels of zinc and metallothionein in liver of several species of the squirrelfish family from Queensland, Australia. *Marine Biology*, **125**, 23–31.

- Hogstrand, C., Reid, S.D. & Wood, C.M. (1995) Ca2+ versus Zn2+ transport in the gills of fresh-water rainbow-trout and the cost of adaptation to waterborne Zn2+. *The Journal of Experimental Biology*, **198**, 337–348.

- House, W.A., (1998) Trace element bioavailability as exemplified by iron and zinc. *Field Crops Research*, **60**, 115-141.

- Hurley, L.S. & Keen, C.L. (1987) Manganese. In: *Trace Elements in Human and Animal Nutrition* (Mertz, W. ed.), 5th edn, Vol. 1, pp. 185–223. Academic Press Inc., New York.

- ICAP, (2009), Sector study: Aquaculture, ICAP.

- Ikeda, Y., Ozaki, H. & Uematsu, K. (1973) Effect of enriched diet with iron in culture of yellowtail. *Journal of the Tokyo University of Fisheries,* **59**, 91-99.

- Iwama, G. K., Pickering, A. D., Sumpter, J. P. & Schreck, C. B. (2012) *Fish Stress and Health in Aquaculture*. Cambridge University Press, New York.

- Kawatsu, H. (1972) Studies on the anemia of fish: V. Dietary iron deficient anemia in brook trout, *Salvelinus fontinalis.* Bull. Freshwater Fish. Res. Lab., **22**, 59–66.

- Knox, D., Cowey, C.B. & Adron, J.W. (1982) Effects of dietary copper and copper:zinc ratio on rainbow trout (*Salmo gairdneri*). *Aquaculture*, **27**, 111–119.

- Knox, D., Cowey, C. B. & Adron, J.W. (1984) Effects of dietary zinc intake upon copper metabolism in rainbow trout (*Salmo gairdneri*). *Aquaculture*, **40**, 199–207.

- Korte, S.M., Olivier, B. & Koolhaas, J.M. (2007) A new animal welfare concept based on allostasis. *Applied Animal Behaviour Science*, **86**, 225–250.

- Koutsogiannopoulos, D.D. (2010) List of species. In :*The fishes of Greece* (in Greek), pp. 61-477.Tati Publications, Athens, Greece.

- Lahey, M., Gubler, C., Chase, M., Cartwright, G. & Wintrobe, M. (1952) Studies on copper metabolism. Hematologic manifestations of copper deficiency in swine. *Blood*, **7**, 1053- 74.

- Lall, S.P. (1989) The minerals. In: *Fish Nutrition* (Halver, J.E. ed.), 2nd edition, pp. 219–257. Academic Press, San Diego, CA.

- Lall, S.P. (2002) The minerals. In: *Fish Nutrition* (Halver, J.E. & Hardy, R.W. eds), 3rd edition, pp. 259–308. Academic Press, San Diego, CA.

- Lall, S.P. & Bishop, F.J. (1977) Studies on mineral and protein utilization by Atlantic salmon (*Salmo salar*) grown in sea water. Technical Report No. **688**, *Fisheries and Marine Service*, Environment Canada.

- Lall, S.P. & Hines, J. A. (1987) Iron and copper requirement of Atlantic salmon Salmo salar grown in sea water. Paper presented at the International Symposium on Feeding and Nutriton of Fish, Bergen, Norway, 23-21 August 1987.

- Lee , Y.H., Layman, D.K., Bell, R.B. & Norton, H.W. (1981) Response of glutathione peroxidase and catalase to excess dietary iron in rats. *Journal of Nutrition* , **111**, 2195–2202.

- Lim, C. & Klesius, P.H. (1997) Responses of channel catfish (*Ictalurus punctatus*) fed iron-deficient and replete diets to Edwardsiella ictaluri challenge. *Aquaculture*, **157**, 83–93.

- Lim, C., Klesius, P.H., Li M, H. & Robinson, E.H. (2000) Interaction between dietary level of iron and vitamin C on growth, haematology, immune response and resistance of channel catfish (*Ictalurus punctatus*) or *Edwardsiella ictaluri* challenge. *Aquaculture*, **185**, 313–327.

- Lojek, A., Ciz, M., Marnila, P., Duskova, M.& Lilius, E.M. (1997) Measurement of whole blood phagocyte chemiluminescence in the Wistar rat. *Journal of Bioluminescence and Chemiluminescence* ,**12**, 225-231.

- Lorentzen, M. & Maage, A. (1999) Trace element status of juvenile Atlantic salmon *Salmo salar* L. fed a fish-meal based diet with or without supplementation of zinc, iron, manganese and copper from first feeding. *Aquaculture Nutrition*, **5**, 163-171.

- Lorentzen, M., Maage, A. & Julshamn, K. (1998) Supplementing copper to a fish meal based diet fed to Atlantic salmon parr affects liver copper and selenium concentrations. *Aquaculture Nutrition*, **4**, 67-72.

- Lucas, J. & Southgate, P. (2003) *Aquaculture, farming aquatic animals and plants,* pp. 1-46*.* Blackwell Publications, UK.

- Lupatsch, I., Kissil, G.W. & Sklan, D. (2003) Defining energy and protein requirements of gilthead seabream (*Sparus aurata*) to optimize feeds and feeding regimes. *The Israeli Journal of Aquaculture*, **55**, 243–257.

- Maage, A. & Julshamn, K. (1993) Assessment of zinc status in juvenile Atlantic salmon (*Salmon salar*) by measurement of whole body and tissue levels of zinc. *Aquaculture*, **117,** 179–191.

- Mann, J. & Truswell, S. (2007) Organic and inorganic essential nutrients. In: Essentials of Human Nutrition, 3rd edition, pp.100-232. Oxford University Press, Oxford, New York.

- Maniatis, G. & Danchev, S. (2011) Sector study: *Aquaculture*. Foundation for Economic and Industrial Research, Athens, Greece.

- Maret, W. & Sandstead, H.H. (2006) Zinc requirements and the risks and benefits of zinc supplementation. *Journal of Trace Elements in Medicine and Biology*, **20**, 3–18.

- Mayer, P., Estruch, V., Blasco, J. & Jover, M. (2008) Predicting the growth of gilthead sea bream (*Sparus aurata*) farmed in marine cages under real production conditions using temperature-and time-dependent models. *Aquaculture Research*, **39(10)**, 1046–1052.

- McPhee, S.J.& Ganong, W.F. (2005) Pathophysiology of Disease: An Introduction to Clinical Medicine, McGraw-Hill Medical.

- Mills, E.S. (1930) The treatment of idiopathic (hypochromic) anaemia with iron and copper. *Canadian Medical Association Journal*, **22**, 175-8.

- Montero, D., Izquierdo, M.S., Tort, L., Robaina, L. & Vergara, J.M. (1999) High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. *Fish Physiology and Biochemistry*, **20**, 53–60.

- Morris, E.R. (1987) Iron. In: *Trace Elements in Human and Animal Nutrition* (Mertz, W. ed.), 5th edn, Vol. 1, pp. 79–142. Academic Press Inc., New York.

- Murai, T., Andrew, J.W. & Smith, R.G.Jr. (1981) Effects of dietary copper on channel catfish. *Aquaculture*, **22**, 353-357.

- Nengas, I. (2007) Personal communication key speaker in the Alltech's 23rd International Feed Industry Symposium, in May 2007 in Lexington Kentucky, USA, in the Aquaculture sessions.

- New, M. (1999) Global aquaculture:current trends of challenges for the 21st century. *World Aquaculture*, **30**, 8-13.

- Nikoskelainen, S., Lehtinen, J. & Lilius, E.M. (2002) Bacteriolytic activity of rainbow trout (*Oncorhynchus mykiss*) complement. *Developmental and Comparative Immunology,* **26**, 797-804.

- Nose, T. & Arai, V. (1979) Recent advances in studies on mineral nutrition of fish in Japan. In: *Advances in Aquaculture* (Pillay, T.V.R. & Dill, W.A. eds.), pp. 584–590. Fishing News Books Ltd., Farnham, England.

- Oates, P.S. & West, A.R. (2006) Heme in intestinal epithelial cell turnover, differentiation, detoxification, inflammation, carcinogenesis, absorption and motility. *World Journal of Gastroenterology*, **12**, 4281–4295.

- Olivia-Teles A. (2012) Nutrition and health of aquaculture fish. *Journal of Fish Diseases*, **35**, 83-108.

- O'Dell, B.L., (1982) Biochemical basis of the clinical effects of copper deficiency. In: *Clinical, Biochemical and Nutritional Aspects of Trace Elements* (Prasad, A.S. ed.), pp. 301–313. Alan. R. Liss Inc., New York.

- Papanastasiou, D.P. (1976) Sparidae. In:*Fish Catch* (in Greek), Volume 1, pp.423-485. Ion Publications, Peristeri, Athens.

- Pavlidis, M.A. & Mylonas, C.C. (2011) *Sparidae: Biology and Aquaculture of Gilthead Sea Bream and Other Species*, Blackwell Publishing Ltd., UK.

- Petridis, D. & Rogdakis, L. (1996) The development of growth and feeding equation for seabream, Sparus aurata L., culture. *Aquaculture Research*, **27**, 413–419.

- Pickering, A.D. (1992), Rainbow trout husbandry: management of the stress response. Aquaculture, **100**, 125-139.

- Pietrangelo A. (1998) Hemochromatosis 1998: is one gene enough?. *Journal of Hepatology*, **29**,502-509.

- Pimentel, J.L., Greger, J.L., Cook, M.E. & Stehl, J.L. (1992) Iron metabolism in chicks fed various levels of zinc and copper, *The Journal of Nutritional Biochemistry*, **3**, 140-145.

- Ramirez-Cardenas, L., Costa, N.M.B. & Reis, F.P. (2005) Copper-iron metabolism interaction in rats. *Nutrition Research*, **25**, 79-92.

- Rasmussen, K.J. (1994) Spray-dried blood in diets to Atlantic salmon (*Salmon salar*). *Fisk. Dir. Skr., Ser. Ernæring*, **6,** 151–161.

- Ravndal, J., Lovold. T., Bentsen, H.B., Roed, K.H., Gjedrem , T. & Rorvik K.A. (1994) Serum iron levels in farmed Atlantic salmon: family variation and associations with disease resistance. *Aquaculture*, **125**, 37-45.

- Reilly, C. (2004) *The Nutritional Trace Metals,* pp.35-134*.* Blackwell Publishing, UK.

- Rigos, G., Samartzis, A., Henry, M., Fountoulaki, E., Cotou, E., Sweetman, J., Davies ,S. & Nengas, I. (2010) Effects of additive iron on growth, tissue distribution, haematology and immunology of gilthead sea bream, *Sparus aurata*. Aquaculture International, **18**, 1093-1104.

- Rotllant, J., Pavlidis, M., Kentouri, M., Abad, M.E. & Tort, L. (1997) Non-specific immune responses in the red porgy *Pagrus pagrus* after crowding stress. *Aquaculture*, **156**, 279–290.

- Rotllant, J., Balm, P.H.M., Ruane, N.M., Pérez-Sánchez*,* J., Wendelaar-Bonga, S.E & Tort, L. (2000a) Pituitary ProOpioMelanoCortin-derived peptides and Hypothalamus- Pituitary-Interrenal axis activity in gilthead seabream (*Sparus aurata*) during prolonged crowding stress, differential regulation of ACTH and MSH release by CRH and TRH. *General and Comparative Endocrinology*, **119**, 152– 163.

- Rotllant, J., Arendts, R.,Mancera, J.M., Flik, G., Wendelaar-Bonga, S.E & Tort, L. (2000b) Inhibition of HPI axis response to stress in gilthead sea bream (*Sparus aurata*) with physiological plasma levels of cortisol. *Fish Physiology and Biochemistry*, **23**, 13–22.

- Rotllant, J., Balm, P.H.M., Pérez-Sánchez*,* J., Wendelaar-Bonga, S.E & Tort, L. (2001) Pituitary and interrenal function in gilthead sea bream (*Sparus aurata* L., Teleostei) after handling and confinement stress. *General and Comparative Endocrinology*, **121**, 333–342.

- Rottmann, R.W., Francis-Floyd, R. & Durborow, R. (1992) *The Role of Stress in Fish Disease*, Southern Regional Aquaculture Center Publication, UK.

- Rumsey, G.L. & Ketola, H.G. (1975) Amino acid supplementation of casein in diets of Atlantic salmon (*Salmo salar*) fry and of soybean meal for rainbow trout (Salmo gairdneri) fingerlings. *Journal of the Fisheries Research Bord of Canada*, **32**,422-426.

- Saari, J., Bode, A. & Dahlen, G. (1995) Defects of copper deficiency in rats are modified by dietary treatments that affect glycation. *Journal of Nutrition*, **125**, 2925- 34.

- Sakamoto, S. & Yone, Y. (1976) Requirement of red sea bream for dietary Fe-I. *Report of the Fisheries Research Laboratory*, Kyushu University, **3**, 53-58.

- Sakamoto, S. & Yone, Y. (1978a) Requirement of red sea bream for dietary iron . II. *Bulletin of the Japanese Society of Scientific Fisheries,* **44**, 223-225.

- Sakamoto, S. & Yone, Y. (1978b) Iron deficiency symptoms of carp. *Bulletin of the Japanese Society of Scientific Fisheries*, **44**, 1157-1160.

- Sakamoto, S. & Yone, Y., (1979) Availabilities of three iron compounds as dietary iron sources for red seabream. *Nippon Suisan Gakkaishi*, **45**, 231-235.

- Sandström, B., Davidsson, L., Cederblad, A. & Lönnerdal, B. (1985) Oral iron, dietary ligands and zinc absorption. Journal of Nutrition, **115**, 411–414.

- Sangiao-Alvarellos, S., Laiz-Carrión, R., Guzmán, J.M., Martin del Río, M.P., Miguez, J.M., Mancera, J.M. & Soengas, J.L. (2003) Acclimation of *S. aurata* to various salinities alters energy metabolism of osmoregulatory and nonosmoregulatory organs. *American Journal of Physiology*, **285**, R897–907.

- Satoh, S., Takeuchi, T., Narabe, Y. & Watanabe, T. (1983a) Effects of deletion of several trace elements from fish meal diets on growth and mineral composition of rainbow trout fingerlings. *Bulletin of the Japanese Society for the Science of Fish*, **49**, 1909±1916.

- Satoh, S., Yamamoto, H., Takeuchi, T. & Watanabe, T. (1983b) Effects on growth and mineral composition of carp of deletion of trace elements or magnesium from fish meal diets. . *Bulletin of the Japanese Society for the Science of Fish*, **49**, 431±435.

- Schreck, C.B. (1981) Stress and compensation in teleostean fishes: response to social and physical factors. In: A.D., P. (Ed.), *Stress and Fish*, pp. 295–321. Academic Press, London.

- Schreck, C.B. (1982) Stress and rearing of salmonids. *Aquaculture*, **28**, 241-49.

- Sealey, W.M., Lim, C. & Klesius, P.H. (1997) Influence of dietary level of iron from iron methionine and iron sulfate on immune response and resistance of channel catfish to *Edwardsiella ictaluri*. *Journal of the World Aquaculture Society*, **28**, 142–149

- Shearer, K., Christiansen, R., Daae, B. & Torrissen, O.J. (1993) Evaluation of four semi-purified diets for juvenile Atlantic salmon (*Salmo salar*). In: *Fish Nutrition in Practice* (Kaushik, S.J. & Luquet, P. eds), **61**,913-921. INRA Editions, Paris

- Sherman, A.R. (1992) Zinc, copper and iron nutriture and immunity. Journal of Nutrition, **122**, 604-609.

- Shiau, S.Y. & Su L.W. (2003) Ferric citrate is half as effective as ferrous sulfate in meeting the iron requirement of juvenile tilapia, *Oreochromis niloticus · O. aureus. Journal of Nutrition,* **133**, 483–488.

- Marks, D.B., Marks, A.D. & Smith, C.M. (2004) *Basic Medical Biochemistry: A Clinical Approach*. Lippincott Williams and Wilkins, USA.

- Smith, S. & Medlicott, M. (1944) The blood picture of iron and copper deficiency anemias in the rat. *American Journal of Physiology*, **141**,354-8.

- Spray, D.J., Hodson, P.V. & Wood, C.M. (1988) Relative contributions of dietary and waterborne zinc in the rainbow trout, *Salmo gairdneri. Canadian* Journal of Fisheries and *Aquatic* Sciences, 45, 32-41.

- Stefanidou, M., Maravelias, C., Dona, A., & Spiliopoulou, C. (2006) Zinc: a multipurpose trace element. *Archives of Toxicology*, **80**, 1–9.

- Tacon, A.G.J. (1992) Nutritional fish pathology. Morphological signs of nutrient deficiency and toxicity in farmed fish. *FAO Fish Technical Paper.* No. **330**. Rome, FAO, p 75.

- Thompson, E.D., Mayer, G.D., Balesaria, S., Glover, C.N., Walsh, P.J. & Hogstrand, C. (2003) Physiology and endocrinology of zinc accumulation during the female squirrelfish reproductive cycle. *Comparative Biochemistry and Physiology,Part A*, **134**, 819–828.

- Thompson, E. D., Mayer, G. D., Walsh, P.J. & Hogstrand, C. (2002) Sexual maturation and reproductive zinc physiology in the female squirrelfish. *The Journal of Experimental Biology*, **205**, 3367–3376.

- Thomson, A.B.R. & Valberg, S. (1972) Intestinal uptake of iron, cobalt and manganese in the iron-deficient rat. *American Journal of Physiology*, **223**, 1327–1329.

- Tort, L., Pavlidis, M. A. & Woo N.Y.S. (2011) Stress and welfare in sparid fishe*. In: Sparidae: Biology and Aquaculture of Gilthead Sea Bream and Other Species* (Pavlidis, M. & Mylonas, C.C eds), pp.85-94. Blackwell Publishing Ltd., UK.

- Tort, L., Puigcerver, M., Crespo, S. & Padrós, F. (2002) Cortisol and haematological response in sea bream and trout subjected to the anaesthetics clove oil and 2-phenoxyethanol. *Aquaculture Research*, **33**, 907–910.

- Tort, L., Rotllant, J., Liarte, C., Acerete, L., Hernández, A., Ceulemans, S., Coutteau, P. & Padros, F. (2003) Effects of temperature decrease on

feeding rates immune indicators and histopathological changes of gilthead sea bream Sparus aurata fed with an experimental diet. *Aquaculture*, **229**, 55–65.

- Turnbull, J.F., Norht, B.P., Ellis, T., Adams, C.E., Bron, J., MacIntyre, C.M. & Huntingford, F.A. (2008) Stocking density and the welfare of farmed salmonids. In: *Fish Welfare* (ed. E.J. Branson), pp. 111–120. Blackwell Publishing Ltd., Oxford, UK.

- Turnland, J.R., Keyes, W.R., Anderson, H.L. & Acord, L.L., (1989) Copper absorption and retention in young men at three levels of dietary copper by use of the stable isotope [65]Cu. *American Journal of Clinical Nutrition* , **49**, 870-8.

- Vallee, B. L. (1986)   A synopsis of zinc biology and pathology. *In Zinc Enzymes* (H. Gray, ed.), Vol. 1, pp. 1–15. Birkha¨user, Basel.

- Van Der Salm, A.L., Pavlidis, M., Flik, G. & Wendelaar Bonga, S.E. (2006) The acute stress response of red porgy, *Pagrus pagrus* kept on a red or white background. *General and Comparative Endocrinology*, **145**, 247–253.

- Vander, A., Sherman, J. & Luciano, D.S. (1997) *Human Physiology: The Mechanisms of Body Function,* 8th edition, Volume 2. McGraw-Hill Education, New York.

- Vangen, B. & Hemre, G.I. (2003) Dietary carbohydrate, iron and zinc interactions in Atlantic salmon (*Salmo salar*). *Aquaculture,* **219**, 597-611.

- Watanabe, T., Kiron, V. & Satoh, S. (1997) Trace minerals in fish nutrition. *Aquaculture,* **151**, 185-207.

- Wendelaar-Bonga, S.E.W. (1997) The stress response in fish. *Physiological Reviews*, **77**, 591–625.