UNIVERSIDADE DO ALGARVE
FACULDADE DE CIÊNCIAS E TECNOLOGIA

INCORPORATION OF SELENIUM ON MICROALGAE AS SUPPLEMENT TO ARTEMIA AND ZEBRAFISH

Tamára Filipa da Costa Santos

Dissertation
Aquaculture and Fisheries Master

Supervisors

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

" aimlessly, devoted,
devoted to the moment"

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RESUMO

O selénio (Se) é considerado um mineral essencial com benefícios tanto para a saúde humana como animal, atribuidos às suas propriedades antioxidantes. Este micronutriente é utilizado como suplemento nutricional em aquacultura, como forma de promover tanto o crescimento como a capacidade antioxidante dos organismos. O Se apresenta ainda funções na prevenção de deformações esqueléticas e redução dos danos musculares. As microalgas apresentam-se como organismos fonte de micronutrientes essenciais, tal como PUFA e carotenóides, com funções no ciclo de transferência do Se, convertendo a sua forma inorgânica (selenito e selenato), a orgânica (selenometionina). O principal objetivo deste trabalho foi produzir biomassa algal enriquecida em Se, para posterior suplementação de diferentes níveis tróficos. Com este intuito, microdietas formuladas com diferentes fontes e concentrações de Se, foram comparadas para determinação no crescimento e desenvolvimento em larvas de peixe zebra (*Danio rerio*). Nas culturas de *Isochyrsis galbana,* enriquecida com 20 mg Se L^{-1} no meio, obteve-se uma concentração de selénio total de 120 mg Kg^{-1} DW, contudo foi observado uma inibição no crescimento para concentrações de selenato superiores a 25 mg Se L^{-1}. A microalga, *I. galbana* enriquecida em Se foi posteriormente introduzida nos cultivos de *Artemia franciscana* (AF), que obteve valores de Se total de 20 mg kg^{-1} DW em comparação com AF produzida num meio controlo, não enriquecido (2 mg Se kg^{-1} DW). Em termos de comparação da biodisponibilidade de microdietas enriquecidas em Se proveniente de sais inorgânicos, levedura ou de biomassa algal, o tratamento utilizado com Se inorgânico foi considerado tóxico para os estágios larvares de peixe zebra, alcançando 100% de mortalidade com uma concentração de 5 mg Se kg^{-1}DW. Não foram verificadas diferenças significativas para os tratamentos com levedura ou microalga suplementadas, quando utilizada uma concentração de 0.5 mg Se kg^{-1} DW. Contrariamente a biomassa de microalga enriquecida em Se com uma concentração de 2 mg Se kg^{-1}DW, obteve resultados significativos em termos de aumento de crescimento, peso e redução do número total de deformações. O presente estudo, com base nos resultados descritos, concluiu que a espécie *I. galbana,* representa uma fonte promissora para a produção biotecnológica de biomassa enriquecida em selénio, destinada à nutrição animal sobre a forma de suplementos.

Palavras-chave: Selénio; selenometionina; *Isochrysis galbana*; *Artemia spp.*; Peixe-zebra; Bioacumulação; Deformações esqueléticas;

ABSTRACT

Selenium (Se) is an essential element known to promote the health of humans and animals. This micronutrient is commonly used in aquaculture as a feed additive to enhance growth and larval antioxidant capacity and prevent skeletal deformities. Microalgae contain valuable micronutrients (e.g. PUFA, carotenoids) and are able to convert inorganic to organic Se, making them a suitable aquaculture feed source. Thus, the aim of this work was to produce Se-enriched *Isochrysis galbana* microalgal biomass to improve the contents of this element at different trophic levels in aquaculture. For this purpose, Se-enriched yeast and Se-enriched microalgae in microdiets were assessed in terms of the biological performance and osteological development of zebrafish (*Danio rerio*) larvae. *I. galbana* was able to incorporate up to 150 mg Se Kg^{-1} DW when exposed to 20 mg Se L^{-1} in the medium, whereas concentrations higher than 25 mg Se L^{-1} inhibited growth. After feeding Se-enriched *I. galbana* to *Artemia* spp., the latter accumulated up to 20 mg Se Kg^{-1} DW compared to the non-enriched control (2 mg Se Kg^{-1} DW). Inorganic Se salts were found to be toxic to the larval stages at 5 mg Se kg^{-1}. There were no significant differences between the treatments containing 0.5 mg Se kg^{-1} supplied through yeast and microalgal sources. In addition, the diet with Se-enriched microalgal biomass with a concentration of 2 mg Se kg^{-1} enhanced length, weight and significantly decreased skeletal deformations in zebrafish as compared to diets containing Se-enriched yeast or non-supplemented diets. Taken together, the results of the present work strongly suggest that *I. galbana* can be used to produce Se-enriched biomass for fish nutrition in the form of feed supplements.

Keywords: Selenium; Selenomethionine; *Isochrysis galbana; Artemia* spp.*;* Zebrafish; Bioaccumulation; Skeletal development;

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ABBREVIATIONS LIST

AAS	Atomic absorption spectrometer
AF	Artemia franciscana
AFDW	Ash free dry weight
ANOVA	Analysis of variance
CC	Cellular concentration
CCMAR	Centro de ciências do mar
DHA	Docosahexaenoic acid
DPF	Days post fertilization
DW	Dry weight
EC	European commission
EDTA	Ethylenediamine tetraacetic acid
EPA	Eicosapentaenoic acid
FA	Fatty acids
FAME	Fatty acid methyl esters
GC-MS	Gas chromatography with mass spectrometry
GSH-Px	Glutathione peroxidase
HPLC-ICP-MS	High performance liquid chromatography coupled with inductively coupled plasma mass spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
IPQ	Instituto português da qualidade
LES	Laboratório de engenharia sanitária
Met	Metionine
MRL	Maximum residue limit
MUFA	Monounsaturated fatty acids
n.d	not detected
NIST	National Institute of Standards and Technology, U.S. Department of Commerce
OD	Optical density
PBS	Phosphate buffer sodium
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SD	Standard lenght

SD	Standard deviation
Se	Selenium
SeCys	Selenocysteine
SeMet	Selenomethionine
SFA	Saturated fatty acids
TFA	Total fatty acids
TL	Total length
$tRNA^{Met}$	Metionine transfer RNA

1
INTRODUCTION

Selenium (Se) is an essential trace mineral that, due to its antioxidant and anticancer properties (Thiry *et al*., 2012), has substantial health benefits for both humans and animals (Umysová *et al*., 2009)

Nutritionists recommend an increase in the consumption of products rich in Se. In case of insufficient supply through diet, Se must be obtained via supplementation products, increased consumption of food naturally rich in Se or by previously enriched products.

Se is used as feed additive in aquaculture in order to achieve the mineral concentration required for organism development (Elia *et al*., 2011) and for its role in immunity and fish health (Rider *et al*., 2009). This micronutrient is usually used in its inorganic forms: selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}), but higher bioavailability and lower toxicity are attributed to the organic form: selenomethionine (SeMet; Doucha *et al*., 2009). Experimental studies suggest that natural supplements, such as Se-enriched biomass of microalgae, are more effective for Se-supplementation when Se is in the bioavailable form of SeMet (Schrauzer, 2000; Gojkovic *et al*., 2014).

Vertebrates and invertebrates are unable to produce SeMet, which makes them dependent on lower trophic level organisms, such as algae, to synthesize and supply it (Williams *et al*., 1994). Thus, algal communities play a key role in the biotransformation (Besser *et al*., 1993; Bowie *et al*., 1996; Dobbs *et al*., 1996; Riedel *et al*., 1996; Reunova *et al*., 2007) and bioaccumulation of Se throughout the food net (Williams *et al*., 1994) as shown in **Figure 1**.

SeMet obtained from algal biomass can be used either as natural food supplement in order to fulfill the requirements of Se in farmed fish (Thiry *et al*., 2012) or to maintain a suitable Se status in humans (Schrauzer, 2000; Brown & Arthur, 2001; Ahsan *et al*., 2014; Hatfield *et al*., 2014).

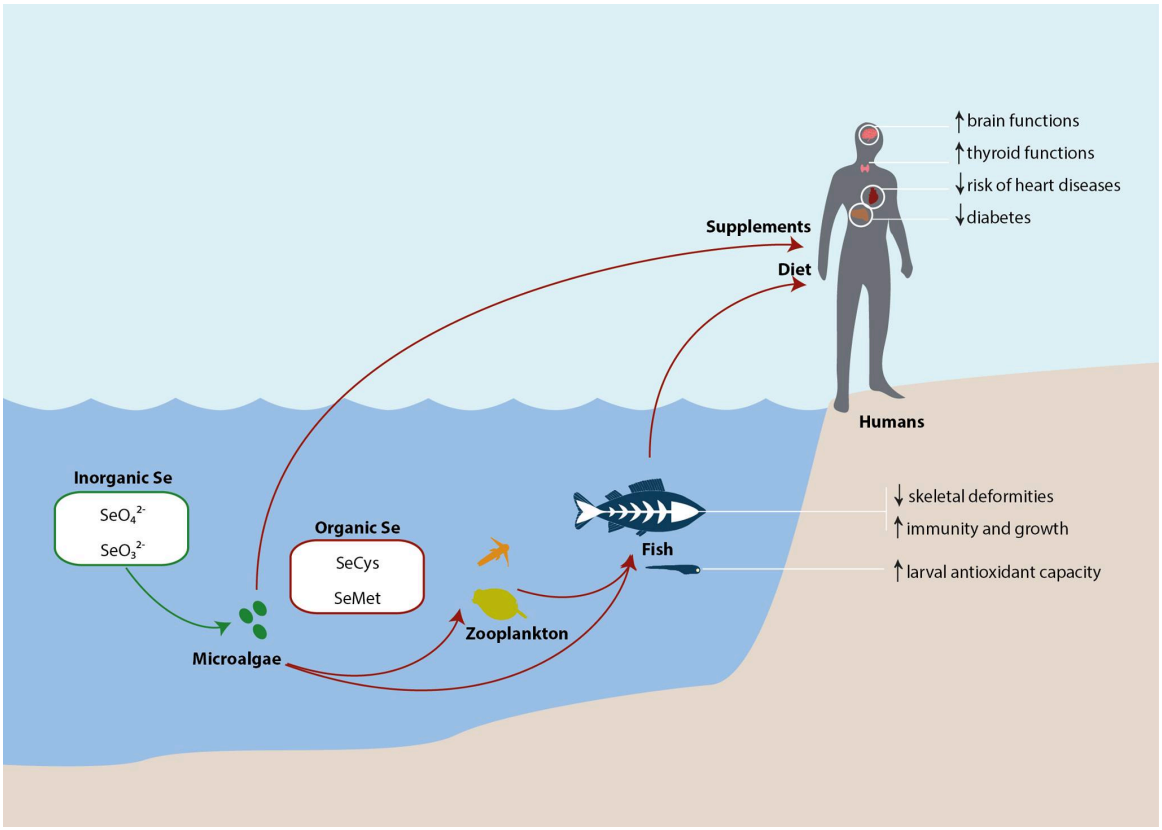

Figure 1 Se trophic cycle in the aquatic food web (adapted from Hasanzzaman *et al*., 2010; Latorre *et al*., 2013). Microalgae assimilate dissolved Se in the form of selenate (SeO_4^{2-}) or selenite (SeO_3^{2-}) from the environment and converted to organic Se (SeMet and SeCyst). Circulation of Se between water to phytoplankton, zooplankton and final consumers occurs via biological transformation of inorganic forms (green pathway) to organic Se (red pathway), with benefits to either fish or humans.

Isochrysis galbana T-ISO was selected due to its high level of polyunsaturated fatty acids (PUFA), in particular of the high-value docosahexaenoic acid (DHA) (Beaudoin *et al*., 2002). Therefore, microalgae Se-enriched biomass can provide high levels of organic-Se

coupled with other valuable micronutrients (e.g. fatty acids, carotenoids; Li *et al*., 2003; Umysová *et al*., 2009; Fournier *et al*., 2010; Vitová *et al*., 2011),

In this context, the present thesis focused on the production of Se-enriched microalgal biomass for different biotechnological purposes, mainly for food supplements and for feed manufacture for the aquaculture industry.

The experimental work assessed the capacity of the marine alga *I. galbana* T-ISO to incorporate inorganic Se. Afterwards, microalgal biomass was used in the supplementation process of live and inert feeds aiming to increase the productivity of cultured aquatic animals and determine the implication of Se in the morphological and osteological development of a vertebrate model - zebrafish. The process of transference along the food chain was also considered of major relevance, since fish is becoming an interesting source of Se for humans (Thiry *et al*., 2012).

1.1. **SELENIUM**

Se was discovered in 1817 by the Swedish chemist Jöns Jacboc Berzelius and was named after the Greek goddess of the moon "Sêlêne" (Lenz & Lens, 2009). It belongs to group VI of the periodic table, and has four oxidative states (-2, +1, +2 and +6), presenting therefore redox functions, reducing peroxides radicals (O_2^{2-}) to neutral forms (O_2; Pacini *et al*., 2013). Se activity is mainly related to selenoproteins (Gojkovic *et al*., 2013), which act as integral part of the immune and antioxidant systems as well as of the metabolism of the thyroid hormone, (Brown & Arthur, 2001).

Se is a micronutrient essential for normal life processes (Köhrle, 2004; Wang *et al*., 2007) and for the preservation of an optimal health status (Rayman, 2000; Brown & Arthur, 2001; Rider *et al*., 2009). Se-antioxidant defense in animal cells maintains the integrity of the cellular membrane and reduces the oxidative damage of biological compounds namely lipids, lipoproteins, and DNA (Rayman, 2000).

However, the limit that defines the beneficial and toxic Se concentrations in vertebrates is narrow (Dörr *et al*., 2008a, 2013b). Se toxicity is dependent on its speciation, target species, type of exposure and environmental aspects (EPA, 2004), and small

concentration variations can have significant consequences on health and performance of the organisms (Hedaoo *et al*., 2008; Ahsan *et al*., 2014).

In aquatic environments, it is possible to find Se in inorganic forms as selenate and selenite (Dörr *et al*., 2008) and their proportion depends on the pH and presence of organic matter (Schiavon *et al*., 2012). Selenite dominates under reducing conditions while selenate is present in alkaline waters (Plant *et al*., 2004; Chapman *et al*., 2011; Schiavon *et al*., 2012). Both inorganic Se species present high bioavailability and bioaccumulation potential for microalgae biotechnology (Lenz & Lens, 2009).

Se organic forms include selenocysteine (SeCys) and selenomethionine (SeMet) (Dörr *et al*., 2008) and these are the most commonly Se forms found in fish. Usually SeMet contents increase with the degree of exposure to Se (Phibbs *et al*., 2011; Pacini *et al*., 2013). SeMet is considered to be more bioavailable for metabolic processes, since it is readily incorporated into proteins, (Kouba, 2014; Le & Fotedar, 2014).

SeCys is part of the active center of the enzyme glutathione peroxidase (GSH-Px), which is a very important antioxidant enzyme involved in the protection of cells from damage caused by free radicals (Rotruck *et al*., 1973; Wang *et al*., 2007). In addition, GSH-Px protects tissues by reducing peroxides (Wang *et al*., 2007) preventing the propagation of free radicals and reactive oxygen species (ROS; Brown & Arthur, 2001). The activity level of this enzyme can also be an effective way to estimate the bioavailability of Se (Wang *et al*., 2007).

Recent studies highlight the benefits of organic-Se when compared with sodium selenite due to its increased uptake, low capacity to induce lipid peroxidation, higher capacity to induce GSH-Px (Rider *et al*., 2009) and less toxicity at higher doses (Wang & Lovell, 1997).

Although organic-Se compounds are considered as the best long-term solution to prevent Se deficit, the inorganic-Se, in the form of selenite, has the ability to act faster to fulfill Se requirements (Thiry *et al*., 2012). Moreover, some authors still recommend this inorganic form due to its lower price, because the highest costs for aquaculture production are usually associated with feed purchase (Chiu *et al*., 2010). Conversely, recent studies argue that supplementation of Se from natural food materials in the form of organic-Se is safer and suitable as compared with consumption of inorganic-Se (Huang *et al*., 2007).

1.2. **SELENIUM IN AQUACULTURE**

1.2.1. **MICROALGAE**

Marine microalgae production is mainly linked to animal feed in the aquaculture sector due to its high content of PUFA, pigments and antioxidants. Moreover, these organisms are considered as a promising source for new products and may generate added value compounds with potential biological activities (Pulz & Grass *et al*., 2004; Spolaore *et al*., 2006).

Algal communities play a key role in the cycle of Se since they are able to incorporate selenate and selenite from the water column and partially convert it into organic-Se (Geoffroy *et al*., 2007).

Se is incorporated into the food chain through bioaccumulation and biomagnification (Phibbs *et al*., 2011) and its exposure at the base of the food web is crucial to the aquatic systems defining the Se concentrations in higher consumers (Ponton & Hare 2013). Resulting concentration in the algal biomass can be much higher than that in the surrounding media (Yamaoka *et al*., 1999; Li *et al*., 2003; Kramárová *et al*., 2012). In tissues of consumers, it can reach concentrations 2000 times higher than those present in lower trophic organisms (Lenz & Lens, 2009).

Microalgae exhibit greater bioaccumulation of Se, reaching higher cellular concentrations as compared to macroalgae (Geoffroy *et al*., 2007; Reunova *et al*., 2007; Umisová *et al*., 2009; Fournier *et al*., 2010).

Se distribution in algal cells varies among species and can be incorporated into amino acids, proteins, soluble carbohydrates and lipids (Bottino *et al*., 1984; Vandermeulen & Foda, 1988; Doblin *et al*., 1999; Li *et al*., 2003). In *Arthrospira platensis* and *Dunaliella salina* selenite accumulation was associated with proteins; well lipids seem to be primarily in *P. tricornutum*, (Wang *et al*., 2003).

Se has been identified as an important component of selenoenzymes in several marine unicellular algae. Aquatic organisms have the highest content of selenoproteins and, so far, 12 have been identified in the green alga *Chlamydomonas reinhardtii*, one in the diatom *Thalassiosira pseudonana* and two in the haptophyte *Emiliania huxleyi* (Araie & Shiraiwa

2009). The highest number, however, was found in *Ostreococcus tauri* and *Ostreococcus lucimarinus* in which 26 and 29 selenoproteins have been identified, respectively (Lobanov *et al*., 2009). In contrast, no selenoproteins have been found in yeast and land plants, suggesting that Se may be a non-essential nutrient to these organisms (Novoselov *et al*., 2002).

Previous studies indicate that inorganic-Se uptake can affect the growth of marine phytoplankton (Umysová *et al*., 2009; Reunova *et al*., 2007; Schiavon *et al*., 2012), although the accumulation and response of microalgae to Se is highly dependent on the species (Dazhi *et al*., 2003; Schiavon *et al*., 2012), concentration and oxidation state of Se used (Pastierova *et al*., 2009; Umysová *et al*., 2009; Schiavon *et al*., 2012). For example, Danbara & Shiraiwa (1999) reported that *E. huxleyi* did not grow in absence of Se, but showed a normal growth in a medium with 10 nM of selenite. Li *et al.* (2003) also observed enhanced growth of *A. platensis* with the addition of 500 μM selenite.

Se can act as an essential micronutrient at low concentrations (Price *et al*., 1987; Price & Harrison, 1988; Pelah & Cohen, 2005). However, at high doses, it may present toxicity (Wong & Oliveira, 1991; Pelah & Cohen, 2005), leading to photosynthesis inhibition, decrease in algal growth rates or increased cellular damage caused by excess ROS production (Pelah & Cohen, 2005; Umysová *et al*., 2009; Fournier *et al*., 2010).

Inhibition of growth by selenate uptake has previously been observed in different microalgae strains. In *D. salina*, Se concentrations of 5 and 10 mg Se L^{-1} cuased a decrease in growth rates, leading to the collapse of the cultures (Reunova *et al*. 2007). In contrast, Pelah & Cohen (2005) observed that *Chromochloris zofingiensis* could tolerate sodium selenite up to 100 mg L^{-1} while the same concentration was found to be lethal to *Scenedesmus quadricauda* (Vitová *et al*., 2011).

Studies demonstrate that algae are able to distinguish between Se species, and even if both forms are accumulated by microalgae, some show higher susceptibility to selenate over selenite, as described for *Chlorella vulgaris* (Simmons & Wallschläger, 2011). However, for *Chaetoceros* sp., *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* the reverse is true (Vandermeulen & Foda, 1985, 1988; Besser *et al*., 1993 Riedel & Sanders, 1996; Riedel *et al*., 1996).

Although high concentration of both inorganic-Se forms can cause oxidative stress, Pastierova *et al*. (2009) found higher toxic effects using selenite than with selenate for *S.*

quadricauda, Desmodesmus subspicatus, C. vulgaris and *Pseudokircheriella subcapitata* (Vitová *et al*., 2011).

The different toxicity levels induced by the different inorganic-Se forms can be related to divergent transport mechanisms for each anion in the microalgae (Umysová *et al*., 2009). Selenate intake is regulated by the sulfate transport system, and is directly dependent and proportional to Se concentration and inversely related to sulfate concentration in the medium (Araie & Shiraiwa, 2009); for selenite, two distinct transport systems were identified, both highly dependent on Se concentration: at low concentration performed by a specific but rapidly saturated ATP-dependent active system, while at higher concentrations uptake was done by a passive non-specific transport system (Morlon *et al*., 2006; Araie & Shiraiwa, 2009; Fournier et al., 2010). Although, Se metabolism in microalgae is not yet fully understood, both ions may share the same metabolic pathway, being reduced to selenide (Se^2; Shrauzzer, 2000) and converted to SeCyst and SeMet. The latter is non-specifically incorporated into proteins, as $tRNA^{Met}$ does not differentiate between methionine (Met) and SeMet (Figure 2; Kouba, 2014; Schrauzer. 2000; Daniels, 1996; Le & Fotedar, 2014).

Beneficial effects of Se to microalgal cultivation are described in some microalgal groups, and it has been demonstrated that growth of some species is enhanced or even dependent on the presence of this micronutrient. Growth rates of the cyanobacterium *A. platensis* were enhanced by the addition of selenate to the medium in the range of 0.5 to 40 mg L^{-1} (Li *et al*., 2003). Reunova *et al*. (2007) observed increased growth in populations of *D. salina* with Se concentrations between 0.01 and 0.5 mg L^{-1}.

Se uptake by algae may also be constrained by the presence of macronutrients such as phosphorus (P) and sulfur (S; Lee & Wang, 2001; Schiavon *et al*., 2012), and an antagonistic effect has been suggested between sulfate and selenate (Williams *et al*., 1994; Fournier *et al*., 2010; Schiavon *et al*., 2012). For example, the green alga *Selenastrum capricornutum* (Williams *et al*., 1994; Riedel & Sanders, 1996) presented lower selenate intake with the increase of sulfate concentration in the medium, supporting the theory of direct competition between sulfate and selenate for transport proteins (Fournier *et al*., 2010; Schiavon *et al*., 2012). Also, Umysová *et al.* (2009) found higher sensitivity to inorganic Se in environments with sulfur deficit in *S. quadricauda* (Fournier *et al*., 2010).

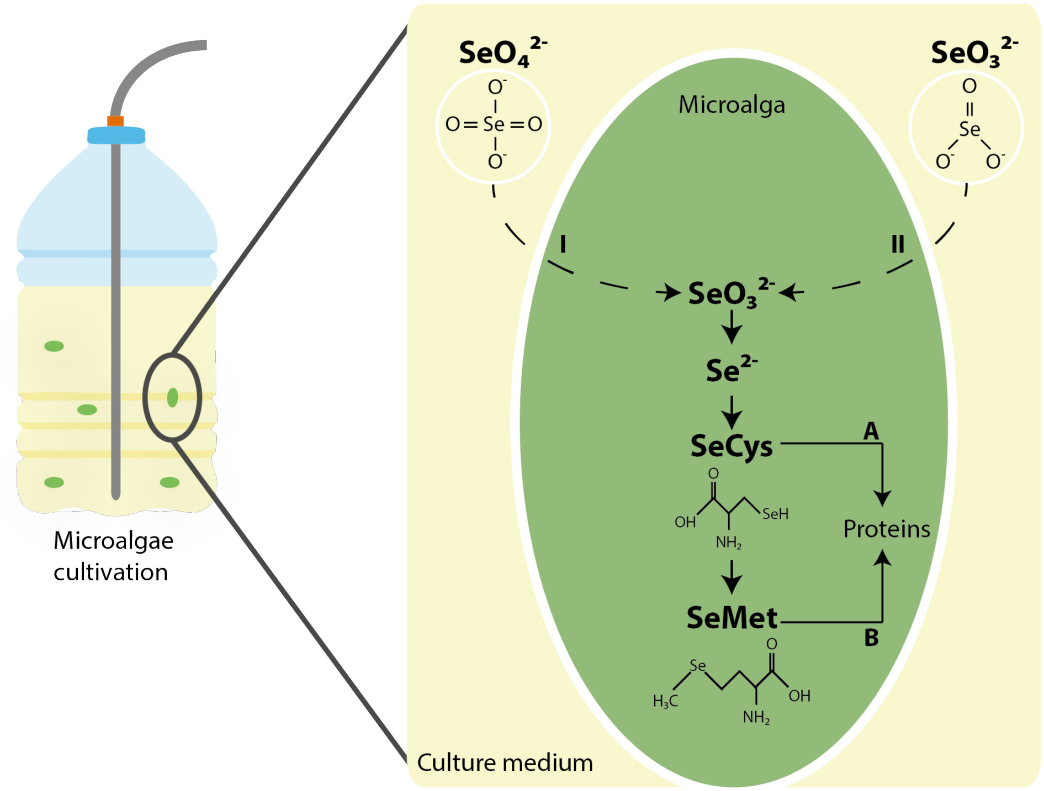

Figure 2 Schematic overview of proposed selenium metabolism in microalgae (adapted from Araie & Shiraiwa, 2009; Zhu *et al*., 2009). Selenate and selenite metabolic uptake in the microalgae cell. [I]: selenate-sulfate transport system; [II] – ATP dependent selenite transport system or non-specific, passive transport system. [A]- SeCys specific incorporation into proteins [B]- SeMet non-specific incorporation into proteins.

Microalgae uptake dissolved inorganic-Se in a dose-dependent manner and can accumulate significantly high Se concentrations. A dose-dependent response was reported for *Chlorella sorokiniana*, which accumulate 3 µg Se g^{-1} after 100 h of exposure to 0.05 mg L^{-1} selenite (Gómez-Jacinto *et al*., 2012).

Recently, researchers have focused their attention for the production of organic Se-enriched food. Gojkovic *et al.* (2013) showed that *C. sorokiniana* could be enriched in SeMet in the presence of selenate, suggesting that this strain can be used as a biological vehicle for Se amino acids. Moreover, at a medium concentration of 40 mg L^{-1} of selenate the microalga reached a SeMet concentration of 140 mg kg^{-1} DW; Umysová *et al.* (2009) observed that *S.*

quadricauda was able to accumulate 300 mg kg^{-1} DW of SeMet in the presence of 50 mg L^{-1} of selenate; and *S. platensis* was able to convert inorganic Se into organic-Se (Li *et al*., 2003) emphasizing its potential for the production of Se-enriched microalgae (Gómez-Jacinto *et al*., 2012).

Se-enriched microalgal biomass production, can help to reduce inorganic Se-supplementation in commercial feeds, being a carrier of valuable selenoaminoacids, leading to either an increase in the productivity and health of aquatic organisms or as a nutritional supplement to humans.

1.2.2. **ZOOPLANKTON**

Se accumulated by aquatic producers, is transferred into intermediate trophic levels consumers (e.g. zooplankton) with further transference and accumulation in higher predators (Fan *et al*., 2002).

For fish aquaculture a proper feed plan is crucial for optimal development and survival rates in larval rearing. Therefore, the selection of nutritive and easily available food sources is important. Due to the small size and undeveloped digestive system of larvae, nutritional options for the larval rearing period are very limited and the use of live feeds (e.g. rotifers and artemia) is for most species, the only choice (Shields *et al*., 1999).

Fish larvae n the natural environment feed mainly on natural marine zooplankton-copepods, however a continuous and intensive copepods production is still not possible (Stottrup *et al*., 2000). The alternative remains in the relative ease production of live feeds and although rotifers and brineshrimp do not fulfill the nutritional requirements of fish larvae, they can be enriched prior feeding. (Penglase *et al*., 2011). Rotifers are small metazoans, with *Brachionus plicatilis* and *Brachionus rotundiformis* being the major marine species used in aquaculture for the production of more than 60 species of marine fish larvae (Penglase *et al*., 2010). Brineshrimp such as *Artemia* spp. has also been found to be a suitable feed for a diverse group of marine organisms, presenting adequate size, short generation time, high digestibility and fecundity rate (Hamre *et al*., 2013). Brineshrimp are considered non-selective particle feeders, being able to incorporate different products prior to feeding the larvae. This

aquatic crustacean represents a way to transfer Se-enriched algae, through "bioencapsulation" to higher vertebrate consumers.

Usually diets used in hatcheries do not supply a balanced nutritional diet, since life feeds do not meet all fish nutritional requirements. One of the main nutritional differences between rotifers and copepods is its mineral content. The higher nutritional difference is assigned to Se (Hamre *et al*., 2008; Penglase *et al*., 2010). Hamre *et al*. (2008) found Se values in rotifers substantially lower than in copepods, with the first in the range (0.08– 0.09 mg kg^{-1} dry weight (DW)) and the second (3–5 mg kg^{-1} DW), while Se requirements for juvenile fish are established between 0.25–0.7 mg Se kg^{-1} (DW) (NRC, 1993; Lin & Shiau, 2005; Penglase *et al*., 2010). Thus, rotifers provide insufficient Se to fulfill these needs (Penglase *et al*., 2011).

However, previous studies indicate that changing the nutritional value of live feeds to a stage closer to copepods can increase both quality and larval rearing success for marine fish (Park *et al*., 2006; Penglase *et al*., 2011). Penglase *et al*. (2011) has also suggested that such enrichment may be most effective when the mineral is delivered as an ingestible food particle instead of the usual water supplementation with the Se in the inorganic form.

1.2.3. **FISH PRODUCTION**

Se is an essential trace mineral required for normal growth and physiological function of fish (Wang & Lovell, 1997) playing an important role in aquaculture productivity (Dörr *et al*., 2013).

This micronutrient is commonly used as an additive in order to increase diet quality (Dörr *et al*., 2008), enhancing larval antioxidant capacity and preventing diseases (Saleh *et al*., 2014), muscle atrophy and skeletal deformations (Wang *et al*., 2013). The European Commission (EC) established for this element a maximum residue limit (MRL) of 0.5 mg kg^{-1} in aquaculture feeds (Directive 70/524/CEE) in order to prevent or reduce the risks of nutritional additives for animal and human health (Regulation 882/2004 EC) and still no minimal dose is legally recommended.

Aquatic animals like zooplankton, absorb inorganic Se directly from the water (Chiu

et al., 2010) through the exoskeleton (Dörr *et al*., 2013). Fish however, can uptake both organic and inorganic Se from the diet (Chiu *et al*., 2010). In higher vertebrates the anterior intestine is the principal way for assimilation, and the liver the main organ for Se metabolism (Hodson & Hilton, 1983; Pacini *et al*., 2013), although the kidney was also indicated as the Se target tissue for different fish species (Elia *et al*., 2011; Huang *et al*., 2012; Pacini *et al*., 2012).

The association between SeMet and proteins leads to an increase in Se concentration in animal tissues (Burk & Hill, 1993; Wang *et al*., 2007), making fish muscle a proper Se source for human nutrition (Wang *et al*., 2007).

Se insufficiency is known to decrease growth (Wang *et al*., 2007), reduce feed efficiency and decrease plasma and hepatic GHS-Px activity in rainbow trout (*Oncorhyncus mykiss*; Hilton *et al*., 1980), Atlantic salmon (*Salmo salar*; Wang & Lovell, 1997) and channel catfish (*Ictalurus punctatus*; Wang & Lovell, 1997).

However, Se requirements are dependent on the Se source present in the diet (Rayman, 2000). Organic compounds are described as more bioavailable than inorganic sources (Wang & Lovell, 1997) for hybrid striped bass (Jaramillo *et al*., 2009) and yellowtail kingfish (Le & Fotedar, 2014). The capacity of SeMet to improve fish immune capacity has also demonstrated in channel catfish with higher antibody production (Wang & Lovell, 1997). This can be associated to SeMet higher incorporation and digestibility in fish when compared to selenite (Wang & Lovell 1997; Lorentzen *et al*., 1998; Cotter *et al*., 2003; Rider *et al*., 2009; Le & Fotedar, 2014). Indeed, Se derived from SeMet has been reported to increase GPx activity, of grouper (*Epinephelus malabaricus*; Lin & Shiau, 2009), cobia (*Rachycentron canadum*; Liu *et al*., 2010), common-carp (*Cyprinus carpio*; Jovanovic *et al*., 1997) and channel catfish (Wang & Lovell, 1997). Nevertheless, sodium selenite (Copat *et al*., 2014) and Se-enriched yeast are the most used sources of Se in animal feed supplementation.

Se requirement in wild fish can vary between 0.1-0.5-μg g^{-1} DW, however cultivated fish need higher concentrations due to Se low bioavailability from feed (Rider *et al*., 2009) and lipid oxidation, resulting from PUFA-enriched feeds (Saleh *et al*., 2014).

Se absorption from formulated feeds can be low due to binding between Se and heavy metals (Le & Fotedar, 2014), for example, the formation of insoluble copper–Se compounds (Lorentzen *et al*., 1998).

Husbandry related stressors (e.g. confinement, handling, high stocking density) increase the demand for antioxidant enzymes (GSH-Px) and dietary supplementation may be necessary to meet the Se requirements in fish (Kucukbay *et al*., 2009). Inclusion of antioxidant factors is critical in farmed fishes not only to reach optimal growth rates but also to maintain a healthy immune system and an optimal oxidative status (Sealey & Gatlin, 2001). Gilthead seabream larvae **(***Sparus aurata*) feed with Se-enriched diets (11.65 mg kg $^{-1}$ DW) showed significantly improved survival rate and stress resistance (Saleh *et al*. 2014), as for European seabass (*Dicentrarchus labrax*) the addition of Se in the diets show to control the injuries caused by ROS leading to a reduction in half of the muscle injuries (Betancor *et al*., 2012).

Incapability to supplement Se in basal diets may be determinant to the health of livestock (Rider *et al*., 2009) leading to significant losses in the aquaculture sector from disease outbreaks (Rider *et al*., 2009).

Se was also described as essential for the correct fish development acting in the reduction of structural deformities. Insufficient levels of this trace mineral, can lead to effects in the ontological development of fish, as reported for common carp with signs of muscle atrophy and skeletal abnormalities (Wang *et al*., 2013).

1.2.3.1. **SKELETAL DEFORMITIES**

Zebrafish has been recently established as vertebrate model organisms for medical research (Lawrence, 2007), frequently used in molecular and developmental research (Fishman, 2001). The relevance of this teleost fish is also increasing in other areas of research fields: e.g animal behavior, fish physiology, and toxicology (Lawrence, 2007), bringing advantages when compared to other model organisms (Fishman, 2001).

A number of favorable characteristics, including small size, high fecundity, transparency during early embryogenesis and rapid development and regeneration, has made this species a potential model for human disease (Lamason *et al*., 2005; Lawrence, 2007). Zebrafish has still become one of the most used model organisms, for the study of bone development, growth and remodeling (Fleming *et al*., 2005).

Bird & Mabee (2003) defined zebrafish axial skeleton as being composed of 31 vertebrae, which are divided into 4 weberian, 10 pre-caudal and 17 caudal vertebra including 3 caudal fin vertebra modified in order to support the caudal fin. Caudal fin represents the most affected area for zebrafish deformities (Bensimon-Brito *et al*., 2010). Internal skeleton of the caudal fin is formed by hypurals (1 to 5), parhypural, and modified haemal arches (Gavaia *et al*., 2006).

Morphological anomalies represent one of the most important problems of the marine aquaculture sector (Georgakopoulou *et al*., 2010) observed with higher incidence in intensive rearing conditions compared to natural environment (Boglione *et al*., 2001; Georgakopoulou *et al*., 2010; Gil-Martens, 2010). Koumoundouros *et al*. (2010) reported that 7 to 20% of aquaculture larvae were affected by skeletal abnormalities, yet in the study from Boglione *et al*. (2013) deformities reached values close do 90% of total larvae.

Marine fish larvae are highly susceptible to skeletal anomalies, as they undergo major functional and anatomic modifications during the early developing stages (Cahu *et al*., 2003). Deformities have been reported to occur as a result of environmental quality (Gil-Martens, 2010), genetics (Gjerde *et al*., 2005) and nutritional factors (Ornsrud *et al*., 2004). Adequate rearing conditions and nutrition is of major importance for improved larvae quality and determine the requirements (e.g. mineral and vitamins) of reared earl life stages for sustaining growth and development (Gil-Martens, 2010). Skeletal deformities represent also an important ethical issue for fish welfare. Aquaculture organisms can suffer high levels of stress and the swimming and feeding capacity can be compromised (Hansen *et al*., 2009; Gil-Martens, 2010). In aquaculture-farmed fish, the most common malformations of the vertebral column are lordosis, kyphosis, scoliosis, and fusions in vertebrae (Boglione *et al*., 2013).

Direct negative effects on both the market value and the production cost are often associated with the decrease of the biological performances of hatchery-reared fish, as survival decrease (Boglione *et al*., 2001, 2013), growth depression (Hansen et al., 2009) and higher susceptibility to diseases, stress and bacteria (Koumoundouros et al., 2010). The percentage of larvae that can survive from a skeletal malformation is very low (Andrades *et al*. 1996). This is associated with high economical consequences for the hatchery, since fish with malformations have low or no market value (Gil-Martens, 2010).

1.3. **IMPACT OF SELENIUM ON HUMAN HEALTH**

Se is an essential nutrient to human biology and nutrition (Brown & Arthur, 2001). Several studies have been carried out regarding the role and importance of this element in human health and its absence is linked to Se-dependent metabolic processes and occurrence of certain diseases (Brown & Arthur, 2001; Rayman, 2004; Copat *et al*., 2014).

Being part of at least 30 human selenoproteins, Se is involved in important metabolic pathways, as the immune defense system, thyroid hormone metabolism, antioxidant defense systems and reproductive performance (Kumar & Priyadarsini, 2014).

Recent evidences have reinforced the role of Se in the prevention of disorders such as cancer, inflammatory diseases, neurodegeneration, infertility and infections diseases (Doucha *et al*., 2009). Moreover, the lack of this mineral can lead to heart disease and hypothyroidism (Pelah & Cohen, 2005).

Se bio-effects depend on its concentration; inadequate supply may result on long-term health implications, which affect 0.5 to 1 billion people worldwide (Haug *et al*., 2007).

In Europe, the recommended daily intake is between 60-70 μg per day in adults, although this concentration is not obtained from the usual diet in many European countries (Rayman, 2000). So, to raise this value, animal feeds are often enriched with Se supplements, frequently in the inorganic form (Doucha *et al*., 2009).

The maximum recommended Se consumption has been set for to the US Food and Nutrition Board, at 400 μg Se day^{-1} in the USA (Thiry *et al*., 2012) and 300 μg Se day^{-1} in Europe (SCF, 2000). The Se essentiality or toxicity only differs by one order of magnitude, reinforcing the need to control the Se supply (Lenz & Lens, 2009).

1.4. **OBJECTIVES**

The main aim of this work was to assess the potential of Se-enriched microalga biomass on the growth performance and osteological development of zebrafish. In order to achieve the main objective, several specific goals were established:

I. Optimize the incorporation of inorganic Se (selenite, SeO_4^{2-}) in the marine microalga *Isochrysis galbana.* Research was centered on the defining the optimum selenate concentration for enhanced incorporation of Se in microalgal biomass.

II. Produce enough Se-enriched microalgal biomass for the formulation of the microdiets. Incorporate Se-enriched alga in brine shrimp (*Artemia* spp.) nutrition.

III. Evaluate the effect of different Se sources and concentrations on the model organism zebrafish (*Danio rerio*) using formulated microdiet.

2
MATERIAL & METHODS

The experimental work was performed between February 2014 and June 2015 at: 1) Centre of Marine Sciences (CCMAR), University of Algarve (UALG), Faro; 2) the Sanitary Engineering Laboratory (LES), UALG, Faro; and the National Health Institute Dr. Ricardo Jorge, Lisbon, Portugal. All organisms, namely *Isochrysis galbana* T-ISO, *Artemia franciscana* and *Danio rerio* were provided by CCMAR through an established cooperation between the research groups MarBiotech and EDGE. The experimental work of this project was divided in three main parts:

I: Optimization of selenate (SeO_4^{2-}) incorporation in the marine microalga *I. galbana* T-ISO;

II: Production of Se-enriched microalgal biomass and incorporation in *Artemia franciscana* for the formulation of experimental microdiets.

III: Assessment of the effect of Se enriched microdiets in the zebrafish (*Danio rerio)*.

2.1. **MICROALGAE**

2.1.1. **EXPERIMENTAL DESIGN**

In the first part of the work, different growth parameters were determined, namely optical density (OD), cellular concentration (CC) and dry weight (DW), in order to optimize Se incorporation in *I. galbana*. Calibration curves for *I. galbana* (CC *vs*. OD and DW *vs*. OD) were established to facilitate the growth optimization of cultures.

To investigate Se accumulation, the marine microalgae was subjected to three independent trials, summarized in Figure 3:

A. Cultures were exposed to increasing selenate concentrations (0, 5, 25, 50 and 100 mg Se L^{-1}) to assess the toxicity of Se in this microalgal strain. Since Se toxicity has not been established for *I. galbana*, the Se concentrations used in the trial were chosen using known toxic levels for other microalgal species (Pelah & Cohen, 2005; Reunova *et al*. 2007; Vitová *et al*., 2011).

B. Cultures were exposed to a narrower range of concentrations (0, 5, 10, 15, 20 mg Se L^{-1}) to assess the Se concentration required for maximum incorporation in the biomass without compromising microalgal growth.

C. Cultures were scaled up and grown in medium with optimized concentration of Se (20 mg Se L^{-1}) to obtain the amount of biomass needed to produce enough amount of microdiet for later experiments.

D. A comparative biochemical analysis between microalga cultivated in Se-supplemented medium and a non-supplemented cultivation medium (control) was performed.

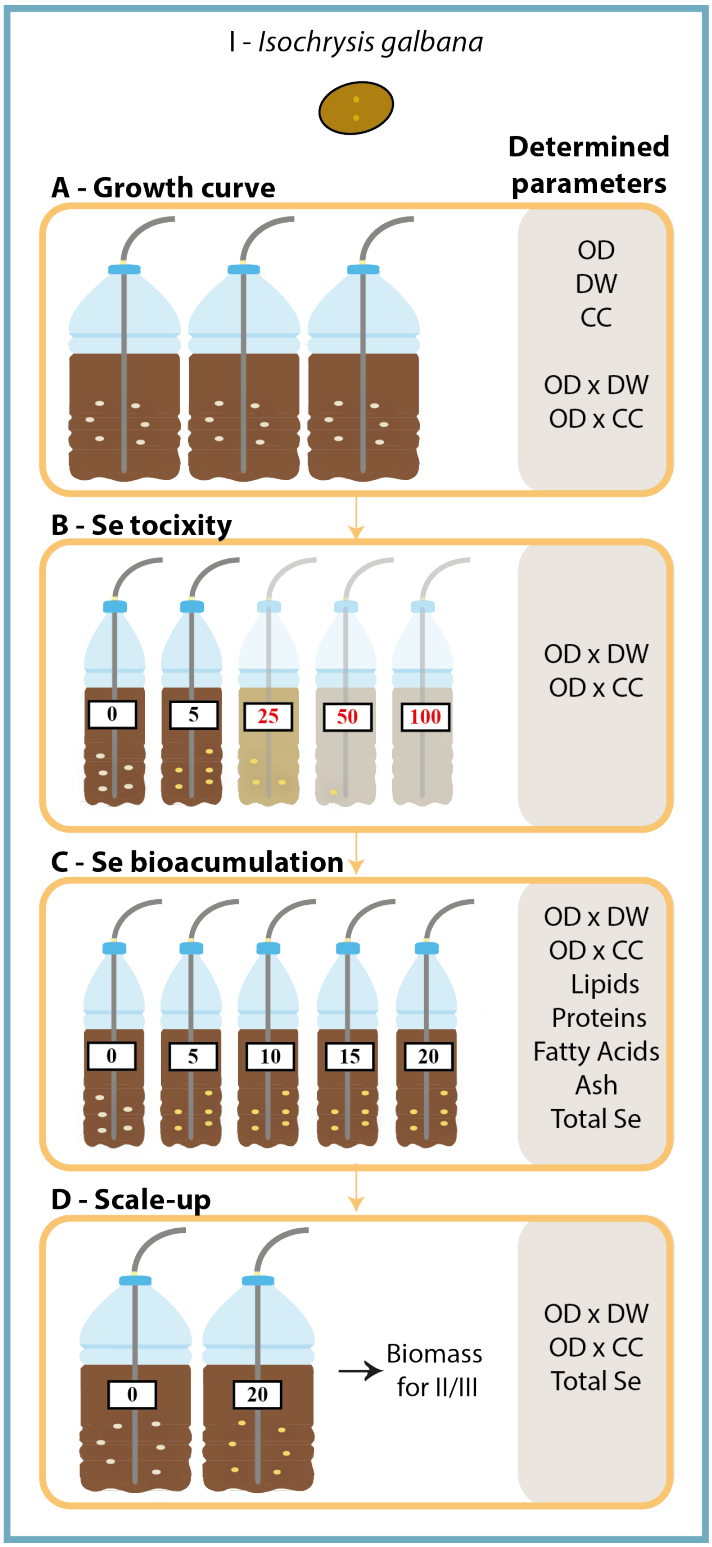

Figure 3 Experimental design (I) and parameters determined in the different assays performed to produce *I. galbana* Se-enriched biomass A) Growth curves were established; B) cultures were exposed to increasing selenate concentrations (0, 5, 25, 50 and 100 mg Se L^{-1}) to establish the lethal Se concentration: C) in the second assay a narrower range of concentrations (0, 5, 10, 15, 20 mg Se L^{-1}) was tested in order to determine the maximum Se incorporation in the biomass; D) to obtain Se-enriched biomass for the following assays, cultures were grown with 20 mg Se L^{-1};. OD-optical density; DW- dry weight; CC-cellular concentration.

2.1.2. **CULTURE CONDITIONS**

Prior to inoculation, all materials were irradiated under UV and the seawater used was sterilized in an autoclave at 120 ºC to avoid culture contamination. Batch cultures of the marine microalga *I. galbana* were performed under controlled conditions in a specialized growth chamber (Aralab Fitoclima s 600 PL clima plus 400) at 20 ºC under continuous light (100 μmol m^{-2} s^{-1}) and aeration. Cultures in the exponential phase of growth were used as inoculum and the initial concentration of each treatment was established at $1x10^6$ cells mL^{-1}.

The culture medium used in this work was a modified ALGAL medium (1:1000) prepared with sterile seawater (Table 1), as described in Pereira *et al*. (2011).

Throughout the experiments, the initial volume was marked on the culture system and the water evaporation was compensated with distilled water. After reaching the stationary phase, microalgal cultures were harvested by centrifugation at 10,000 *g* for 15 min (Beckman Coulter Avanti J-25 High-Performance centrifuge). Obtained biomass was later freeze-dried and stored in vacuum desiccators until further analysis.

Table 1 Composition of the modified ALGAL medium (concentrated 1000×) used throughout this work.

Component	Concentratio
Macronutrients solution (M)	
$NaNO_3$	2
KH_2PO_4	0.1
Micronutrients solution (mM)	
$ZnCl_2$	1
$ZnSO_4.H_2O$	1
$MnCl_2.4H_2O$	1
$Na_2MoO_4.2H_2O$	0.1
$CoCl_2.6H_2O$	0.1
$CuSO_4.5H_2O$	0.1
EDTA-Na	6.4
$MgSO_4.7H_2O$	2
Iron solution (mM)	
$FeCL_3.6H_2O$	20
EDTA-Na	20

2.1.3. **MICROALGAL CULTIVATION WITH SELENATE**

All growth experiments were carried out at the same controlled conditions with autoclaved seawater and ALGAL medium. The only difference in the treatments was the Se concentration used as selenate (SeO_4^{2-}). A selenate stock solution was prepared by dissolving sodium selenate in autoclaved seawater to achieve an initial concentration of 1 g L^{-1} (Na_2SeO_4, anhydrous, Sigma-Aldrich).

Selenate toxicity determination in *I. galbana* was performed in 1.5 L plastic bottles with a final volume of 1 L. Microalga was exposed to increasing selenate concentrations of 0, 5, 25, 50 and 100 mg Se L^{-1}.

Afterwards, in the same experimental conditions, microalgae were cultured in medium supplemented with sub-lethal concentrations of inorganic Se tolerated by the algae (0, 5, 10, 15, 20 mg Se L^{-1}). Total Se determination and biochemical analysis were performed after the cultures reached late stationary phase (day 21).

Subsequently, cultures were scaled-up into two different treatments: control (non-supplemented cultivation medium) and for the highest selenate concentration tolerated by the algae (20 mg Se L^{-1}). Cultures were grown in 5-L plastic bottles until stationary phase (day 15) and the obtained biomass was used for the enrichment ofAF spp. and for the preparation of microdiets used in the zebrafish supplementation trial. Differences in total Se accumulation (summarized in section 2.4) and biochemical composition between microalga cultivated in Se-supplemented medium and a non-supplemented cultivation medium were evaluated.

2.1.4. **GROWTH PARAMETERS**

Cultures were grown in 5-L plastic reactors under the aforementioned conditions (section 2.1.2). All experiments were performed in triplicate and each bottle was inoculated with 500 mL of *I. galbana* T-ISO, 3 L of sterile seawater and modified ALGAL medium (1:1000). Cultures were maintained until the stationary phase (day 17) was reached.

Under aseptic conditions and without water replacement, 40 mL samples were collected every two days from each culture in order to perform the OD, CC, and DW analysis.

Upon individual determination of the growth parameters, a relationship between CC and OD and between DW and OD was established. Absorbance was measured at 750 nm in triplicates and the following linear equations were obtained: $CC = (2x10^7 \text{ x } OD750) - 758588$ with a $r^2 = 0.983$ (Figure 4) and for $DW = (0.879 \text{ x } OD) + 0.0142$ with a correlation coefficient $r^2 = 0.991$ ($p < 0.01$; Figure 7). These correlations enabled the estimation of CC and DW on further cultures trough the values of absorbance.

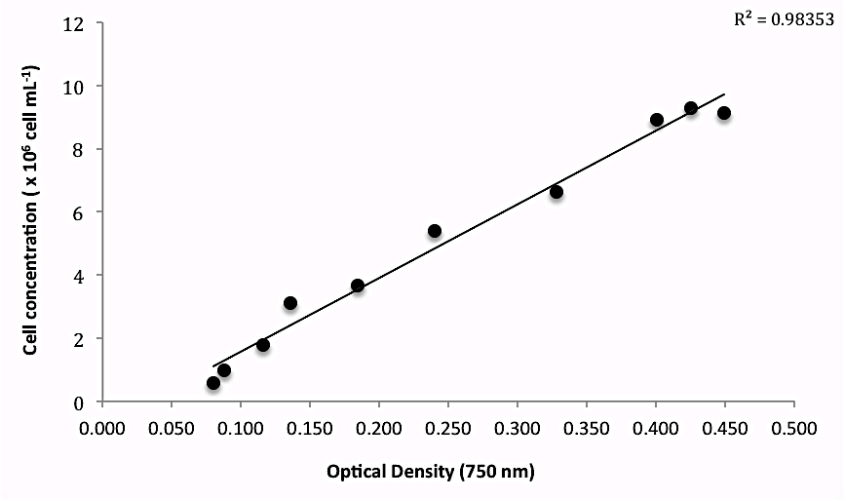

Figure 4 Relationship between optical density measured at 750 nm and cellular concentration (cells mL^{-1}).

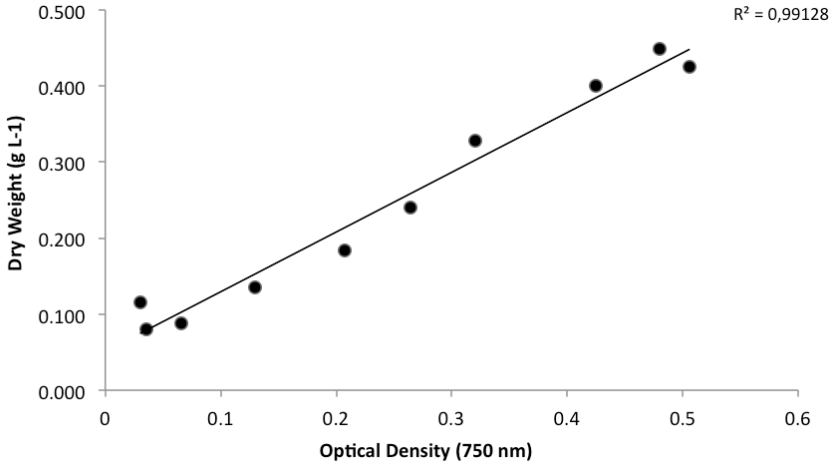

Figure 5 Relationship between optical density measured at 750nm and dry weight (g L^{-1}).

2.1.4.1. **CELLULAR CONCENTRATION**

CC was measured by optical microscopy, using a Neubauer chamber. Samples of 1 mL were taken from each replicate and fixed with Lugol's solution (1:1000). Ten µL of microalgal culture, were strongly homogenized and transferred to each chamber so that the cell number in every count was between 30 and 300 cells, following the manufacturer's specifications. When the cell count was higher, samples were diluted with seawater. Samples were counted in quadruplicate and CC for each count was calculated using the following equation [1]:

$$\text{Cellular Concentration (cells mL}^{-1}) = cell\ number \text{ x } 10^{4} \text{ x } dilution \quad [1]$$

2.1.4.2. **OPTICAL DENSITY**

The OD of cultures was estimated at 540 (OD540) and 750 nm (OD750) using a microplate reader (BioTek Synergy 4). In order to measure the OD, 250 µL of undiluted algal culture were pipetted per well in a 96-well plate and the absorbances were measured at both wavelengths. The absorbance selected was the 750 nm for the high correlation coefficient find between growth parameters.

2.1.4.3. **DRY WEIGHT**

Glass microfiber filters (1.2 µm) were washed with 10 mL ammonium formate (31.5 g L^{-1}) using a vacuum filtration system and left to dry for 24 hours at 60 ºC in an oven. Afterwards, the filters were placed in the desiccator for 15 minutes and weighed (initial weight). For each replicate, 10 mL of sample were filtered, rewashed with 10 mL ammonium formate and dried at 60 ºC for 48 hours, or until constant weight was obtained, and their final weight was recorded (Zhu & Lee, 1997). DW was calculated using the following equation [2]:

$$Dry\ weight\ (\mathrm{g\ L^{-1}}) = \frac{\text{Initial weight} - \text{Final weight}}{\text{Volume of the sample}} \qquad [2]$$

2.1.5. **BIOCHEMICAL ANALYSIS**

2.1.5.1. **MOISTURE AND ASH**

To assess moisture content, 10 mg of dry biomass were placed in porcelain crucibles and dried in an oven at 105 ºC for 24 hours. Subsequently, they were placed in a desiccator for 15 minutes and weighed to obtain the DW. Moisture content in the samples resulted from

the difference between initial biomass weight and DW.

Afterwards, samples were burned for 8 hours in a muffle furnace at 560 ºC for 6 hours. The crucibles were later cooled down until constant weight was achieved. Furthermore, ash free DW (AFDW) was determined by deducting both moisture and ash weight from the initial biomass weight (Widbom, 1984).

2.1.5.2. **TOTAL LIPIDS**

Total lipids were determined gravimetrically using the Bligh and Dyer (1959) method with modifications. Briefly, 30 to 40 mg of freeze-dried microalga samples were weighed in test tubes. Afterwards, 0.8 mL of distilled water were added and allowed to rest for 20 minutes. Subsequently, 2 mL methanol and 1 mL chloroform were added and the mixture was homogenized with an IKA Ultra-Turrax disperser in an ice bath for 60 seconds. Later, 1 mL of chloroform was added and samples were homogenized for 30 seconds, followed by the addition of 1 mL of distilled water and a 30-s homogenization. Samples were then centrifuged for 10 min at 2000 *g* and the organic phase was transferred into a new tube. Finally, 1 mL chloroform was transferred to pre-weighed test tubes.

Lipid extracts were evaporated at 60 ºC in a dry bath overnight, allowed to cool in a desiccator for 3 hours and the final weight was registered. The percentage of total lipids in samples was determined using the following equation [3]:

$$\%\text{Total Lipid} = \left[\frac{(final\ weight - initial\ weight) \times total\ vol.\ choroform\ (2\ mL)}{\frac{Vol.\ evaporated\ choroform\ (1\ mL)}{Sample\ DW}}\right] \quad [3]$$

2.1.5.3. **FATTY ACID PROFILE**

The fatty acid methyl esters (FAME) profile was determined resorting to gas chromatography coupled with mass spectrometry (GC-MS) through a modified protocol from Lepage and Roy (1984), as described in Pereira et al. (2012). Concisely, 20 mg of freeze-dried microalgae were transferred into derivatisation vessels. Samples were later homogenized with an IKA Ultra-Turrax disperser in a mixture of acetyl chloride and methanol (20:1, v/v) for 90 s. Then, 1 mL of hexane was added to the mixture and heated to 90 °C in a water bath for 60 minutes. Afterwards, samples were allowed to cool and 1 mL of distilled water, and 4 mL hexane were added. The mixture was vortexed for 1 minute in order to allow mass transfer from the polar to the non-polar phase, followed by phase separation by centrifugation (2000 *g* for 5 min). The organic phase was transferred to new vials and remaining microalgal biomass was further extracted 4 times with 4 mL of hexane. Extracts were then washed with excess anhydrous sodium sulfate to remove any residual water and filtered using 0.45-μm syringe filters. Finally, hexane was evaporated until dryness under a gentle nitrogen gas flow and further resuspended in 500 μL of gas chromatography-grade hexane. Samples were kept at -20 º C until further analysis.

Extracts were analyzed using an Agilent GC-MS (Agilent Technologies 6890 Network GC System coupled with a 5973 inert Mass Selective Detector) using an Agilent Tech DB-5MS column (length: 25 m; internal diameter: 0.250 mm; film: 0.25 μm). The injection temperature was set for 300 ºC and helium was used as carrier gas at a constant flow of 0.8 mL min^{-1}.

Compounds were identified by comparison of the retention times of standard samples (Supelco 37 FAME Mix, Sigma-Aldrich) and the mass spectra compared to the NIST library. FAME determination was performed by the elaboration of individual calibration curves for all FAME detected (**Table 2**) using four dilutions from the initial standard. All samples were analyzed in triplicate.

Table 2 Calibration curve and correlation coefficient for each detected FAME (y: peak area; x: concentration μg g^{-1}).

FAME	CALIBRATION CURVE	R^2
C14:0	$\mathbf{y} = 1.162.\ 10^5\boldsymbol{x} + 8.663.\ 10^5$	**0.945**
C15:0	$\mathbf{y} = 1.221.\ 10^5\boldsymbol{x} + 4.082.\ 10^4$	**0.957**
C16:1	$\mathbf{y} = 1.034.\ 10^5\boldsymbol{x} - 3.304.\ 10^5$	**0.958**
C16:0	$\mathbf{y} = 1.122.\ 10^5\boldsymbol{x} + 1.000.\ 10^6$	**0.943**
C18:3	$\mathbf{y} = 9.204.\ 10^5\boldsymbol{x} - 5.455.\ 10^4$	**0.971**
C18:2	$\mathbf{y} = 9.450.\ 10^4\boldsymbol{x} - 3.853.\ 10^5$	**0.968**
C18:1	$\mathbf{y} = 1.761.\ 10^5\boldsymbol{x} + 5.188.\ 10^5$	**0.950**
C18:0	$\mathbf{y} = 1.232.\ 10^5\boldsymbol{x} + 5.071.\ 10^4$	**0.963**
C20:5	$\mathbf{y} = 8.785.\ 10^5\boldsymbol{x} - 6.058.\ 10^5$	**0.983**
C20:2	$\mathbf{y} = 8.791.\ 10^4\boldsymbol{x} - 5.173.\ 10^5$	**0.969**
C20:1	$\mathbf{y} = 1.116.\ 10^5\boldsymbol{x} + 5.268.\ 10^4$	**0.954**
C22:6	$\mathbf{y} = 5.188.\ 10^4\boldsymbol{x} - 6.761.\ 10^5$	**0.999**
C22:1	$\mathbf{y} = 1.849.\ 10^5\boldsymbol{x} - 9.670.\ 10^5$	**0.965**
C22:0	$\mathbf{y} = 1.102.\ 10^5\boldsymbol{x} - 6.501.\ 10^5$	**0.963**

2.1.5.4. **PROTEIN CONTENT**

Crude protein content was determined by measuring total nitrogen in a CHN elemental analysis (Vario EL III, Elementar). Total nitrogen content was multiplied by 4.59 to obtain the total protein content on the biomass. This conversion factor was proposed by Lourenço et al. (2004) for *I. galbana.*

2.1.6. **TOTAL SELENIUM DETERMINATION**

2.1.6.1. **ATOMIC ABSORPTION SPECTROMETER**

Total Se analyses were done using a graphite furnace atomic absorption spectrometer (AAS) in the Laboratório de Engenharia Sanitária (LES; Faro, Portugal) certified for multi-element analyse by the Portuguese Institute of Quality (Instituto Português da Qualidade - IPQ). The limit of detection (LD) of AAS was 0.2 mg kg^{-1}.

Powdered samples (0.500 g) from *I. galbana* were subjected to a microwave assisted acid digestion procedure (Milestone Ethos Plus lab station with HPR-1000/10S high pressure segmented rotor) under optimized conditions and according to a temperature-controlled program (Table 3). The final digestion temperature was 200 ºC in perfluoroalkoxy (PFA) vessels, which were equipped with temperature and pressure sensors.

Samples were treated with 6 mL of 65% pure nitric acid (HNO_3) and 2 mL of 30% hydrogen peroxide (H_2O_2), in triplicate, transferred to a 10-mL volumetric flask and the volume completed with deionized ultrapure Milli-Q water (Q-POD Element, Milestone). Digestion blanks were run in parallel with the samples.

Total Se in the acid digests was measured using a graphite furnace atomic absorption spectrometer under operating conditions. Accuracy and precisions of the method was confirmed using certified reference material for the element tested.

Table 3 Microwave digestion program for determination of total selenium in microalgae.

Stage	T (ºC)	Time (min)	Power (W)
1	200	10	1000
2	200	20	1000
3	150	11	1000

2.1.6.2. **INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY**

Inductively coupled plasma mass spectrometry (ICP-MS; XSeries 2, Agilent Technologies) was performed to assess the total Se content. This analysis was performed in the Food and Nutrition Department (DAN) from the National Health Institute Doutor Ricardo Jorge (INSA; Lisboa, Portugal).

Se detection limit with ICP-MS was 0.5 μg kg^{-1}. Procedures that followed the experimental conditions are summarized in Table 4.

Samples were spiked by high purity ICP stock standard 100 mg L^{-1} (multi-element solution IX, Merck) with different concentrations of metals for the recovery repeatability test, in order to verify the analytical method. Germanium (Ge), Yttrium (Y) and Indium (In) were selected as internal standards for analytical signal correction. Isotopes ^{77}Se, ^{78}Se, ^{80}Se and ^{82}Se were simultaneously monitored, but only ^{77}Se was used for quantitative analyze.

Algal biomass, control (CTRL) and Se-enriched *I. galbana* (20 mg Se L^{-1}) were used (50 mg DW) in triplicate for the microwave-assisted acid digestion procedure (Milestone Ethos Plus), identical to the procedure described before (section 2.4.1.1), under the parameters defined in Table 3.

As a result of digestion, colorless samples were obtained, which were transferred to volumetric flasks. Final volume was achieved with HNO_3 (2%), 100 mL to Se-enriched algae and 25 mL to control biomass.

AF. and fish biomass were processed under the same microwave-assisted acid digestion protocol (Milestone Ethos Plus), performing the temperature-controlled program described in Table 5. AF (0.100 g DW) and Zebrafish (0.050 g DW) were digested using 4 mL of ultrapure HNO_3, 3 mL of ultrapure H_2O_2 and 1 mL of deionized Milli-Q water. Clear solutions were obtained in all samples.

The AF samples that resulted from the digestion were poured into volumetric flasks and the volume was completed with 25 mL of 2% HNO_3, in the control samples (no addition of Se) and 100 mL in the Se-enriched AF.

There were four different experimental groups regarding zebrafish (CTRL; LEV; MA1; MA2). After digestion, the final volume was adjusted to 25 mL with HNO_3 (2%). Afterwards, total Se was determined.

Table 4 ICP-MS working conditions for total Se determination, operating with Argon (Ag).

Elements of measuring system	**Working parameters**
Extraction	-133.3
Pole Bias	12.0
Focus	-0.1
Hexapole Bias	-3.0
Nebulizer flow rate (L min^{-1})	0.83
Forward Power (W)	1404
Cool gas flow rate (L min^{-1})	14.9
Auxiliary gas flow rate (L min^{-1})	0.97
Sampling Depth	125
Standard Resolution	110
High Resolution	120
Analogue Detector	1900
PC Detector	3080
Readings	3

Table 5 Microwave digestion program for total selenium determination in AF and zebrafish samples.

Stage	**Time (min)**	**T (ºC)**	**Power (W)**
1	10	180	1150
2	5	180	0
3	6	210	1150
4	5	210	0
5	6	210	650

2.2. **LIVE FEEDS**

2.2.1. EXPERIMENTAL DESIGN

AF was cultivated under two different treatments: T-ISO-Se^{+} and without Se enrichment, T-ISO-Se^{-} (control). In the Se enrichment treatment, AF was subjected to a standard short-term enrichment for 12 hours, using 20 mg L^{-1} of selenate enriched microalgae (Figure 6).

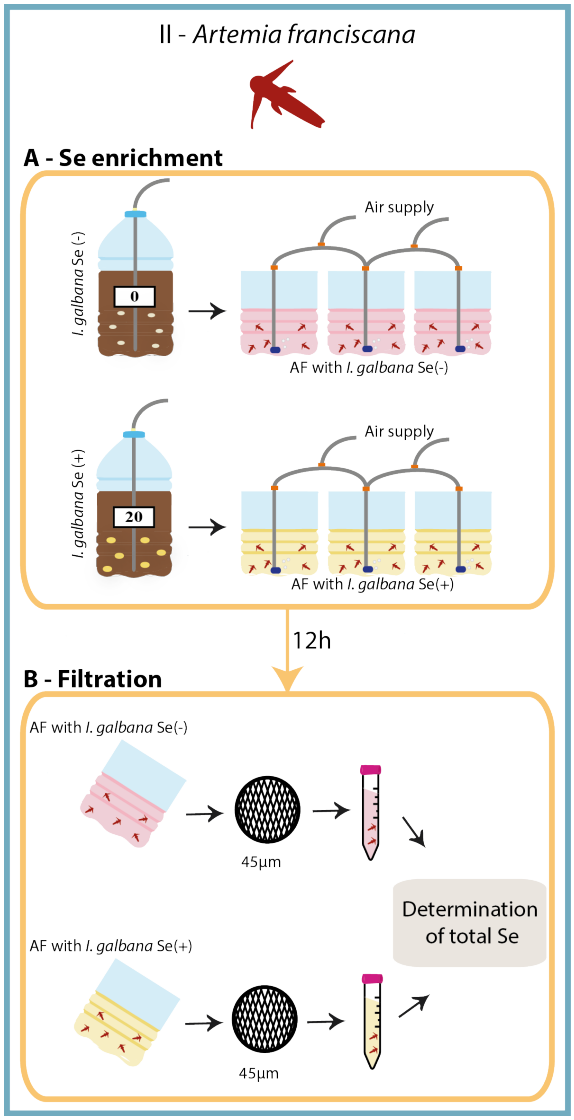

Figure 6 Experimental designs established for: A- the enrichment of *Artemia franciscana* with *I.galbana* Se-enriched biomass (Se^{+}) and compared to a treatment with *I. galbana* without Se enrichment. B-Artemia was latter filtered with a 45 µm mesh, freeze dried and stored in a dessicator until determination of total Se.

2.2.2. **CULTURE CONDITIONS AND REARING**

Artemia. franciscana (AF) cysts were incubated at 28 ºC in seawater (35 ppt) under strong continuous aeration for 24 hours. After reaching the Instar II stage 150,000 metanauplii were transferred to 5-L plastic bottles to obtain a final volume of 3 L. The two treatments were done in triplicate, in separated systems, and subjected to a short-term enrichment with the enriched microalgae using 6 x 10^6 cells/mL of *I. galbana* T-ISO. Upon enrichment, brine shrimp samples were collected with a 45-µm mesh, rinsed with distilled water, concentrated, transferred to new flasks and freeze-dried until further analysis.

2.3. **MICRODIET PREPARATION**

Inert diets for all the treatments were prepared by SPAROS Lda. (Olhão, Portugal). Feed basic formulation contained 61% crude protein, 13% crude fat, 0.5% fiber, 7.2% ash and 19.0 MJ Kg^{-1} gross energy. Diets were prepared by mixing powder ingredients and fish oil and the resulting mash was humidified and agglomerated. The resulting pellets were dried, powdered and separated to the appropriated size. Standard ingredients and proximate composition of the experimental diets are described in Table 6.

A trial was conducted with 5 different experimental diets containing the same basal mix composition, but with a different formulation in terms of Se content and source. Among the treatments two diets, (MA [1]; MA [2]), were formulated with the addition of Se-enriched *I. galbana* biomass, in order to obtain 0.5 and 2.0 mg Se Kg^{-1}, respectively. For the remaining treatments were included in equal proportion non-enriched microalga biomass and the Se content had different sources (inorganic salts, enriched yeast) to a final Se content of 0.5 mg Se Kg^{-1}. The summarized dietary treatments tested in the experiment were:

CTRL - non-enriched microalgae;
MA (1) - enriched T-ISO (0.5 mg Se Kg^{-1});
MA (2) - enriched T-ISO (2 mg Se Kg^{-1});
LEV - inorganic Se enriched yeast (0.5 mg Se L^{-1}) and non-enriched T-ISO;
SS - inorganic Se (0.5 mg Se Kg^{-1}) and non-enriched T-ISO;

Table 6 Standard diets composition (%)**.**

Ingredients	Basal Mix (%)
MicroNorse	52.7
CPSP 90	15
Fish gelatin	4
Wheat gluten	7
Gelatinized peas (Aquatex 8071)	5
Vit & Min Premix PV01	2
Brewer's yeast	5
Soy lecithin – Powder	7
Antioxidant powder (Paramega)	0.2
Sodium propionate	0.1
NaH_2PO_4	2

2.4. **ZEBRAFISH**

2.4.1. EXPERIMENTAL DESIGN

Microdiets using Se-supplemented microalgal biomass were compared with standard feed plans containing inorganic Se in zebrafish larvae. Se effect was assessed on the performance and osteological development of zebrafish (Figure 7).

Each treatment was done in triplicate using 100 individuals, with a total of 1500 larvae. Feeding had the duration of 30 days after fertilization (DPF), allowing the fish to reach the juvenile stage.

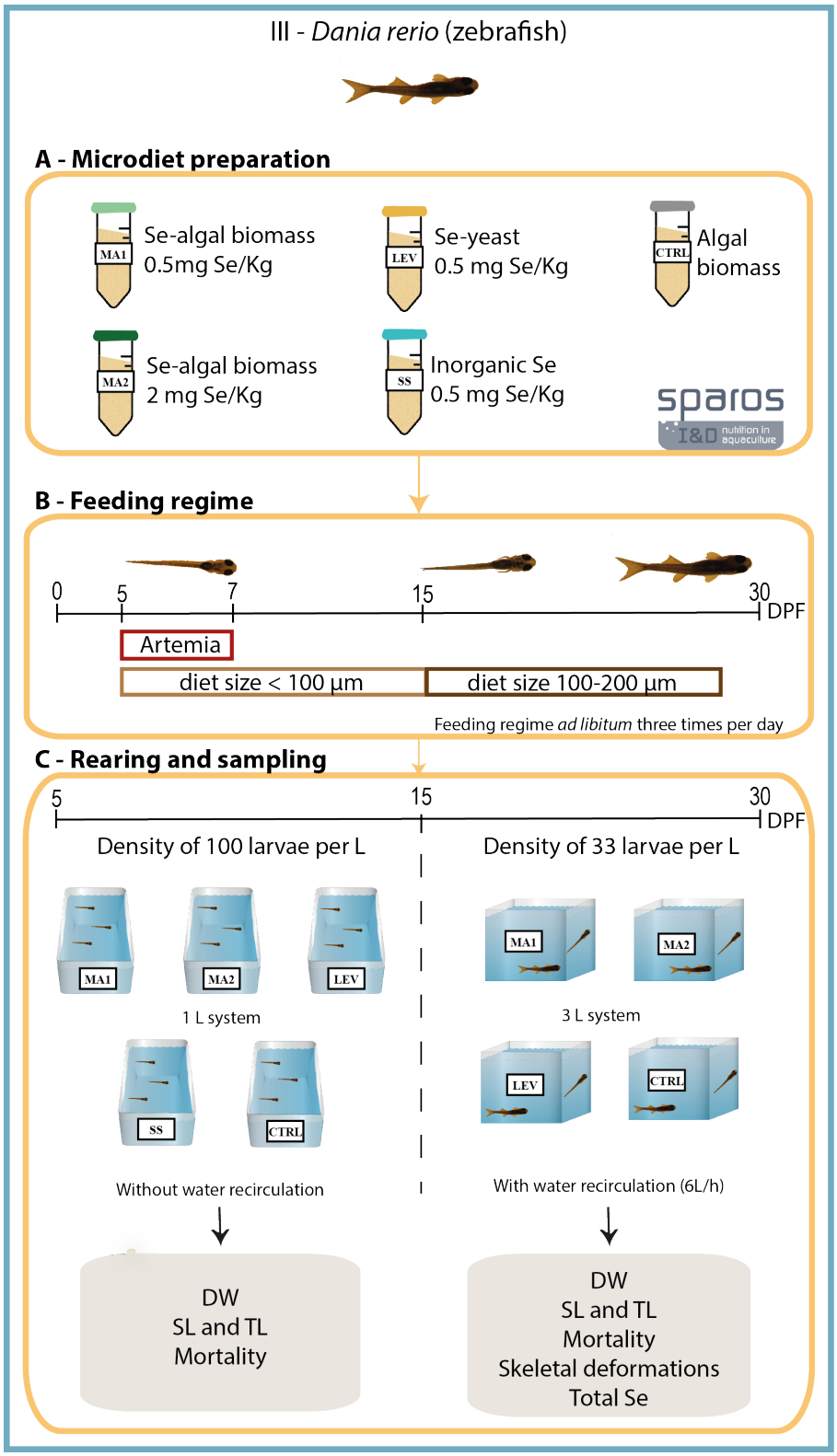

Figure 7 Experimental design used to assess the effect of different microdiets on the model vertebrate, zebrafish. A-Produced microdiets with different sources and concentrations of Se; B-Temporal feeding regime established with AF and microdiets for 31 DPF. C- Rearing system used in the course of the assay, larvae were grown at a density of 100 larvae l-1 until 15 DPF and later transferred into 3 L systems at a final density of 33 larvae L-1. Larvae samples were collected at 15 DPF and 31 DPF for later analyses.

2.4.2. **LARVAE REARING**

Adult breeding stocks of wild-type zebrafish (AB strain) were maintained in 3L-freshwater tanks in a water recirculation system with a renewal rate of 7.3 L h^{-1}. Freshwater was treated by a reverse osmosis system (ZebTEC®, Tecniplast, Italy). Water parameters were maintained at controlled pre-set conditions: 28 ± 0.5 ºC, 680 µS of conductivity and pH of 7.5.

The broodstock was mated and the eggs incubated in 1-L reproduction tanks and further maintained at 28 ºC (without water recirculation). Methylene blue (0.01%) was added in order to reduce bacterial growth, as described in Brand et al. (2002).

After the 5 initial days, 100 larvae were randomly relocated to the experimental system that consisted in 15 rectangular tanks of 1 L. Each tank was randomly placed and assigned to one of the five treatments (CTRL; LEV; SS; MA1; MA2). Tanks were cleaned daily, mortality monitored and rearing water was fully exchanged every 2 days.

Afterwards, larvae at 15 days post fertilization (DPF) were redistributed to 3-L glass tanks connected to a recirculating system. The recirculating flow rate was maintained at 100 mL min^{-1} so that the entire volume was renewed two times per hour. Water parameters were maintained under the aforementioned conditions and a 14:10 light-dark photoperiod cycle was established. At this stage, tanks were cleaned three times a day, in order to remove debris and keep the water parameters constant. Temperature, conductivity and pH were monitored daily and nitrate and ammonia levels were controlled weekly and maintained at recommended levels (< 0.1 mg L^{-1}).

2.4.3. **LARVAE SAMPLING**

Larvae were sampled at 5, 15 and 30 DPF to analyze morphometric parameters, such as weight, total length (TL) and standard length (SL). On each DPF, 10 larvae were randomly selected, and subjected to a lethal tricaine dose (Sigma-Aldrich). All larvae were photographed individually (Sony Powershot G12, Sony, Japan) and TL and SL measured using an image analyses software (Image-J). Samples were freeze-dried and further weighed

in a precision balance to determine its DW.

All organisms were collected at the end of the experiment (30 DPF) and divided: 15 individuals were used for staining and determination of skeletal deformities (described in section 2.3.5), and the remaining biomass was stored at -20 °C and later freeze-dried for chemical analysis (described in section 2.4).

2.4.4. **NUTRITION**

Larvae were firstly fed with newly hatched AF nauplii until 7 DPF. For that purpose, 1g L^{-1} of AF cysts were cultured for 24 hours and maintained in seawater at a salinity of 35 ‰, 28 °C and strong aeration. Newly hatched AF were collected and rinsed with water on a 150-µm sieve to eliminate bacteria, and further diluted to prevent the fish from undergoing an osmotic shock.

Microdiets were first administrated at 5 DPF in a co-feeding regime with AF.; inert diets were given two times a days intercalated with one meal composed by newly hatched AF. When the majority of larvae presented the necessary mouth size (7 DPF), the feeding scheme was changed and larvae were entirely fed *ad libitum* with the treatment diets, three times a day, until the end of the experiment. Individuals with 5-15 DPF were fed with diets with 100 µm pellet size and after this period and onwards, were replaced to 100-200 µm pellets. Feeding period had a total duration of 30 days until the juvenile stage.

2.4.5. **SKELETAL DEFORMITIES**

Larvae from the final sampling (30 DPF) were used to determine skeletal abnormalities. Collected fishes were stained using mount bone and cartilage double staining procedure, as described in Walker & Kimmel (2007) and adapted from Gavaia *et al*. (2000).

Organisms were anesthetized with a lethal dose of tricaine and fixed in a 4% buffered paraformaldehyde (PFA) for 24 hours at 4°C. Larvae were later washed with 0.1 M phosphate buffer saline (PBS), pH 7.4, followed by ethanol (70%) and maintained at 4 ºC.

Afterwards, samples were removed from the ethanol solution and submitted to a hydration process through sequential baths of decreasing ethanol concentrations (96–70-50-30-15%) for 15 minutes. Samples were finally rinsed with distilled water.

The staining protocol consisted in two phases: firstly using alcian blue 8GX solution for cartilage and secondly with alizarin red S for bone staining. In the first phase, 5 mL of alcian blue 8GX (0.1%) were applied for 30 minutes, while the second phase of the procedure included the removal of excess dye with ethanol and incubation of the samples in 1% KOH for 1 hour. The hydration process was repeated and the organisms retained in KOH (1%). A solution of alizarin red S and 1% KOH (1:50) was applied overnight and subsequently a final cleaning with 1% KOH was performed.

As the last step, the stained specimens were subjected to a series of glycerol baths (25-50-75%) in distilled water, each for a period of 3-4 hours. Samples were preserved at room temperature at the highest glycerol concentration (75%) until observation.

Subsequently stained fish were observed for skeletal abnormalities in the cranial region, vertebral column and caudal fin complex (stereomicroscope Leica MZ6; LeicaMicrosystems, Wetzlar, Germany).

2.5. **STATISTICAL ANALYSES**

Data was analysed using using one-way ANOVA for growth and lipid correlations with a confidence level of 99%.

Data were evaluated using one-way ANOVA with Tukey's multiple comparison tests for growth correlations, biochemical analyses, number of deformities and presence of deformities by area. Data related to larvae, namely total and standard length and weight were analyzed by repeated measures ANOVA. Significant level for all tests was $p < 0.05$. Statistical analysis and graphs were performed in the statistic program GraphPad Prism 5.

3
RESULTS & DISCUSSION

3.1. MICROALGAE

3.1.1. SELENATE EFFECT ON GROWHT

In order to optimize the concentration for Se supplementation, microalgal cultures were first cultivated in the presence of selenate at concentrations ranging from 0 to 100 mg Se L^{-1}. Cultures were grown for 5 days with an initial CC of $1x10^6$ cells mL^{-1}. Obtained results revealed a strong growth inhibition in concentrations above 25 mg Se L^{-1}, in fact both concentrations of 50 and 100 mg Se L^{-1} promoted the collapse of microalgal cultures, showed in Figure 8 by constant cellular concentration for the 5 days trial. At concentrations below 25 mg Se L^{-1}, cells tolerate selenate and cultures were able to grow, although the growth rate was reduced significantly.

As previously reported, Se requirement is highly species-dependent and high levels are known to display toxicity in microalgae (Boisson *et al.*, 1995). Inhibition of growth by Se uptake had been observed previous in different microalgae strains, Reunova *et al*. (2007) reported that concentrations of 5 and 10 mg Se L^{-1} led to a decrease in the growth rates causing the collapse of *D. salina* cultures.

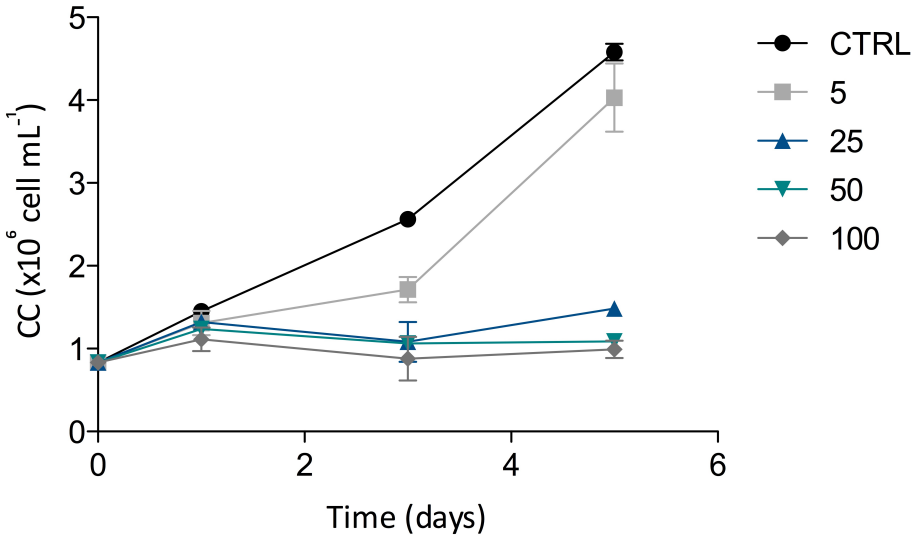

Figure 8 Growth of *I. galbana* in nutrient solutions with different selenate concentrations in the medium. Optical density measured at 750 nm during a cultivation period of 5 days and cellular concentrations (CC) estimated for 5 different treatments (CTRL:Control; 5 mg Se L^{-1}; 25 mg Se L^{-1}; 50 mg Se L^{-1}; 100 mg Se L^{-1}). Data are given as mean values ± SD of the means (n = 3).

However, certain microalgae strains can tolerate increasing concentrations of Se, Umysová *et al*. (2009) had demonstrated that *S. quadricauta* tolerates a maximum Se concentration of 50 mg L^{-1}, while Vitová *et al*. (2011) had reported 100 mg L^{-1} as the lethal Se concentration for this strain. Moreover, C. *zofingiensis* (Pelah & Cohen, 2005) and *Cricosphaera elongata* (Boisson *et al*., 1995) also tolerate high concentrations of Se, 100 mg Se L^{-1} of had also been reported as the limit concentration for the growth of both strains.

Upon establishing the lethal Se concentration for *I. galbana*, microalgae were grown in a narrower range of selenate concentrations, between 0 and 20 mg Se L^{-1}, until they reach the stationary phase of development (Figure 9).

I. galbana growth followed the normal pattern for microalgae cultures, with a prolonged lag phase until day 5. Afterwards cultures started to grow exponentially, reaching the higher cellular concentration at day 19, except for the highest Se treatment (20 mg Se L^{-1}), which occurred at day 17. Control cultures displayed a CC of $1.27x10^7$ cells ml^{-1} and a DW of 0.619 g L^{-1} at day 19. The culture subjected to the higher Se concentration (20 mg Se L^{-1}) had at day 17 a CC of $1.04x10^7$ and a DW of 0.544 g L^{-1}.

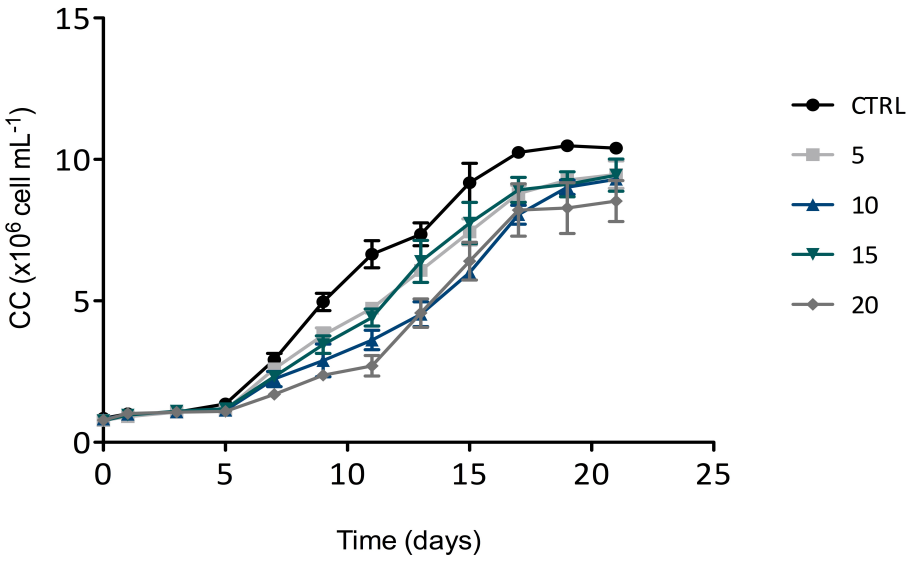

Figure 9 Cellular concentration of *I. galbana* grown in media with different selenate concentrations (CTRL:Control; 5 mg Se L^{-1}; 10 mg Se L^{-1}; 15 mg Se L^{-1}; 20 mg Se L^{-1}). Data are given as mean values ± SD deviation (n = 3).

Growth decreased slightly with the addition of sodium selenate although all concentrations maintain a viable microalgal culture. At lower concentrations (5 and 10 mg Se L^{-1}), selenate showed no markedly effects on the growth of this strain, Li *et al*. (2003) had reported a higher growth for *A. platensis* with the addition of low Se concentrations (0.5 to 40 mg Se L^{-1}) to the cultures, although growth in *I. galbana* cultures was not enhanced with Se-enrichment of the medium.

Therefore, to produce Se-enriched biomass a sub-lethal selenate concentration of 20 mg Se L^{-1} was established. Stable growth was obtained for both treatments Se-added cultures were able to maintain cell viability until the end of the 15 days trial.

3.2. SELENIUM BIOACCUMULATION

I. galbana exposed to selenate incorporate this element in different degrees, depending on the concentration of exposure (Figure 10). Increased Se concentration (0-20 mg Se L^{-1}) in the culture resulted in the increase of total Se in microalgal cells.

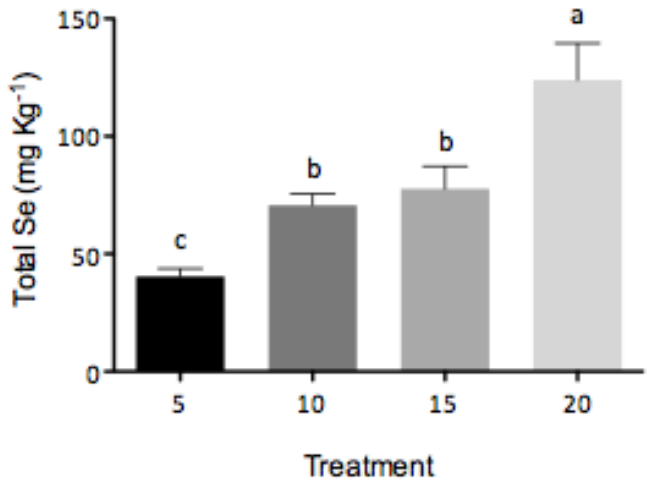

Figure 10 Total Se content (mg Kg $^{-1}$ of DW) of I. galbana grown in media with different Se concentrations (5 mg Se L^{-1}; 10 mg Se L^{-1}; 15 mg Se L^{-1}; 20 mg Se L^{-1}). Bars represent means ± SD (n = 3). Bars labelled with different letters are significantly different (ANOVA in conjunction with Tukey's test for multiple comparisons $p < 0.05$).

Concentrations used were established as non-toxic, with no serious hazard to the vital activities of cultures. Control cultures had a Se content inferior to the quantification limit detectable by EEA (<0.2 mg Kg $^{-1}$ DW).

The lowest Se treatment (5 mg Se L^{-1}), led to an accumulation of 41 mg Se Kg $^{-1}$ DW, which was 3 times lower than the treatment with the highest Se concentration, registering a total Se content of 123 mg Se Kg $^{-1}$ DW. Treatments with 10 and 15 mg Se L^{-1} obtained similar values for total Se (70.71 and 77.68 mg Se Kg $^{-1}$ DW respectively). It was considered that the highest concentration of Se in the medium allowed a high exposure and thus a higher intake. A dose-dependent response had also been reported for *C. vulgaris* (Sun *et al*., 2014) and *C. sorokiniana* (Gómez-Jacinto *et al*., 2012).

Bioaccumulation of Se has observed in other species for example *C. sorokiana* accumulated 3 mg Se Kg^{-1} DW after 100 h of exposure to 0.05 mg L^{-1} selenite (Gómez-Jacinto *et al*., 2012) and *C. vulgaris* had resisted to 75 mg Se L^{-1} with a total Se concentration of 857 mg Kg $^{-1}$. The superior accumulation of C. vulgaris when compared with *I. galbana* could be related with the higher tolerance of the species to Se (75 mg Se L^{-1}) in opposition to 20 mg Se L^{-1} used in this work.

According to our data, *I. galbana* can tolerate and accumulate Se efficiently with the addition of 20 mg Se L^{-1} to the culture medium. Therefore, microalgae biomass used to the formulation of microdiets was produced using this Se concentration. Se concentration in the obtained biomass was determined by AAS and ICP-MS (Table 7). Although, ICP-MS was considered a more sensitive detector to the quantification of trace elements (Thiry *et al*., 2012), both methods gave similar results.

Table 7 Comparison between total Se content (mg Kg^{-1}) between AAS and ICP-MS for *I. galbana* control with no Se addition (ISO-CTRL) and for ISO-20 (cultivated under 20 mg Se L^{-1}). Data represented by means ± SD (*n* = 3).

Treatment	Total Se –AAS	Total Se -ICP-MS
ISO-CTRL	<0.200	0.901 ± 0.062
ISO-20	152.035 ± 0.435	154.133 ± 6.270

3.3. BIOCHEMICAL ANALYSIS

3.3.1. AFDW AND PROTEIN CONTENT

The biochemical composition of microalgae cultures is essential to establish their potential for aquaculture purposes (Kivomars *et al*., 2012). The biomass proximate composition was analyzed in cultures at the stationary phase for both treatments (CTRL, ISO 20) and is presented in Table 2. Values observed for ash content and AFDW of microalga had no significant difference between treatments. The crude-protein content was determined and was expressed in percentage of DW. Se had no significant effect on the crude protein content of samples; control group had 20.1% protein content, similar to the value obtained to cultures with Se (19.7%). Values are in agreement for those reported for this species by previous works (Skrede *et al*., 2011).

3.3.2. TOTAL LIPIDS CONTENT

The total lipid content of cultures reduced with increasing selenate concentration in the medium. Control cultures were able to accumulate 17% of lipids DW, while the total lipid was reduced to 13% DW in the 20 mg Se L^{-1} treatment. Microalgae value for nutritional purposes is highly related to its lipid content and fatty acid composition (Fidalgo *et al*., 1998). Previous works reported a total lipid content for *I. galbana* between 20% and 30% (Zhu *et al*., 1997; Sukenik & Wahnon, 1991), which is higher than the values obtained in this work.

Table 8 Biochemical composition of I. galbana grown without Se addition (ISO-CTRL) and for ISO-20 (cultivated under 20 mg Se L^{-1}). Data represented by means ± SD, n =3.

Composition	ISO-CTRL	ISO-20
Ash (%)	14.38 ± 2.75	13.14 ± 2.96
AFDW (mg)	0.02 ± 0.00	0.03 ± 0.00
Crude Protein (%)	20.14 ± 0.14	19.66 ± 0.15
Total Lipids (%)	17.15 ± 1.65	13.39 ± 0.20

3.3.3. **FAME PROFILE**

The FAME profile was also determined for both control and for the highest Se concentration used in this experiment (20 mg Se L^{-1}; Table 3). Se addition to the microalgal cultures apparently had no significant effects in the fatty acids profile in both cultures. The main fatty acids detected in both cultures were palmitic (C16:0), oleic (C18:1) and docosahexaenoic (C22:6n-3; DHA) acids, which accounted for more than 60% of total fatty acids (TFA). Myristic (C14:0) and palmitoleic (C16:1) acids were also detected at relatively high proportions (7-9% of TFA). The FAME profile of *I. galbana* is mainly composed of polyunsaturated fatty acids (PUFA; ≈50%) rather than monounsaturated (MUFA; ≈25%) and saturated (SFA; ≈25%) fatty acids. This high amount of PUFA led to a ratio of PUFA/SFA of nearly 2 in both cultures, which is highly interesting for nutritional purposes. Additionally, PUFA are mainly composed of n-3 PUFA (>35% of TFA), due to the high content of DHA, and also of eicosapentaenoic acid (EPA) although this PUFA was detected at lower proportion (≈1% of TFA). This resulted in a very low ratio of n-6/n-3 PUFA (0.3), with n-3 fatty acids, with high nutritional value for aquaculture (Strobel*, et al*., 2012). PUFA synthetized by algae, are essential components in the rearing and development of marine fish, mollusks and crustaceans in early stages of development (Fidalgo *et al*., 1998), and their content is a main factor for the selection of microalgae strains for aquaculture production. *I. galbana* has a especial interest for this sector due to their high PUFA content especially for the high percentage of DHA (>25%) as reported by Sánchez (2000) and Fidalgo (1998), which is consistent with the results obtained in the present work. EPA and DHA importance

to aquaculture has been related to their function as structural and functional components of cell membranes and due to the role as precursors of biologically active compounds (e.g. eicosanoids; Sargents *et al*., 1999). Marine fish are unable to produce PUFA in sufficient amounts, obtaining DHA and EPA trough primary producers. (Copeman & Laurel, 2010).

Table 9 FAME profile of microalgae grown under different culture conditions (control conditions and treatment supplemented with Se at 20 mg L^{-1}). Values are represented in percentage of total FAME ± SD deviation. n.d. = not detected, (n = 4).

FAME	**Common name**	**Conditions**	
		Control	**20 mg L^{-1} (SE)**
C14:0	Myristic acid	9.39 ± 0.07	7.08 ± 0.06
C16:0	Palmitic acid	12.69 ± 0.15	15.89 ± 0.22
C18:0	Stearic acid	0.67 ± 0.01	0.80 ± 0.14
C20:0	Arachidic acid	0.33 ± 0.04	n.d.
C22:0	Behenic acid	1.39 ± 0.04	1.14 ± 0.13
Σ SFA		24.45	24.90
C16:1	Palmitoleic acid	8.26 ± 0.16	6.84 ± 0.04
C18:1	Oleic acid	16.81± 0.85	17.14 ± 0.03
Σ MUFA		25.90	24.45
C18:2(n-6)	Linoleic acid	7.70 ± 0.41	8.09 ± 0.01
C18:3(n-6)	Linolenic acid	3.62 ± 0.09	3.62 ± 0.55
C20:2(n-6)	Eicosadienoic acid	0.19 ± 0.01	0.93 ±0 .18
C20:5(n-3)	Eicosapentaenoic acid	1.32 ± 0.05	0.93 ± 0.10
C22:6(n-3)	Docosahexaenoic acid	37.23 ± 1.93	35.229 ± 0.07
Σ PUFA		50.05	48.80
Σ Ω3		38.55	36.16
Σ Ω6		11.50	12.64
Σ PUFA / Σ SFA		2.05	1.96
Σ Ω6 / Σ Ω3		0.300	0.35

3.4. SE TRANSFERANCE UP THE FOOD CHAIN

3.4.1. **ZOOPLANKTON**

Artemia spp. is a non-selective and obligate filter feeder, which can consume detritus, microalgae and bacteria. The nutritional value of these organisms is dependent on both the macronutrients and micronutrients it can accumulate (Vismara *et al*., 2003) that are later transferred to fish larvae (Espinosa & Allam, 2006).

Minerals and PUFA enrichment in live preys, has been identified as a requirement for marine fish larviculture (Hamre *et al*., 2008; Izquierdo & Koven, 2011). Microalgae are considered a better alternative for the enrichment process when compared with commercial enrichments, mainly due to a higher survival rate, nutrient content and salt tolerance (Chakraborty *et al*., 2007; Vikas *et al*., 2012).

Our data demonstrated that short-term enrichment with live Se-supplemented microalga could significantly enhance the Se content in live feeds. Upon a 12-hour enrichment with Se-enriched *I. galbana, Artemia* spp. contained 20 mg Se Kg^{-1} DW, while the control treatment had only 2 mg Se Kg^{-1} DW (**Table 10**). Kim *et al*. (2014) has suggested that the use of Se-enriched rotifers with a Se concentration of 2.2 mg Se Kg^{-1} DW could improve growth and development of sea bream larvae.

Vikas *et al*. (2012) proved the capability of *Artemia* nauplii to incorporate high amounts of EPA and DHA from this microalga. Thus, *Artemia* spp. with incorporated *I. galbana* can provide at the same time a high content of Se and essential fatty acids, namely DHA.

Table 10 Comparison between total Se content (mg Kg $^{-1}$) in A-CTRL (*Artemia* control with no Se addition) and A-Se (Artemia Se-fortified with ISO- cultivated under 20 mg Se L^{-1}). Data represented by means ± SD (n = 3). Means with different letters are significantly differents (Student t test, $p < 0.05$).

Treatment	Total Se (mg Kg $^{-1}$)
A-CTRL	19.880± 0.6343
A-Se	2.022±0.1556

3.5. ZEBRAFISH

3.5.1. **TOTAL SELENIUM**

Total Se was determined in zebrafish biomass for all the four treatments (CTRL - non-enriched microalgae; MA (1) - enriched T-ISO (0.5 mg Se Kg^{-1}); MA (2) - enriched T-ISO (2 mg Se Kg^{-1}); LEV - inorganic Se enriched yeast (0.5 mg Se L^{-1}) and non-enriched T-ISO; SS - inorganic Se (0.5 mg Se Kg^{-1}) and non-enriched T-ISO) at the end of the 31 days trial (Figure 6).

The highest total Se content was determined in the fish fed with feed supplemented with microalgae in order to achieve a Se concentration of 0.5 mg Kg^{-1} – MA1 (9.41 mg Se Kg^{-1} DW), followed by MA2 (2.48 mg Se Kg^{-1} DW), LEV (1.58 mg Se Kg^{-1} DW) and CTRL (1.82 mg Se Kg^{-1} DW). The retention time of Se in fish muscle is generally dependent on its chemical form; fish fed with organic Se having longer retention times, than fish fed with inorganic salts (Wang & Lovell 1997; Nugroho & Fotedar, 2013; Lin *et al*., 2014). Microalgae can convert inorganic Se and accumulate it in the form of organic Se, increasing its bioavailability (Sun *et al*., 2014). Our results are in accordance with this since feeds containing Se-fortified *I. galbana* biomass presented the highest total Se contents while no differences were observed between the control treatment and zebrafish fed with Se-enriched yeast (LEV). Supplementation with Se-fortified algal biomass was therefore more effective than Se-enriched yeast, which supports the evidences of the higher bioavailability of microalgae as a Se source to fish. Surprisingly, fish fed with the microdiet containing 0.5 mg Se Kg^{-1} DW (MA1) showed a markedly higher accumulation than those fed with the

microdiet containing 2 mg Se Kg^{-1} DW (MA2). Contrary to our results, Se uptake by fish has been shown to be dependent on the Se concentration used in diets (Küçükbay *et al*., 2009; Ellia *et al*., 2011; Saleh *et al*., 2014). It is possible that a switch has occurred during the processing of the samples.

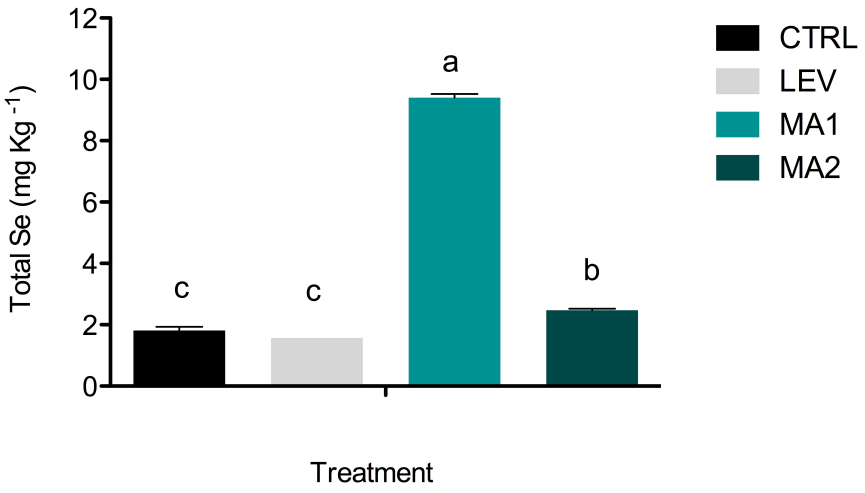

Figure 11 Total Se content (mg Kg^{-1} of DW), determined by ICP-MS, in zebrafish breed with different experimental diets: control diet (CTRL), diet supplemented with 0.5 mg Se Kg^{-1} DW of Se-enriched yeast (LEV) and diets supplemented with Se-fortified *I.galbana* T-ISO, 0.5 mg Se Kg^{-1} DW of (MA1) and 2 mg Se Kg^{-1} DW (MA2). Bars represent means ± SD, (n = 3). Bars labelled with different letters are significantly different (one way ANOVA in conjunction with Tukey's test for multiple comparisons, $p < 0.05$).

3.5.2. **SURVIVAL**

Nutritionally complete diets are fundamental to promote the economic potential of finfish production in aquaculture industry (Penglase *et al*., 2011). Antioxidant nutrients, like Se have been described to enhance survival and growth in fish (Saleh *et al*., 2014). However, the benefits of Se enrichment are highly dependent on speciation and concentration used (Wang & Lovell, 1997).

The results obtained in this experiment, showed 100% mortality for experimental microdiet SS, formulated with inorganic selenite in the concentration of 5 mg Se kg^{-1} DW, indicating excessive dietary Se for this larval stage. Inorganic Se was also toxic to rainbow

trout juveniles fed with 13 mg Se Kg^{-1} DW (Hilton *et al*., 1980) and cod larvae fed with 3 mg Se Kg^{-1} DW (Penglase *et al*., 2010). Whereas, remaining treatments showed no statistical differences for survival (Table 11). Zebrafish larvae fed with Se-enriched yeast or Se-fortified microalga biomass had similar survival rates than those of the control. These results are in agreement with the work for *Solea senegalensis* larvae (Ribeiro *et al*., 2012) and for *Epinephelus malabaricus* (Shiau, 2005).

Table 11 Survival (%) of zebrafish during 31 days trial and subjected to different diets: control (CTRL), diet supplemented with 0.5 mg Se Kg^{-1} DW of Se-enriched yeast (LEV) and diets supplemented with Se-fortified *I.galbana* T-ISO, 0.5 mg Se Kg^{-1} DW of (MA1) and 2 mg Se Kg^{-1} DW (MA2). Values are represented by the means ± SD deviation (n = 3). Different letters are significantly different (One way ANOVA in conjunction with Tukey's test for multiple comparisons, $p < 0.05$).

Diets	Survival (%)
CTRL	53.67 ± 1.70^{a}
SS	0.00 ± 0.00^{b}
LEV	61.67 ± 4.19^{a}
MA1	68.33 ± 8.06^{a}
MA2	65.67 ± 4.03^{a}

3.5.3. **GROWTH PARAMETERS**

Morphological traits, such as standard length (SL), total length (TL) and weight were enhanced by the treatment with the highest concentration of Se (2 mg Se kg^{-1} DW) in microalga biomass (Figure 7). Zebrafish larvae feed with MA2 achieved the highest SL (9.95 mm), TL (12.29 mm) and weight (2.24 g) after 31 days, while the lower value of SL and TL was obtained with the LEV diet (8.95 and 10.77 mm respectively) and the lower weight with the MA1 diet (1.41 g).

Se enrichment has been described to improve growth and development in *Carassuis auratus gibelio (*allogynogenetic crucian carp; Wang *et al*., 2007), *Oncorhynchus mykiss* (rainbow trout; Hilton *et al*., 1980) and *Epinephelus malabaricus* (grouper; Lin & Shiau, 2005). Kim *et al*. (2014) observed also higher growth in *Pagrus major* (red sea bream) fed

with diets containing 2.2 mg Se Kg^{-1} DW. Larvae fed with LEV and MA1 diets had the lower growth values for length (SL; TL) and weight when compared with control and MA2 treatments. These results indicate that LEV and MA1 supplemented diets may not fulfill Se requirements for zebrafish larvae.

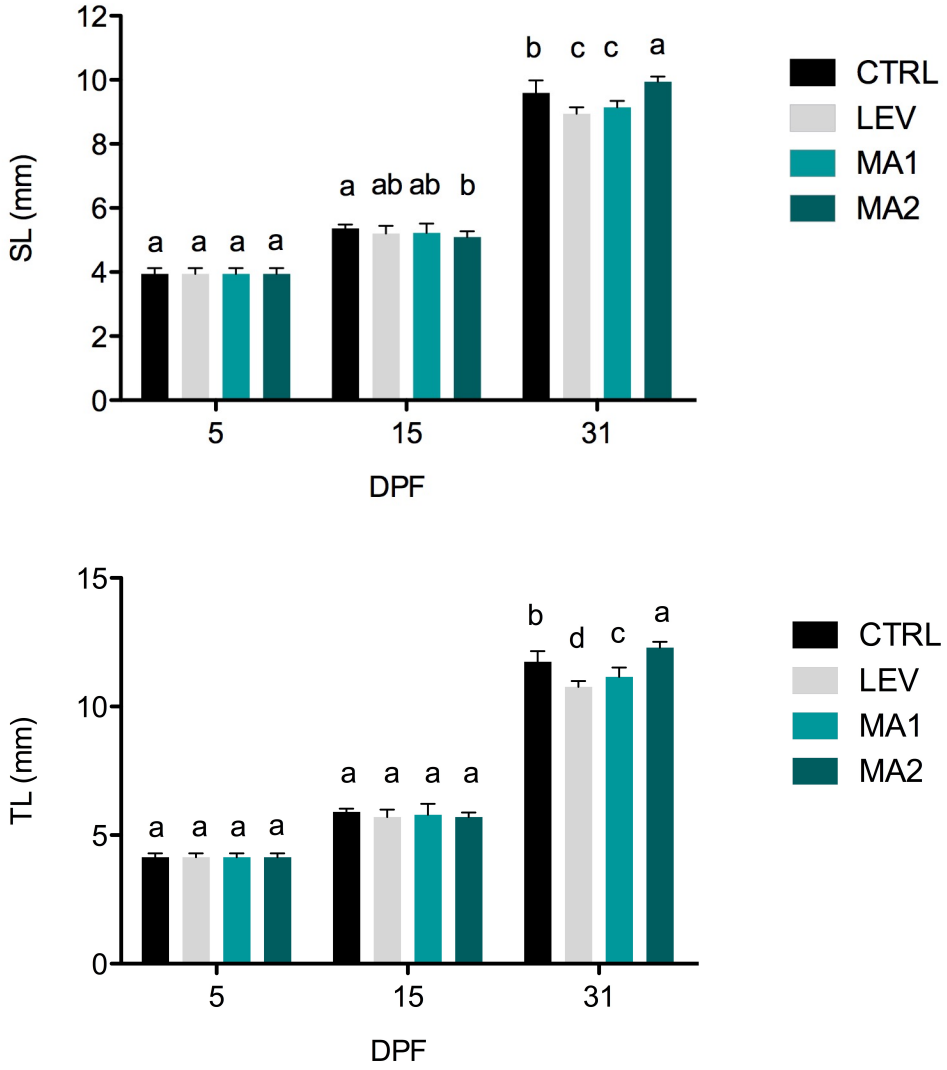

Figure 12 Standart length-SL and Total length-TL (mm) observed in zebrafish larvae fed with different diets: control (CTRL), diet supplemented with 0.5 mg Se Kg^{-1} DW of Se-enriched yeast (LEV) and diets supplemented with Se-fortified *I.galbana* T-ISO, 0.5 mg Se Kg^{-1} DW of (MA1) and 2 mg Se Kg^{-1} DW (MA2). Samples were collected at 5, 15 and 31 days post fertilization (DPF). Bars show means ± SD deviation, (n = 3). Bars labelled with different letters are significantly different (Two-way ANOVA $p < 0.05$).

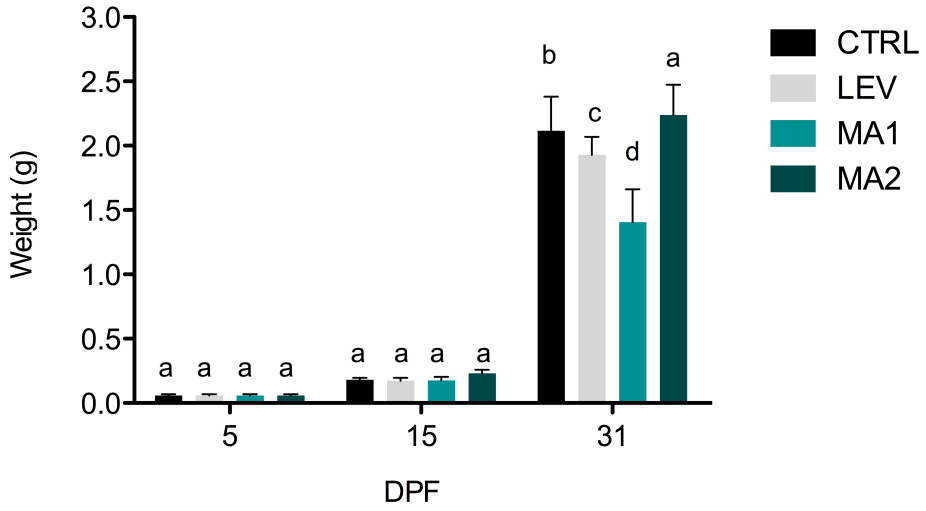

Figure 13 Weight (g) observed in zebrafish larvae fed with different diets: control diet (CTRL), diet supplemented with 0.5 mg Se Kg^{-1} of Se-enriched yeast (LEV) and diets supplemented with Se-fortified *I.galbana* T-ISO, 0.5 mg Se Kg^{-1} of (MA1) and 2 mg Se Kg^{-1} (MA2). Samples were collected at 5, 15 and 30 days post fertilization (DPF). Bars show ± SD deviation (n = 3). Bars labelled with different letters are significantly different (Two-way ANOVA $p < 0.05$).

3.5.4. **LARVAE DEFORMITIES**

Boglione *et al*. (2001) reported high incidence of anomalies in farmed fish, mainly in the head and vertebral axis. In zebrafish, the caudal fin complex has been described as the most affected area (Bensiomon-Brito *et al*., 2010). Deformities can modify the external shape, swimming capacity and feeding behavior of fish with major consequences in the growth rate and welfare status of the organisms (Boglione *et al*., 2013).

Inclusion of Se from algae biomass can be beneficial in fish development during larval stages. Fish fed with Se-enriched microalgae displayed a significant reduction in total skeletal deformities, demonstrated in Table 12. Control and LEV treatments exhibited a similar total number of deformities (103 and 104, respectively), which were significantly higher than MA1 (63) and MA2 (75) values.

Se addition has been reported to improve skeletal development, since the inclusion of antioxidant factors in the diets reduce the high oxidation risk of diets with a high content of EPA and DHA (Lewis-McCrea & Lall, 2007; Saleh *et al*., 2014) reducing the incidence of muscle injury caused by the production of reactive oxygen species (Betancor *et al*., 2012).

Se-addition in diets also resulted, in a significant reduction of fin ray's deformities: MA1 (22.33%), MA2 (24.33%) and LEV (55.33%) when compared with control diets (71.00%). This type of injury can cause severe osteological deformities in larvae, altering their swimming capacity and mobility (Koumoundouros, 2010).

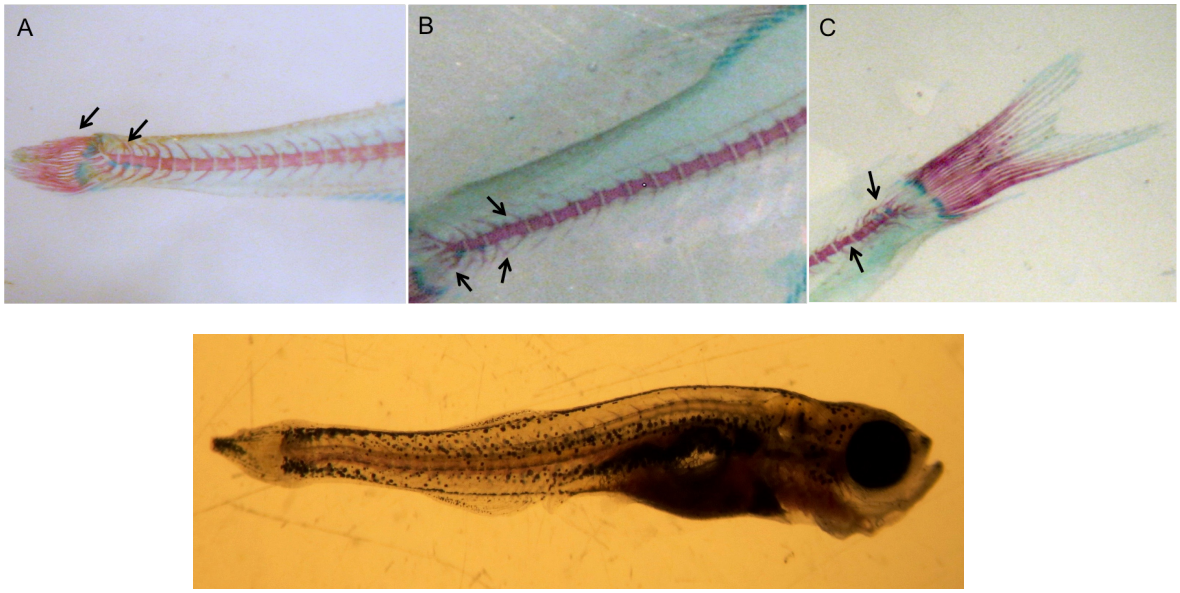

Figure 14 Examples of most common deformities observed in zebrafish larvae under different Se supplemented diets. A) Deformities affecting of caudal fin affecting fin rays and caudal fin vertebrae; B) Fusions affecting caudal vertebra neural and haemal arches and hypurals; C) deformity in neural and haemal arches and compression in haemal vertebra; D) Deformities affecting caudal fin rays.

Table 12 Total number of deformities and average nº of deformities per zebrafish larvae subjected to different dietary treatments: control (CTRL), diet supplemented with 0.5 mg Se Kg^{-1} DW of Se-enriched yeast (LEV) and diets supplemented with Se-fortified *I.galbana* T-ISO, 0.5 mg Se Kg^{-1} DW of (MA1) and 2 mg Se Kg^{-1} (MA2) DW. Values are represented by means ± SD deviation. Different letters are significantly different (One way ANOVA in conjunction with Tukey's test for multiple comparisons, $p < 0.05$)

Treatment	Total nº Deformities	Deformities per fish
CTRL	103 ± 10.11 [a]	2.30 ± 0.23 [a]
LEV	104 ± 8.33 [a]	2.33 ± 0.19 [a]
MA1	63 ± 7.23 [b]	1.41 ± 0.16 [b]
MA2	75 ± 1.00 [b]	1.67 ± 0.02 [b]

In terms of major axial skeleton deformities, Afonso *et al.,* (2000) focused in the importance of Lordosys-Scoliosis-Kyfosis (L-S-K), since their effects are visible on overall body morphology. Percentages of incidence of L-S-K were significantly lower, for all Se enriched diets in comparison with the control treatment (Figure 9). Se-enriched algal biomass diets demonstrated a positive effect in the reduction of deformities reductions: larvae fed with MA1 and MA2 diets exihbeted values of 8.89% and 17.78%, and in larvae fed with LEV the incidence of L-S-K was almost reduced to half the control value (26.67%).

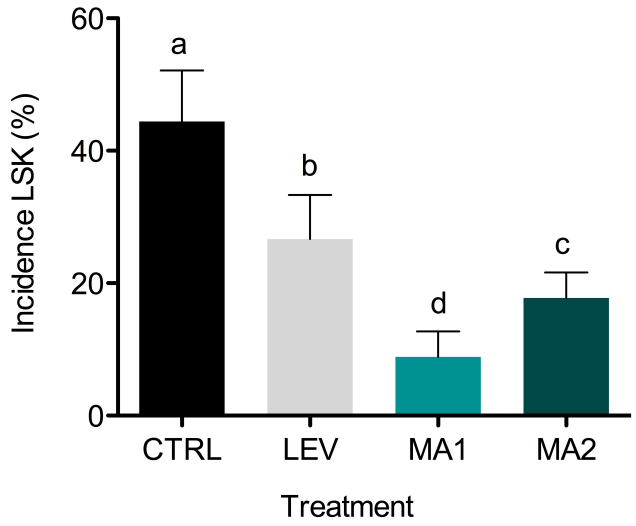

Figure 15 Incidence of Lordosys-Scoliosis-Kyfosis (LSK; %) in the caudal fin area. Zebrafish individuals were subjected to different diets: control diet (CTRL), diet supplemented with 0.5 mg Se Kg^{-1} of Se-enriched yeast (LEV) and diets supplemented with Se-fortified *I.galbana* T-ISO, 0.5 mg Se Kg^{-1} of (MA1) and 2 mg Se Kg^{-1} (MA2). Samples were collected at 31 DPF. Bars show means ± SD deviation(n = 3). Bars labelled with different letters are significantly different (Two-way ANOVA $p < 0.05$).

Deformities in zebrafish were highly reduced in treatments with Se-supplemented microalgal biomass (MA1; MA2; Figure 10). Weberian apparatus (W) and precaudal vertebrae (PC) revelead a similar behavior, with comparable or higer percentage of deformities for CTRL and LEV treatments, respectively, in comparison with MA1 and MA2. Concerning the incidence of caudal vertebrae deformities (C), all diets originated different values of deformities incidence: larvae fed with MA1 showed only 20% of incidence of deformities in caudal vertebrae, while MA2, LEV and CTRL had 28.9%, 58%, and 67.9 % respectively. The incidence of deformities in caudal fin vertebrae (CF) was similar for all treatments, with values around 90%.

A more specific analysis (e.g. parhypural, hypurals 3 to 5, fin rays) showed that treatments MA1 and MA2 caused a significant reduction in the incidence of deformed hypurals from 3- 5, parahypurals and fin rays when compared to control groups (Figure 11).

Globally, these results indicate that the different concentrations and sources of Se strongly affect zebrafish larval growth, survival and incidence of skeletal deformities. Se, as a micronutrient could in fact bring benefits to aquaculture product enhancement, with the reduction of skeletal anormalies and enhanced growth.

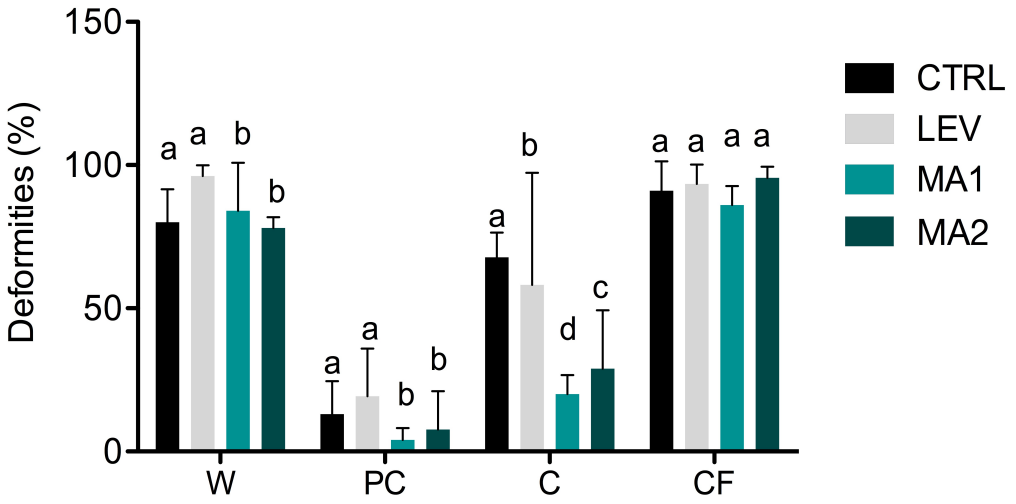

Figure 16 Incidence of deformities (%) by skeletal zones (W- weberian vertebrae, PC- precaudal vertebrae, C- caudal vertebrae and CF –caudal fin vertebrae) in zebrafish subjected to different dietary Se treatments: control diet (CTRL), diet supplemented with 0.5 mg Se Kg^{-1} DW of Se-enriched yeast (LEV) and diets supplemented with Se-fortified *I.galbana* T-ISO, 0.5 mg Se Kg^{-1} DW of (MA1) and 2 mg Se Kg^{-1} DW (MA2). Samples were collected at 31 DPF. Bars show means ± SD deviation (n = 3). Bars labelled with different letters are significantly different (Two-way ANOVA, $p < 0.05$).

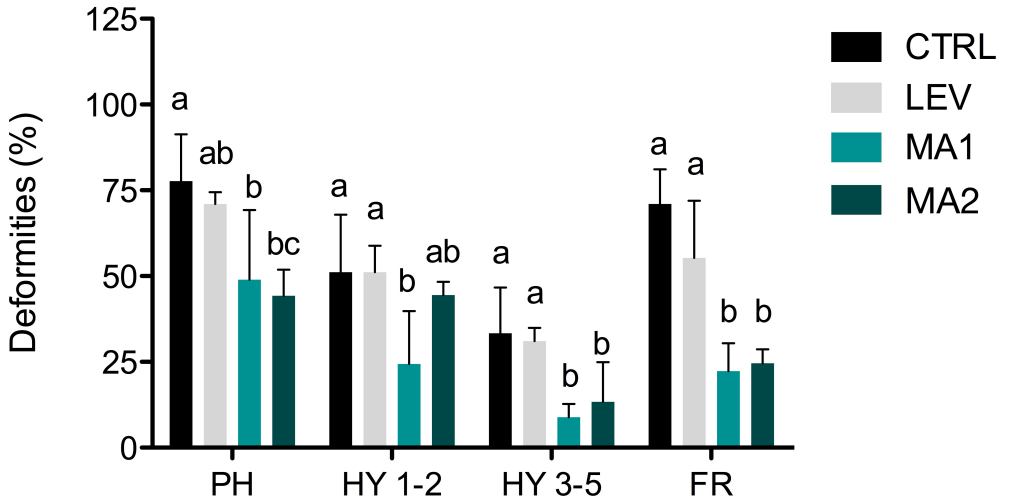

Figure 17 Incidence of deformities (%) by caudal fin structures: [PH]- caudal fin parhypural; [HY 1-2]- caudal fin hypural 1 and 2; [HY 3-5]- caudal fin hypural 3, 4 and 5; [FR]-fin rays. Zebrafish individuals were subjected to different diets: control diet (CTRL), diet supplemented with 0.5 mg Se Kg^{-1} of Se-enriched yeast (LEV) and diets supplemented with Se-fortified *I.galbana* T-ISO, 0.5 mg Se Kg^{-1} of (MA1) and 2 mg Se Kg^{-1} (MA2). Samples were collected at 31 DPF. Bars show means ± SD deviation(n = 3). Bars labelled with different letters are significantly different (Two-way ANOVA $p < 0.05$).

4
CONCLUSIONS

The present study provides a new insight into the impact of selenium on the haptophyte *I. galbana,* especially regarding its toxicity and bioaccumulation. Growth inhibition was found for concentrations of selenate higher than 25 mg Se L^{-1}. The bioaccumulation of Se in the microalga was dose-dependent. *I. galbana* was able to incorporate 150 mg Se Kg^{-1} DW in a medium supplemented with 20 mg Se L^{-1}.

Using live Se-fortified microalgae as an enrichment diet for *Artemia* spp in a short-term experiment of 12 hours with Se-supplemented *I. galbana,* enhanced Se levels in *Artemia* up to 20 mg Se Kg^{-1} DW when compared to control treatment.

Zebrafish larval growth performance was significantly improved by microalgal Se-supplemented diets, especially at the concentration 2 mg Se Kg^{-1} DW. Fish fed with Se-enriched biomass also displayed a significant decrease in skeletal deformities compared with control groups and Se-enriched yeast (LEV) diet. Addition of Se-fortified *I. galbana* biomass to formulated diets, showed a significant drop of Lordosis-Kyphosis-Scoliosis and anomalies in fin rays.

Therefore, *I. galbana* seems to be a promising species for the production of biomass enriched in selenium for different biotechnological purposes. The potential applications of the algal biomass include the maintenance of animal health and productivity, reduction of inorganic selenium supplementation in commercial animal feeds and potential use as food supplement with benefits to human health.

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