Universidade de Aveiro Departamento de Biologia
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Vitória Sofia Almeida Santos Pereira

Efeitos protetores de suplementação alimentar com macroalgas marinhas na integridade genética de dourada (*Sparus aurata*)

Protective effects of seaweed feed supplementation towards genetic integrity in gilthead seabream (*Sparus aurata*)

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica do Doutor Mário Guilherme Garcês Pacheco, Professor Auxiliar c/ Agregação do Departamento de Biologia da Universidade de Aveiro, e da Doutora Sofia Isabel Antunes Gomes Guilherme, Investigadora em Pós-doutoramento do Departamento de Biologia da Universidade de Aveiro.

o júri

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palavras-chave Macroalgas marinhas, peixes, dano genético, potencial anti-genotóxico

resumo

A integridade e estabilidade do ADN são essenciais para a saúde e condição fisiológica geral dos organismos e, em última instância, para a sua sobrevivência. Este tema tem sido negligenciado no que diz respeito a peixes em sistemas de aquacultura, descurando os potenciais impactos de fatores endógenos e exógenos. A manipulação das condições de cultivo para alcançar um crescimento rápido, assim como a ocorrência (intencional ou acidental) de agentes/condições tais como desinfetantes, anestésicos, antibióticos e contaminação aquática podem criar estados de stresse passíveis de afetar a integridade do ADN. Além disso, a longo prazo, esta situação pode comprometer o desempenho do crescimento, o bem-estar animal e reduzir as receitas.

As macroalgas marinhas são uma fonte potencial de compostos naturais de elevado valor, com um amplo espectro de atividades biológicas e benefícios para a saúde. Tem sido sugerido que uma dieta enriquecida com algas melhora o crescimento, metabolismo dos lípidos, atividade fisiológica e a resistência a doenças de várias espécies de peixes. No entanto, o seu potencial anti-genotóxico permanece por explorar.

Assim, este estudo teve como objetivo central avaliar as propriedades anti-genotóxicas de uma ração enriquecida com macroalgas, em dourada (*Sparus aurata*), após um desafio genotóxico (injeção intraperitoneal de 40 $mg.kg^{-1}$ de ciclofosfamida - CP). A ração enriquecida foi suplementada com um total de 5% de algas, incorporando três espécies em partes iguais: *Ulva* spp. (Chlorophyta), *Fucus* spp. (Phaeophyta) e *Gracilaria* spp. (Rhodophyta).

Deste modo, dois grupos de peixes foram alimentados de forma diferenciada nos primeiros 30 dias, até ao momento da injeção, onde foram separados consoante a dieta/estímulo. Posteriormente, de modo a esclarecer o efeito protetor da ração enriquecida, 3 dias após a injeção, metade de cada grupo previamente alimentado com esta dieta viu a sua alimentação alterada para a ração padrão, mantendo-se todos os outros grupos iguais, durante mais 7 dias.

Foi avaliado o dano genético através dos ensaios do cometa e de anomalias nucleares eritrocíticas (ANE), em paralelo com o estudo do envolvimento do sistema antioxidante, como indicação de um estado pró-oxidante, determinando as atividades da superóxido dismutase (SOD), catalase (CAT), glutationa-S-transferase (GST), glutationa peroxidase (GPx) e glutationa redutase (GR), bem como o teor total de glutationa (GSHt). A elucidação do dano oxidativo de ADN foi feita através do ensaio do cometa melhorado pela incubação com enzimas específicas de reparação de ADN, FPG e Endo III, que convertem purinas e pirimidinas oxidadas em ruturas extra de cadeia única, respetivamente.

Os resultados demonstraram que a alimentação enriquecida com algas apresenta propriedades anti-genotóxicas em células sanguíneas de dourada, evidentes em relação às quebras de cadeias de ADN e a lesões cromossómicas, embora pareça mais pronunciada no último tipo de expressão genotóxica. Esse efeito foi particularmente notório no último momento de amostragem, uma vez que foi conseguida uma completa recuperação do dano cromossómico em peixes previamente injetados com CP. Foi ainda evidenciada uma clara atividade de proteção contra o dano oxidativo do ADN, particularmente na presença de um forte insulto genotóxico ocorrido três dias após a injeção de CP, o que foi expresso no impedimento da oxidação de bases purínica pela suplementação de algas. No entanto, os antioxidantes não foram alterados pela dieta suplementada, com exceção da atividade da GST que foi induzida como resposta ao tratamento de CP. Considerando a persistência de efeitos favoráveis, 7 dias após a remoção de algas da dieta foram suficientes para reduzir parcialmente a eficácia da proteção, principalmente no que diz respeito à capacidade de contrariar o dano oxidativo de ADN.

Globalmente, estes resultados são promissores quanto à identificação dos benefícios da inclusão de algas na dieta de peixe, oferecendo uma estratégia potencial para fortalecer a condição dos peixes e, assim, revigorar a atividade aquícola, fornecendo também novos conhecimentos sobre os mecanismos de proteção do ADN em peixes.

keywords Seaweeds, fish, genetic damage, anti-genotoxic potential

abstract

The DNA integrity and stability are essential to organisms' health, fitness and, ultimately, to survival. This matter has been neglected in what concerns to fish in aquaculture systems, disregarding the potential impacts of both endogenous and exogenous factors. The manipulation of rearing conditions to achieve a fast-growing performance, as well as the occurrence (intentional or accidental) of agents/conditions, such as disinfectants, anesthetics, antibiotics and waterborne contaminants, may create stressful conditions passible to affect DNA integrity. Furthermore, in the long-term, this situation may compromise growth performance, animal welfare and a reduction in revenues.

Seaweeds are a potential source of natural and high value compounds, with a large spectrum of biological activities and health benefits. It has been suggested that algae-enriched diet improves the growth, lipid metabolism, physiological activity, and disease resistance of various fish species; however, its anti-genotoxic potential was scarcely evaluated.

Hence, this study aimed to evaluate the anti-genotoxic properties of a macroalgae-enriched diet to provide protection in gilthead seabream (*Sparus aurata*) against a genotoxic challenge (i.p. injection of 40 $mg.kg^{-1}$ cyclophosphamide - CP), as well as to clarify if the potentially favorable effects of algae persist beyond the end of supplementation. The enriched diet was supplemented with 5% of equal parts of three species: *Ulva* spp. (Chlorophyta), *Fucus* spp. (Phaeophyta) and *Gracilaria* spp. (Rhodophyta).

Thus, two groups of fish were differently fed during the first 30 days, until the CP injection, where they were separated depending on the diet/stimulus. Subsequently, in order to clarify the protective effect of the supplemented diet, 3 days after injection, half of each group previously fed with this diet has changed to the standard feed, keeping all the other groups the same way, for another 7 days.

Genetic damage was evaluated through the erythrocytic nuclear abnormalities (ENA) and comet assays and the involvement of the antioxidant system as indication of a pro-oxidant status was assessed by superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR) activities, as well as by total glutathione (GSHt) content. The elucidation of the oxidative DNA damage-protecting activity was made by adopting as diagnostic tool the comet assay improved with DNA lesion-specific enzymes, FPG and Endo III, which convert oxidized purines and pyrimidines into extra DNA single strand breaks, respectively.

The results pointed that algae-enriched feed exhibits anti-genotoxic properties in gilthead seabream blood cells, evident in relation to DNA strand breaks and to chromosomal lesions, though it appeared more pronounced in the latter type of genotoxicity expression. This effect was depicted by fish sampled at the last sampling moment, since a significant recovery of chromosomal damage was evident in fish previously injected with CP. A clear oxidative DNA damage–protecting activity was displayed, particularly in the presence of a strong genotoxic insult occurring three days after CP injection, when purine oxidation was prevented by algae supplementation. Nonetheless, blood antioxidants were not altered by the supplemented diet, with the exception of GST activity that was induced as response to CP treatment. Considering the persistence of favorable effects, 7 days without algae uptake was enough to partially reduce the protection efficacy, mainly in what concerns to the oxidative DNA damage-protecting capacity.

Overall, these results seem to be promising towards the benefits of seaweed inclusion in fish diet, offering a potential strategy to strengthen fish fitness, and thus, to invigorate aquaculture activity, also providing new insights on the mechanisms of DNA protection in fish.

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1. Introduction

1.1. Marine macroalgae as a source of functional ingredients

Marine algae, also called seaweeds, are taxonomically classified in red (Rhodophyta), brown/yellow (Phaeophyta), green (Chlorophyta) and blue-green algae (Cyanophyta). Macroalgae (macroscopic and multicellular species), which include the above three groups of seaweeds other than blue-green algae, have a long history of utilization as direct or processed food across the globe (Holdt and Kraan, 2011).

Macroalgal worldwide production increases 5.7% every year and more than 16.3 million tonnes were produced in 2011 from global capture and aquaculture (FAO, 2014), reflecting an increasing importance in human and animal nutrition (Patarra et al., 2011). Macroalgae are rich in soluble dietary fibers, proteins, minerals, vitamins, antioxidants, and polyunsaturated fatty acids, in parallel with a low caloric value. Though the available amounts of the previous algae components may vary depending on the species, season and area of production (Murata and Nakazoe, 2001), a large number of bioactives has been identified with potential applications in various areas, including pharmaceutical, cosmeceutical, nutraceutical and functional food industries (Mendis and Kim, 2011; Pangestuti and Kim, 2011).

In recent years, the number of studies on marine macroalgae addressing their chemical composition and physiological properties has grown exponentially, and these organisms have become a focus of commercial interest as potential ingredients of functional or health-promoting foods in humans (Patarra et al., 2011). Besides their nutritional value, seaweeds are rarely implicated in allergy risks compared to other marine food products (Fleurence et al., 2012). Nevertheless, seaweeds may also contain a high proportion (30-71% of dry weight) of polysaccharides such as xylans, agar, carrageenan or alginates, which are anti-nutritional factors, limiting the digestibility of protein fractions (Fleurence et al., 2012); however, an initial enzymatic treatment of algae to remove polysaccharides was suggested as a possible way to increase protein accessibility or digestibility from red seaweeds such as *Palmaria palmata* (Fleurence, 2004). In the same way, safety hazards associated with seaweed consumption also include excess content of metals, radioactive isotopes, dioxins and pesticides, which could limit their use in human and animal food products, though can be controlled once vary with

species, collection time, growth phase and collection site (Garcia-Vaquero and Hayes, 2016; van der Spiegel et al., 2013).

Taking into account human nutrition, generally, seaweeds are used as gelling agents and stabilizers in the food and pharmaceutical industries, and recent research has proved their medicinal value against various diseases (Mendis and Kim, 2011). For instance, they are natural sources of hydrosoluble and liposoluble vitamins, such as thiamine (B1) and riboflavin (B2), b-carotene and tocopherols (E), as well as of long-chain polyunsaturated essential fatty acids (PUFAs) from the omega-3 family (Khotimchenko et al., 2002), which may reduce the risk of heart disease, thrombosis and atherosclerosis, improving antiviral protection (Kamat et al., 1992; Plaza et al., 2008; Mendis and Kim, 2011). In the same way, they are also important sources of minerals, such as Ca, P, Na, K and I, as well as other vitamins like A, B12, C and D. Curiously, their high calcium concentration coincides with an easy assimilation (in the form of calcium carbonate) into the body, compared, for instance, to calcium in cow's milk (in the form of calcium phosphate) (Mendis and Kim, 2011).

It has also been reported that some kainoid amino acids (e.g. kainic and domoic acids found in numerous algal species, such as *Digenea simplex* and *Chondria armata*), act as central nervous system stimulants, being currently used in research associated with neurophysiological disorders such as Alzheimer's and Parkinson's disease and epilepsy; however, upon exceeding the safe levels they become neurotoxic (Smit, 2004; Harnedy and FitzGerald, 2011).

The increment of seaweeds in human nutrition is not easy in western countries, particularly in Europe, since it is not part of daily eating habits, as it happens in Asia since ancient times; however, their increasing input can be envisaged.

Recently, macroalgae started being used also in livestock/animal production, namely as feedstuff for horses and poultry in several regions of the world, by the addition of small quantities to the feed and the subsequent assessment of the animal to check for possible prebiotic activity and enhanced animal performance (Garcia-Vaquero and Hayes, 2016).

1.2. Marine macroalgae in fish nutrition

According to FAO (2012), aquaculture has been growing at an average annual rate of 5.6 %, during the last 10 years, producing presently about 47% of the fish consumed worldwide. It is expected to reach 71.0 million tonnes by 2020 (FAO, 2012). Within this framework, feeding has become a critical factor on the management of aquaculture production units, which is strongly related to the fact that fish species selected for their high commercial value are mostly carnivorous, thus requiring a high protein diet content, generally formulated by fish waste or fish solely used for its production (FAO, 1994; Batista, 2008). Such facts have triggered intense research towards the evaluation of alternative sources of protein, with much attention directed to the nutritional aspects and costs. Those alternative sources include animal products, from both invertebrates (zooplankton) and vertebrates (e.g. blood, liver, meat or bone), as well as unicellular organisms (fungi and bacteria), seeds (e.g. soybean, sunflower or cotton), legumes (e.g. soy, beans or peas) and other vegetable products (e.g. corn gluten, wheat or protein concentrates). However, most of these alternatives has a number of drawbacks (Batista, 2008; Garcia-Vaquero and Hayes, 2016). For example, according to Tacon (1994), the availability of protein from unicellular organisms is limited, expensive and of variable quality, while the use of terrestrial animal by-products is also inadvisable because of possible microbial contamination.

Thus, as algae are at the base of the aquatic food chains, representing a food resource that wild fish are adapted to consume (Norambuena et al., 2015), they are receiving increasing attention as a novel feed ingredient in pisciculture (Batista, 2008).

At present, seaweeds with elevated protein content and production rates are under particular scrutiny in view of their potential nutritional benefits (Rupérez and Saura-Calixto, 2001; Valente et al., 2006). Mustafa et al. (1995) found that the inclusion of three different seaweeds (*Ascophyllum nodosum*, *Porphyra yezeoensis* and *Ulva pertusa*) at a level of 5% increased body weight, feed utilization and muscle protein deposition in red seabream (*Pagrus major*) fingerlings. In addition, red algae, such as *Porphyra tenera*, showed to have protein levels higher than those found in high protein legumes (e.g. soybean) (Rupérez and Saura-Calixto, 2001). Still, nitrogen-enriched conditions like the effluents of fish farms, where seaweeds are used as biofilters, can increase their protein content (Valente et al., 2006).

Apart of their potential nutritional value as protein substitutes, algae may also give an important contribute in fish diets as lipid sources (Nakagawa et al., 1987) and all benefits already described above. Several studies have proven that small amounts (2.5 – 10%) of algae in fish diets resulted in positive effects, including increase in growth performance, feed utilization efficiency, physiological condition, disease resistance, improved stress response, carcass quality (Emre et al., 2013; Norambuena et al., 2015; Wassef ate al., 2005), intestinal micro biota and enhanced protein retention during the winter period of reduced feed intake (Norambuena et al., 2015). For example, an inclusion of 3 - 6% of *Macrocystis pyrifera* significantly increased the levels of PUFAs in the muscle of rainbow trout (*Oncorhynchus mykiss*) (Dantagnan et al., 2009) and 5% of *Ulva lactuca* and *Pterocladia capillacea* provided a better growth, feed utilization, nutrient retention, and survival rates of *Nile tilapia* (Wassef et al., 2013).

On the other hand, it has also been noted that the inclusion of macroalgae in fish feeds at high levels might have negative effects (Garcia-Vaquero and Hayes, 2016). Previous studies using *Ulva rigida* meal showed a reduced growth and feed utilization in common carp (*Cyprinus carpio*) (Diler et al., 2007) and Nile tilapia (*Oreochromis niloticus*) fed with 20% of this algae (Azaza et al., 2008). In the same way, *Porphyra purpurea* when included at levels of 16.5% and 33% in the diets of the mullet *Chelon labrosus* was found to decrease growth performance and feed utilization efficiency with increasing levels of seaweed (Davies et al., 1997). These results could be due to substances with anti-nutritional activity, like lectins, tannins, phytic acid, as well as protease and amylase inhibitors (Norambuena et al., 2015).

Organisms are daily exposed to oxidants generated both endogenously and exogenously (Saad et al., 2015) and farming protocols often induce stressful and pro-oxidant conditions (affecting, for instance, the immune system), dietary supplementation with macroalgae may be used to prevent economic losses by improving fish condition and survival (Peixoto et al., 2015) in association with strengthened antioxidant systems (Andrade et al., 2013; Queiroz et al., 2014; Surget et al., 2016).

As some authors concluded that the response to algae inclusion into feed is dose-dependent and species-specific (Norambuena et al., 2015; Valente et al., 2006), further studies would benefit from using specific seaweeds to enlarge our understanding of the effects on different farming fish species (Peixoto et al., 2015), filling an existing knowledge gap.

1.2.1. The case of gilthead seabream (*Sparus aurata*) cultivation and nutrition

1.2.1.1. Species biology and aquaculture production

Gilthead seabream, *Sparus aurata*, is a teleost belonging to the Class Actinopterygii, Order Perciformes and Sparidae Family. It can be found in the Atlantic Ocean, from the British Isles, Gibraltar Strait to Cape Verde, around the Canary Islands, and in all Mediterranean Sea. It is an eminently coastal species, living on rocky or sandy bottoms. Gilthead seabream is a sedentary fish that migrates alone or in small aggregations, moving in early spring towards protected coastal waters, in order to find abundant food and mild temperatures. It is a eurythermal and euryhaline species, i.e. can tolerate significant variations in temperature and salinity, respectively. The feed is based on shellfish (bivalves and gastropods) and crustaceans, although can also feed on small fish, polychaetes and algae (Batista, 2008; Madeira et al., 2016).

Traditionally, gilthead seabream was cultured extensively in coastal lagoons and saltwater ponds, until intensive rearing systems were developed during the 1980s (FAO, 2004). These methods are very different, especially regarding fish farming density and food supply. In extensive systems it is generally reared with mullets, seabass and eels and feed naturally. On the other hand, in semi-intensive systems, the rearing zone is fertilized to increase natural food availability, with a supplement of commercial feed and, for example, some polycultures are created. In fact, seabream (*Sparus aurata*), as accessorily herbivorous with a natural tendency to feed on macroalgae (Bauchot and Hureau, 1990), has been widely used in Portugal in polyculture system with seabass (*Dicentrarchus labrax*) to prevent the excessive growth of macroalgae, a frequent problem in seabass monoculture pounds (Afonso, 2016). Finally, in intensive systems, gilthead seabream is fattened with commercial pellets (European Commission, 2012). It is a very suitable species for aquaculture in the Mediterranean region, due to their good market price, high survival rate and feeding habits (which are relatively low in the food chain), as well as due to the fact that it is possible to control their whole life in captivity (FAO, 2004).

It has been highly captured and cultured in Europe, being its production growing globally (FAO, 2015), as seen in figures 1 and 2.

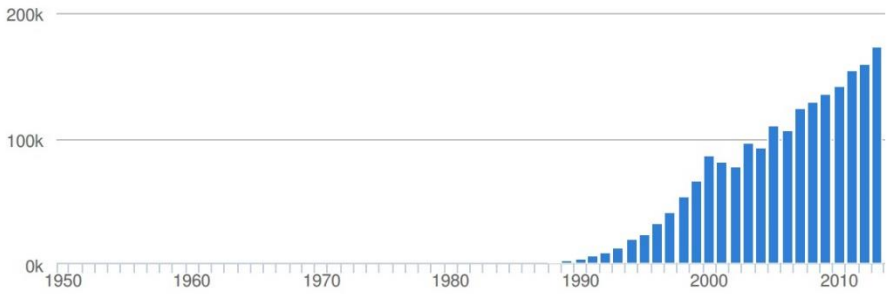

Figure 1: Global aquaculture production of *Sparus aurata* (tonnes) (Adapted from: FAO FishStat cited in FAO, 2015).

This species demonstrated very quickly a high adaptability to intensive rearing conditions, both in ponds and cages, and its annual aquaculture global production increased regularly until 2000, when it reached a peak of over 87 000 tones (Figs. 1 and 2), though keeping growing regularly over time (FAO, 2004). Most production occurs in the Mediterranean region, with Greece being by far the largest producer. Turkey, Spain and Italy are also major Mediterranean producers. In addition, considerable production occurs in Croatia, Cyprus, Egypt, France, Malta, Morocco, Tunisia and Portugal. There is also gilthead seabream production in the Red Sea, Persian Gulf, and Arabian Sea (FAO, 2004; FAE, 2012).

Comparing with the capture fisheries, aquaculture production had an exponential increase over the years, while the former production has remained more or less stable (Barazi-Yeroulanos, 2010), as illustrated in figure 2.

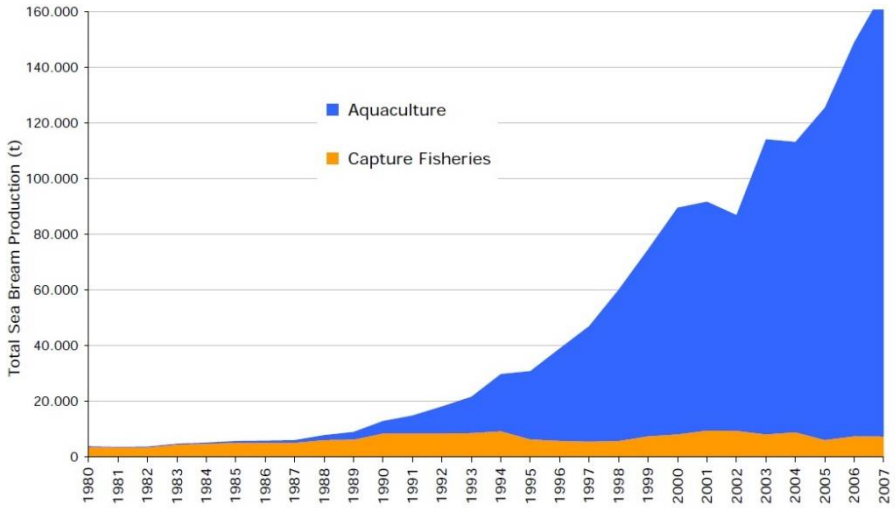

Figure 2: Mediterranean aquaculture and capture fisheries production of gilthead seabream (Adapted from: FAO, 2008; elaborated by APROMAR).

Although Portugal is the third world's largest fish consumer, the weight of national aquaculture in the supply of fish to the Portuguese market is still very low. As regards to fish species produced in 2010, turbot (30 %) topped the list, followed by trout (12 %), gilthead seabream (11 %) and European seabass (5 %) (APA, 2014). According to the Portuguese Association of Aquaculture Producers (APA), the national aquaculture is an activity with a promising future and undeniable potential. In this direction, the significant research capacity and know-how on gilthead seabream cultivation points out this species as a candidate to substantially increase its preponderance within the national aquaculture scenario. However, at the moment, market conditions seem very far from those that pertained in the first half of the 1990s. New marketing strategies for rearing gilthead seabream profitably can include, for instance, small production systems able to increase the value of the product by producing low quantities of higher quality fish (e.g. organic fish) (Colloca and Cerasi, 2005). Hence, a dietary manipulation in gilthead seabream culture, incorporating macroalgae in aquafeeds, is a strategy that deserves attention from researchers and farmers as a mean to improve fish welfare and nutritional value, promoting profit and product differentiation.

1.2.1.2. Nutrition strategies and constraints – macroalgae as alternative feed ingredients

Rotifers are a first food to seabream larvae and are continually offered throughout the 32-35 days larval rearing period. Artemia nauplii in III stage, which are fed to larvae from approximately 18 days old to the end of larval rearing, together with rotifers, are previously enriched at the II stage. Rotifers and artemia enrichment is based on commercial lipid preparations, to enhance their levels of certain essential fatty acids (EPA; DHA) and vitamins that are critically important for good growth, development and survival (Webster and Lim, 2002). In Mediterranean hatcheries baker's yeast and/or microalgae (e.g. *Chlorella* sp., *Isochrysis galbana*, *Pavlova lutheri*, *Dunaliella tertiolecta*) are used to improve the rotifers and artemia survival, as well as water quality in the larval tanks, creating the so-called 'green water' that is used during the initial rearing phases (Caggiano, 2000).

Juveniles at about 45 days old start the weaning with a dry high-protein (50 - 60 %) formulated diet. Feeds are distributed by automatic feeders at 2-hour intervals for small fish (1-3 g) or by hand for larger fish. Grading is necessary at least two or three times per cycle, in order to avoid growth differentiation (Caggiano, 2000; Moretti et al., 1999). Then, the diet used to feed gilthead seabream in fattening may

vary, but generally, contains fish meal (about 70%), wheat flour, fish oil, vitamin and mineral premix (Wassef et al., 2005).

In that way, the long-term sustainability of this feeding strategy may be threatened by its present over-dependence on fish meal and fish oil (FAO, 2002). The preferential use of fish meal is due to its ideal nutritional source to meet the protein and lipid requirements for carnivorous fish, although it is a relatively expensive and limited supply ingredient (Tacon, 1997). In general, fish meal is obtained from resources not used for direct human consumption, namely fish of low commercial value or fish remains from the canning and filleting industry. However, it should be taken into account that variations in the quality of fish meal may affect feed intake and its digestibility and, consequently, animals' performance (Pereira, 2003). For example, a dietary deficiency in pyridoxine resulted in growth retardation, high mortality, hyper-irritability coupled with erratic swimming behavior, and degenerative changes in peripheral nerves (Kissil et al., 1981). In addition, a lack of vitamin C led to extensive tubular damage and inflammatory response of the haemopoietic tissue leading to granuloma (Alexis et al., 1997). Such nutritional deficiency signs are, however, not commonly observed under current farming conditions, since most feeds are adequately supplied (FAO, 2012).

Overall, despite the general growth prospects, in recent years the sector has been with difficulties of saturation in the market, which led to a very sharp decline in prices (Batista, 2008). Investment in research and technological development is undoubtedly a necessary condition for overcoming the problems facing the sector, as has been done with other species of high commercial value and traditional consumption, such as salmon (Guerra et al., 2003).

Growth, health and reproduction of fish are primarily dependent upon an adequate supply of nutrients, irrespective of the culture system in which they are grown (Hassan, 2001). Thus, it is necessary to formulate diets with high protein content, capable of providing high growth rates, but cheaper and with less waste (Pereira, 2003).

Seaweeds are not a main source of energy although they are reported to have nutritional value for fish regarding vitamin, protein and mineral contents (Ortiz et al., 2006). In this way and as discussed above, macroalgae can offer a promising alternative source of feed supplements for seabream rearing, with lower costs involved. However, this possibility has been scarcely explored so far, which is somewhat surprising taking into account the natural propensity of this species to ingest seaweeds. The few studies carried out indicate that feeding *S. aurata* with 10% of *Pterocladia* meal or 5% of *Ulva* meal

produced the best growth performance, feed utilization, nutrient retention and survival (Wassef et al., 2005). In addition, Queiroz et al. (2014) tested recently the use of *Gracilaria* spp., *Ulva* spp., and *Fucus* spp. in two different levels of supplementation (2.5% and 7.5%) or as a mix supplemented diet at 7.5% (2.5% of each algae), finding out that, though growth performance showed no difference, immune and antioxidant responses of *S. aurata* were substantially improved.

1.3. DNA integrity as a key for wellness and survival

The DNA integrity and stability are essential to organisms' health, fitness and, ultimately, to survival (Clancy, 2008). However, DNA is frequently a target for endogenous and exogenous sources of genotoxic stress. In fact, DNA in aerobic organisms is constantly damaged due to its susceptibility to reactive oxygen species (ROS) that may be endogenously formed as part of physiological processes (Oliveira et al., 2010). In what concerns to the exogenous agents, a plethora of agents present in the environment has the potential to alter the structural integrity of the DNA molecule (Geacintov and Broyde, 2010).

In addition to genetic insults caused by the environment, the process of DNA replication during cell division is prone to error. The rate at which DNA polymerase adds incorrect nucleotides during DNA replication is a major factor in determining the spontaneous mutation rate in an organism (Clancy, 2008). It has been estimated that an individual cell can suffer up to one million DNA changes per day (Clancy, 2008). Nevertheless, cells have various repair mechanisms to restore the molecule to its initial structure, no matter whether this damage is caused by the environment or by errors in replication (Clancy, 2008; Costa and Teixeira, 2012).

1.3.1. Genetic damaging events

Considering the importance of an uncorrupted DNA, the future of the cell might be compromised when exposed to a genotoxic agent (Shugart and Theodorakis, 1996). Therefore, when a compound is able to interfere with the DNA molecule, the resulting damage can include base modification, DNA adducts, DNA single- and double-strand breaks and chromosomal aberrations. DNA bases can be modified by several mechanisms, including alkylation, through which the genotoxicants can covalently

bind and alter the structure of the DNA nucleotides (Tretyakova et al., 2012), resulting in abnormal nucleotide sequences, miscoding of messenger RNA, blockade of DNA replication, and breakage of DNA strands (Lancellotti et al., 2016). Moreover, ROS can attack both DNA bases and the deoxyribose backbone of DNA, reacting five times faster with nucleobases, and resulting in base modifications or single- and double-strand breaks (Srut et al., 2015; Weaver, 2008); although DNA breaks can also result from errors at the DNA replication and repair processes (Negritto, 2010). Single strand-breaks are not particularly serious to the DNA molecule, since they can be easily repaired. In opposition, double strand-breaks are probably the most deleterious type of DNA damage that, if not repaired, can lead to cell death (Weaver, 2008). Genotoxic agents can thus induce alterations at the chromosomes number and structure, resulting in structural chromosomal aberrations (clastogenicity) along with numerical chromosomal aberrations (aneugenicity - faulty chromosomal segregation during anaphase), that are the main causes of nuclear abnormalities presented by some cells (Fenech, 2000; Stoiber et al., 2004).

A single alteration in the DNA molecule of an organism could originate serious biological consequences, disrupting normal cell processes and leading to the cell death. The impact of genotoxic chemicals on the DNA integrity is considered one of the first events that occurs in organisms exposed to a genotoxic pressure, highlighting thus the importance of its early evaluation (Frenzilli et al., 2009; Guilherme, 2012). Fish are excellent subjects for the study of the mutagenic and/or carcinogenic potential of waterborne genotoxicants since they can metabolize, concentrate and store them (Obiakor et al., 2010).

The consequences of DNA damage are diverse and usually adverse. Acute effects include changes in DNA metabolism that may trigger cell cycle arrest or cell death, whereas long-term effects include irreversible mutations that can contribute to cancer development (Srut et al., 2015).

As mentioned above, the exposure to a genotoxicant itself might not be enough to provoke severe genetic damage. In fact, the cell has a set of repair systems that allow the balance between DNA lesions and DNA integrity (Friedberg, 2003). DNA repair can be divided into two main mechanisms, i.e. direct reversal of DNA damage or removal of the damaged section (followed by re-synthesis of the excised region) (Weaver, 2008). Thus, nucleotide excision repair (NER) generally deals with severe DNA lesions, recognizing the strand with damage, cutting on either side of it, removing an oligonucleotide (24-32 nucleotides). The removal is followed by DNA polymerase activity, which fills the gap and the nick is sealed by a DNA ligase (Weaver, 2008). On the other hand, base excision repair (BER) appears as the

most prevalent and removes common and subtle changes to DNA bases, being in general similar to the NER. Thus, the damaged base is recognized and removed by an enzyme called DNA glycosylase, leading to a break in the glycosidic bond between the sugar and the damaged base which will result in an apurinic/apyrimidinic (AP) site. Afterwards, an AP endonuclease completes the break which will be filled with a new base by a DNA polymerase. Finally, the nick is sealed by a DNA ligase, repairing the DNA molecule (Weaver 2008).

1.3.2. Approaches for genetic damage evaluation in fish

The adoption of genotoxic endpoints aims to assess an eventual relation between the exposure to a genotoxicant and the resulting effects at the individual. These methodologies are currently understood as suitable tools for the evaluation of acute and chronic exposure to low concentrations of genotoxicants in a wide range of species (Ohe et al., 2004; Scalon et al., 2010), and to detect changes on the health of wild and cultured fish (Madeira et al., 2016).

1.3.2.1. Comet assay

The alkaline Single Cell Gel Electrophoresis (SCGE) protocol, or Comet assay, detects DNA damage in cells of various organs and tissues of organisms exposed to genotoxicants (Garcia-Käufer et al., 2015). It was developed and reported by Singh et al. (1988) and successfully adapted to fish erythrocytes (e.g. Guilherme et al., 2014; Sekar et al., 2011; Çok et al., 2011).

This assay is a standard method used to measure DNA strand breaks, as well as other type of DNA lesions (pyrimidine dimers, oxidized bases, alkylation damage) (Collins, 2004). It is capable of showing each cell as a comet shaped nucleoid, with the head and tail revealing the intensity of the DNA damage, i.e. if a cell presents a concentric nucleoid, with no tail, it has no DNA damage and if it presents a long tail, this is a cell with severe DNA damage (Collins, 2004), as shown in figure 3.

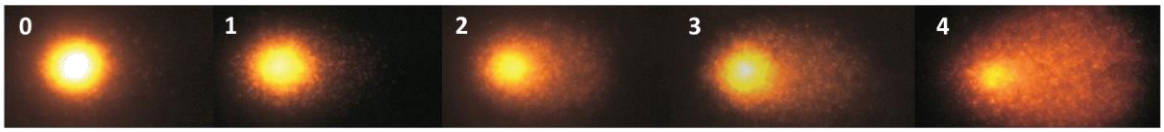

Figure 3: Images of comets stained with ethidium bromide, elucidating the visual scoring classification from 0 (no tail) to 4 (almost all DNA in tail) (Adapted from: Kleinsasser et al., 2004).

According to some authors, the use of this assay has some limitations, since the type of DNA damage detected can be quickly repaired or a result of breaks at alkali labile sites (Lee and Steinert, 2003; Speit and Schütz, 2008). Thus, in order to improve its sensitivity and to shed light on the eventual oxidative cause in the observed damage, is frequently used with an extra step where nucleoids are digested with enzymes that recognize, in particular, the oxidative DNA damage, creating additional breaks. Among the lesion specific enzymes, endonuclease III (EndoIII) and formamidopyrimidine DNA glycosylase (FPG) are often used. EndoIII detects oxidized pyrimidines while FPG allows the detection of the major purine oxidation product 8-oxoguanine (Azqueta et al., 2009; Collins, 2004).

Bearing in mind that the comet assay protocol requires very small cell samples, the technique showed to be suitable for abroad variety of fish sizes, from very small fish up to bigger species. In what concerns to the type of agent tested, the application of comet assay in the field of aquatic genotoxicology has accompanied the evolution of other subareas of environmental toxicology (Lapuente et al., 2015). Therefore, its applicability to both eukaryotic and prokaryotic organism and its use in almost any cell type makes this assay very verifiable, reliable, and relatively rapid in data collection and with realistic correlation. In addition, one of the virtues of this assay is unquestionably its cost-effectiveness, compared to many other techniques (Lapuente et al., 2015).

1.3.2.2. Erythrocytic nuclear abnormalities (ENA) assay

The ENA assay is a standard method applied to organisms with nucleated erythrocytes (e.g. fish) to assess cytogenetic damage, due to the genotoxicant ability to induce chromosomal damage (Guilherme, 2012). Nuclear abnormalities were first described by Carrasco et al. (1990) and later, several authors have linked them to different aetiological factors, such as viral erythrocyte necrosis,

nutritional deficiencies (folic acid deficiency), diets containing oxidized oil, and pollution (Strunjak-Perovic et al., 2009). Nuclear abnormalities in erythrocytes have been used in biomonitoring programs to detect genotoxic effects and chromosomal damage in fish (Azevedo et al., 2012; Teles et al., 2005).

The mechanisms of erythrocyte nuclei deformity formation have not yet been completely explained and there is no consensus about the real causes of these changes. Nevertheless, some authors suggest including this assay in routine genotoxicity tests (Guilherme et al., 2014; Marques et al., 2014; Strunjak-Perovic et al., 2009). Thus, five nuclear lesions categories can be considered: kidney shaped nuclei (K), segmented nuclei (S), lobed nuclei (L), vacuolated nuclei (V) and micronuclei (MN), as illustrated in figure 4. These nuclear deformations are signals of structural chromosome breakage (clastogenicity), as well as total loss of chromosome and mitotic spindle apparatus dysfunction (aneugenicity) (Fenech, 2000), being almost irreparable and considered as less transient alterations, displaying a later appearance, when compared with those detected by the comet assay (Guilherme, 2012).

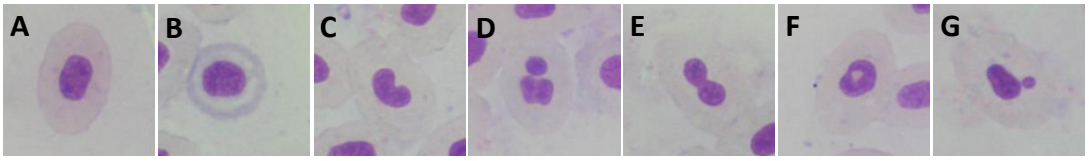

Figure 4: Mature (A) and immature (B) erythrocytes of fish with nuclear normal shape. Mature erythrocytes with nuclear abnormalities: kidney shaped (C), lobed (D), segmented (E), vacuolated (F) and micronuclei (G). Giemsa stain (Adapted from: Maceda-Veiga et al., 2015).

The analysis is described as an efficient cytogenetic technique, due to its ample sensitivity to cytotoxic effects on cells exposed to pollutants (Azevedo et al., 2012), being, probably, the most common method to survey eco-genotoxicity, certainly by its righteous merit (Martins and Costa, 2015).

1.3.3. Potential genotoxic hazards in aquaculture

The manipulation of rearing conditions to achieve a fast-growing performance may create stressful conditions passible to affect DNA integrity (Nagarani et al., 2012; Peixoto et al., 2015), including increased ROS production. According to Livingstone (2001), exposure to metals (Al, Cd, Cr, Hg, Ni), NO_2,

O_3, and SO_2^-, as well as hypoxia and hyperoxia, may induce ROS over-generation, leading to overwhelmed antioxidant defenses and DNA damage. This matter has been neglected in the context of intensive aquaculture, disregarding the potential impacts of both endogenous (e.g. age, feeding behavior, food consumption) and exogenous (e.g. chemicals present in the water, seasonal and daily changes in dissolved oxygen and water temperature) factors. In the long-term, this situation may compromise growth performance, animal welfare and a reduction in revenues (Fazio et al., 2015; Leal et al., 2011).

Oxidative stress is the consequence of an imbalance between pro-oxidants and antioxidants (Fazio et al., 2015), i.e. results from the excessive production of ROS in a way that the buffering effect of antioxidant agents is not enough to prevent their damaging effects, leading to a disturbance in cell homeostasis and possible cell death (Blier, 2014; Madeira et al., 2016). Elevated levels of ROS and/or depressed antioxidant defenses may result in DNA oxidation and increased steady-state levels of unrepaired DNA (Guilherme, 2012). The monitoring of different markers of oxidative stress could be powerful tools to evaluate the metabolic and general health status of fish. This would be a relatively simple procedure to estimate if the selected phenotypes, as well as the nutritional or environmental conditions they experience, allow metabolic optimality at cellular and tissue levels (Blier, 2014).

Growth is a critical time in the life of all organisms, but most animals do not grow at their maximal rate (Mangel and Stamps, 2001). Many organisms exhibit faster growth during recovery from total or partial food deprivation than they do during periods of continuous food availability. The response, which tends to restore the original growth trajectory, is called compensatory growth (Jobling, 1994). Fish have their highest rates of growth (20-50%/day) in their larval stages, after which growth rate decreases in the juvenile (5-10%/day) and adult stages (1-3%/day). One of the reasons why animals may not achieve their highest growth rates is because the increase in metabolic activity needed to fuel rapid growth may cause damage to the organism (e.g. some studies, such as Alonso-Álvarez et al., 2007 have reported positive correlations between growth rate and oxidative stress in the form of ROS). Rollo (2002) suggested that the growth hormone axis may exacerbate free radical processes, aging, cancer (via reducing apoptosis and promoting growth), and autoimmunity or inflammation in mammals. For these reasons, the intensive production of fish is a potential source of concern with respect to the appearance of pathogens and, mainly, to the spreading of diseases (Leal et al., 2016), likely to be preceded by disturbances on DNA structure.

Ozone is a powerful oxidizing agent widely used in aquaculture, particularly in recirculating systems (Summerfelt, 2003), for the purpose of the treatment of fish, disinfection of eggs, sterilization of water, to improve the water quality and decomposition of odorous compounds in natural water (Zhang et al., 2016). However, ozone is a very unstable molecule and an overdose of ozonation can occur as short-term events (Silva et al., 2011). It was reported that fish exposure to ozone may induce the formation of ROS that results in oxidative damage (Ritola et al., 2002). Moreover, Silva et al. (2011) revealed that the exposure of juvenile turbot (*Scophthalmus maximus*) to ozonated water induces genetic damage, which remained during the post-exposure period.

In the same way, ultraviolet (UV) irradiation is also being widely applied within aquaculture systems (Summerfelt, 2003), in spite of being recognized as a natural stressor to most forms of life, mainly as a result of its pro-oxidant potential (Ibrahim et al., 2015). UV irradiation can be used to destroy ozone residuals (catalyzing the conversion of O_3 to O_2) and to denature the DNA of microorganisms, causing the microorganisms to die or lose their function. Applying UV irradiation for disinfection can be both less costly and less complex than using ozone; however, UV irradiation may not work in situations where turbid water (and associated poor UV transmittance) may be encountered (Hunter et al., 1998). Ibrahim et al. (2015) showed negative impacts of ultraviolet-A radiation on antioxidant and oxidative stress biomarkers of African catfish (*Clarias gariepinus*), as well as a significant increase in the values of DNA fragmentation with an upward trend in the exposure groups.

Formalin (aqueous solution of formaldehyde stabilized with methanol) is another, and one of the most used, disinfectant in aquaculture. It is used to eliminate infectious agents but may be responsible for negative effects on fish and water quality. Formalin is able to react with functional groups of several biological macromolecules, such as proteins, polysaccharides and glycoproteins, as well as DNA and RNA (Leal et al., 2016). Yildiz and Ergonul (2010) demonstrated that formalin exposure represents potentially a stressful event for gilthead seabream and European seabass when considered the elevated plasma cortisol, glucose, disrupted hydromineral balance, altered CRP and ceruloplasmin. Although the lack of information about formalin effects on fish DNA, some non-fish system studies have demonstrated that high concentrations of formaldehyde can induce mutations, for example, in mouse lymphoma cells, by induction of chromosomal aberrations (Speit and Merk, 2002). Based on this information, the risk for fish should not be overlooked.

An additional problem is the treatment with anesthetic agents as they are widely used on farmed fish to control stress during handling and confinement operations, such as netting, weighing, sorting, vaccination, transport and slaughter, although it is necessary in order to ensure fish welfare (Ferreira et al., 2017). Tricaine methanesulfonate (MS222) is the most widely used fish anesthetic and it is approved by the U.S. Food and Drug Administration (FDA) for use on aquatic organisms (FDA, 2011). MS222 has high solubility in any matrix, high potency, complete recovery, the ability for induction of a range of depths of anesthesia, and it also can be administered via immersion or injection. Nevertheless, concerns have been raised once it showed to be behavioral aversive for fish (Readman et al., 2013) and also reducing pH by 0.5-1.0 units (low pH has been described as being irritant to fish), decreasing neural sensitivity (Palmer and Mensinger, 2004). To prevent this effect, the administered anesthetic solutions should be buffered using sodium bicarbonate or Tris-buffer at pH 7.0-7.5 (Ross and Ross, 2008). Barreto et al. (2007) showed that bath exposure with MS222 does not induce primary DNA damage when evaluated in juvenile Nile tilapia (*Oreochromis niloticus*) and, in the same way, Gontijo et al. (2003) reported that the anaesthetic benzocaine (structurally similar to MS222 - both are analogues of procaine) is not genotoxic to fish; however, another procaine analogue, carbisocaine, has been shown to have genotoxic activity on single-celled Eukaryote algae *Euglena gracilis* (Siekel, 1990). Taking into account the scarcity of studies in fish, the risk of MS222 should not be excluded. Thinking further, essential oils extracted from the medicinal plants are an alternative option for fish anesthesia. Essential oils indicated higher efficiency compared to chemicals, mitigating the side-effects that are often associated with synthetic substances. For example, *Cinnamomum zeylanicum* was evaluated as equal competent anesthetic agent satisfying the criteria of the ideal anesthetic, reducing DNA strand breakage and indicating anti-stress, anti-genotoxic and geno-protective effect in gilthead seabream (*Sparus aurata*) (Golomazou et al., 2016).

Also antibiotics should be taken into account. They are biologically active at low concentrations (ng L^{-1} to µg L^{-1}), acting as potential micropollutants of the environment and are used to treat microbial infections or to prevent diseases (Burkina et al., 2015). Antibiotic residues may contaminate aquatic systems and their extensive use in fish farming may lead to their presence in its active form in the environment (Ambili et al., 2013), partly due to its poor bioavailability by oral route as well as by inadequate and irregular absorption from the gastrointestinal tract (Singh et al., 2005). The antibacterial agent oxytetracycline (OTC), extensively used in aquaculture practices all over the world,

showed to be capable of inducing damage through oxidative and reductive (redox) processes, as well as increase the production of ROS, which consequently exert oxidative damage (Nakano et al., 2015). In the same direction, Rodrigues et al. (2016) showed that OTC can cause oxidative effects in rainbow trout's tissues and genotoxicity, in levels already reported to occur in the wild.

Regarding the provenance of water in aquaculture, the accidental occurrence of contaminants is a reality, even at low levels, and there are a plenty of chemical agents that can induce DNA damage, even if light. Fish are extremely sensitive to many waterborne toxicants, because these affect the gills by increasing the permeability to water and ions in its epithelium. The compensatory responses of the fish will significantly increase the energy required for maintenance of water and ion homeostasis, and this will result in reduced growth and reproduction impairments (Bonga and Lock, 1991). Hexabromocyclododecanes (HBCDs) are the third most widely used brominated flame retardants in the world (Yang et al., 2012) and are ubiquitous environmental contaminants, widely distributed in aquatic systems including the marine environment and marine organisms. The currently available toxicity data of HBCDs on aquatic organisms are mainly focused on fish. In fact, HBCDs induce the generation of ROS and result in developmental toxicity in both freshwater and marine model fish (e.g. in the zebrafish *Danio rerio* and the marine medaka *Oryzias melastigma* [Du et al., 2012 and Hong et al., 2014]).

Chlorothalonil (tetrachloroisophtalonitrile) is a fungicide that is widely used on agricultural crops around the world and as such, it is also a ubiquitous aquatic contaminant. Despite its high usage, the effects of this fungicide on non-target aquatic organisms have not been fully investigated; however, Garayzar et al (2016) found that chlorothalonil showed increased transcriptional subnetworks related to cell division and DNA damage and decreased expression of gene networks associated with reproduction, immunity, and xenobiotic clearance.

On the other hand, Guardiola et al. (2016) analyses mercury (Hg), an environmental contaminant that causes acute and chronic damage to multiple organs, in *Sparus aurata* and Hg was seen to accumulate in liver and muscle, inducing histopathological damage to skin and liver; fish exposed to MeHg showed a decreased biological antioxidant potential and increased levels of the reactive oxygen molecules and Guilherme et al. (2008) demonstrated that elevated ENA frequency in *Liza aurata* is in concomitance with increased blood Hg levels. Another risk concerning Hg is its presence in fish meal (e.g. Johnston and Savage, 1991 reported mean Hg concentrations in fish meal of 20 to 7.700 ppb).

1.3.4. The protective effects of macroalgae against a genotoxic challenge

As the protective effect of many seaweed species suggests the presence of anti-oxidative and anti-genotoxic constituents in their tissues, the interest in macroalgae as a potential and promising source of natural ingredients has increased during the last years (Celikler et al., 2009; Yuan and Walsh, 2006).

In this context, Nagarani et al. (2012) suggested that *Kappaphycus alvarezii* (Rhodophyta) extract exhibits potent anti-genotoxicity effects in fish (*Therapon jarbua)* against DNA damage induced by mercury (Hg) and thus, recommended its use as a supplement in fish diet, also suggesting benefits for humans ingesting Hg-contaminated fish. Zinadah et al. (2013) showed that when flathead grey mullet (*Mugil cephalus*) diet contains 10-20 % *Ulva lactuca* or *Caulerpa prolifera* a considerable effect on stress responses and DNA protection was achieved. However, despite these studies, there is a notorious lack of information regarding fish.

In a work from a research team, a macroalgae mixture revealed anti-genotoxic and anti-mutagenic effects in *Drosophila melanogaster* (Valente et al., 2014). In addition, recent *in vitro* data suggested protective effects of red (*Palmaria palmate* [Yuan and Walsh, 2006] and *Porphyra* sp. [Know and Nam, 2006]) and brown *(Laminaria setchellii, Macrocystis integrifolia* and *Nereocystis leutkeana)* (Yuan and Walsh, 2006) algae against different cancer types. The benefits of algal extracts in human lymphocytes (*in vitro*) was also evaluated, showing no clastogenic or cytotoxic effects, in parallel with strong anti-genotoxic, anti-clastogenic and protective effects against the chemotherapeutic agent mitomycine-C (e.g. Celikler et al., 2008 have proved it with *Ulva rigida* and *Fucus vesiculosus* and Celikler et al., 2009 with *Codium tomentosum*).

Notwithstanding the publications above mentioned, it is manifest a scarcity of information about macroalgae protective effects, given their diversity, as well as a lacuna on the understanding of the mechanisms underlying their anti-genotoxic properties.

1.4. Goals and thesis structure

Bearing in mind the attention devoted to the issue, it seems to exist a dominant idea (implicitly rather than explicitly) that DNA integrity in farming fish is not routinely compromised. However, as demonstrated above, several agents/conditions intentionally or accidentally associated to fish-farming

practices may pose a substantial risk to the structural integrity of the DNA molecule. Thus, in order to fulfill a knowledge gap, a central goal was defined for the present thesis concerning the evaluation of the anti-genotoxic properties of a macroalgae-enriched diet in gilthead seabream (*Sparus aurata*). The tested diet was supplemented with three different species - *Ulva* spp. (Chlorophyta), *Gracilaria* spp. (Rhodophyta) and *Fucus* spp. (Phaeophyta) – in a total percentage of 5 % (incorporating equal percentages of each algae).

Emanating from the previous general goal, the following specific objectives were considered:

- Assessment of the protective properties of macroalgae supplementation towards two types of genetic damage in blood cells, viz. DNA strand breaks (primary and reparable damage), measured with the comet assay, and chromosomal damage (potentially more permanent damage), measured through the erythrocytic nuclear abnormalities (ENA) assay;
- Elucidation of the oxidative DNA damage–protecting activity, adopting as diagnostic tool the comet assay improved with DNA lesion-specific enzymes;
- Clarification of the involvement of antioxidant system modulation on the potential defense mechanisms under investigation, evaluating enzymatic and non-enzymatic antioxidants;
- Discrimination between a beneficial action in relation to a baseline genomic integrity or coping with an exogenous genotoxic challenge, using a model genotoxicant – cyclophosphamide;
- Evaluation if the potentially favorable effects of algae persist beyond the end of supplementation.

The information obtained from this research would enable the formulation of a fish diet with seaweed supplementation and, consequently, a lower proportion of fish meal and lower feed cost production. In addition, this study can improve aquaculture production of algae and fish, being thus of paramount importance the within the framework of a “blue growth” development model.

2. Material and Methods

2.1. Chemicals

DNA lesion-specific repair enzymes, namely formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (EndoIII), were purchased from Professor Andrew Collins (University of Oslo, Norway).

Cyclophosphamide and all the other chemicals were obtained from the Sigma-Aldrich Chemical Company (Madrid, Spain).

2.2. Fish and holding conditions

Gilthead seabream (*Sparus aurata* L.) specimens, with an initial average body weight of 104±35 g and total length of 19±2 cm, were supplied by the semi-intensive fish farm Materaqua, Lda. (Ílhavo, Portugal). Fish condition was assessed along the experiment through the Fulton's condition factor (K), according to the expression $K = (W \times 100)/L^3$, where W = weight (g) and L = total length (cm).

Prior to the experiment described below, fish were acclimatized to the experimental tanks/conditions, including to the standard diet (S), for one week. The first experimental period of 30 days, and consequently acclimation, took place in 8000 L tanks, while 1000 L tanks were adopted in the post-injection periods (see figure 5). All the tanks were kept under a natural photoperiod, as open systems (each thank was independently supplied by a flow-through seawater system pumping from Aveiro lagoon, corresponding to a water renewal rate of 6 times per day), with the following physico-chemical conditions: salinity 35, temperature 17±1 ˚C, nitrite 0.05±0.02 mg L^{-1}, ammonia 0.5±0.4 mg L^{-1} and dissolved oxygen 12.5±1.4 mg L^{-1}.

Fish well-being and all the procedures were supervised by a certified operator, in accordance with national and international guidelines (Directive 2010/63/EU) to ensure minimal animal use and discomfort.

2.3. Experimental diets

Two diets were prepared (2.0-mm pellet size), composed by the same basic ingredients, corresponding to a standard diet (S) - formulation adjusted according to recommendations for *S. aurata*, and a diet enriched with algae (A). Algae supplementation concerned a total percentage of 5 %, incorporating three different species - *Ulva* spp. (Chlorophyta), *Gracilaria* spp. (Rhodophyta) and *Fucus* spp. (Phaeophyta) - in an equal percentage (approx. 1.67 %). Algae were reared at ALGAPlus, Lda. (Ílhavo, Portugal), an integrated multi-trophic aquaculture (IMTA). Diets were produced by SPAROS, Lda. (Faro, Portugal), and the respective formulations are presented in table 1.

Fish were hand-fed once a day (10 a.m.), at a daily rate of 3% (as percentage of fish biomass).

Table 1: Formulation and chemical composition of the experimental diets

	Dietary treatments	
	Standard diet	Algae-enriched diet
Ingredients (%DM)		
Fishmeal LT 70	27	26.5
Fishmeal 60	20	20
CPSP 90	3	3
Blood meal	5	5
Soy protein concentrate	8	8
Wheat Gluten	10	10
Wheat meal	7.5	3
Pea starch	5.1	5.1
Fish oil	13	13
Vit & Min Premix	1	1
Binder	0.4	0.4
MIX Macroalgas	0	5
As fed basis		
Crude protein (%DM)	50.3	50.3
Crude fat (%DM)	17.7	17.6
Fiber (%DM)	0.6	0.4
Starch (%DM)	7.8	5.1
Ash (%DM)	12.1	12
Total P (%DM)	1.4	1.4
Gross Energy (kJ g^{-1} DM)	20.8	20.8

DM = dry matter

2.4. Experimental design and sampling

A lot of 130 fish was divided into two tanks as follows: 55 fish corresponding to the group reared with a standard diet (S); 75 fish corresponding to the group reared with the algae-enriched diet (A) (see figure 6). Following acclimation, and just before the experiment beginning, 10 fish (randomly collected from both tanks) were sampled and used as the initial reference group (time zero; t_0). As a first step of the experiment, fish were reared for 30 days either with the S or A diet. Then, fish from both experimental groups (S and A) were intraperitoneally injected with 40 mg kg^{-1} cyclophosphamide (CP), corresponding to S_{CP} and A_{CP} groups, or with a saline solution, and sampled at days 3 and 10 post-injection (p.i.), keeping the diet unaltered. Additionally, in order to evaluate whether the potentially favorable effects of algae remain after the end of supplementation, a subgroup of fish previously fed with algae-enriched diet was submitted to a diet alteration at day 3 p.i., being then fed with the standard diet for 7 days (sampled at day 10 p.i.). This diet reversion was applied to both CP treated (A_{CP}/S) and untreated (A/S) groups.

Fish were not fed on the day before sampling. At each sampling time point, 10 fish were sampled per experimental group (n=10). Immediately after collection, fish were anesthetized with 0.5 mgL^{-1} tricaine methanesulfonate (MS-222) for approximately 15 min (Gilderhus and Marking, 1987). After being weighed (to nearest 0.1 g) and measured (total length; to the nearest 0.1 cm), fish were sacrificed by cervical transection.

Fish blood was drawn from the posterior cardinal vein, using heparinized (27 mg mL^{-1} heparin) Pasteur pipettes, and placed into 2 mL microtubes (two microtubes per fish). Thus, one microtube containing 0.002 mL of blood diluted in 1 mL of chilled PBS (pH=7.4; 0.01M), constituted the cell suspension for comet assay, and the other, with the remaining blood volume, was assigned for antioxidants analysis. Aliquots for comet assay were kept cold up to further procedures, while the aliquots for antioxidants determination were immediately frozen in liquid nitrogen. Additionally, blood smears were immediately prepared for ENA assay.

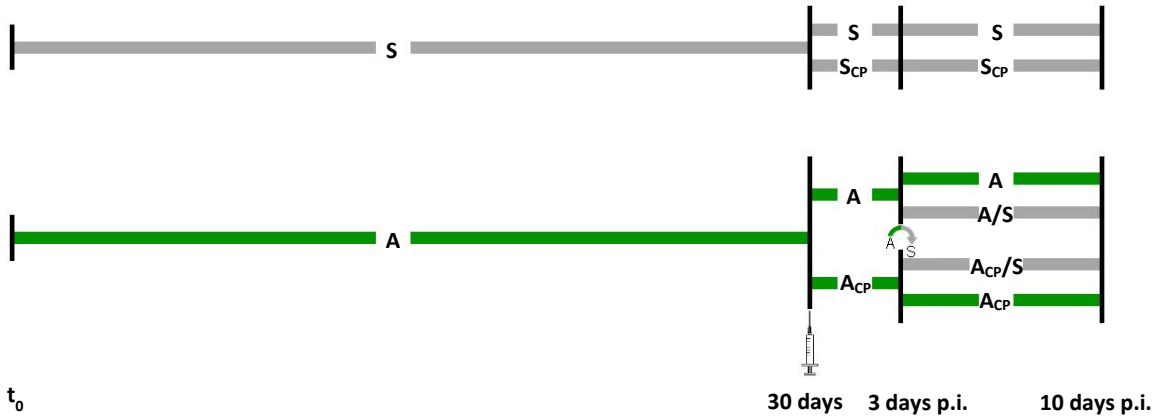

Figure 5: Schematic overview of the experimental design. Each horizontal line represents an experimental group: grey lines correspond to fish groups fed with a standard diet (S), while green ones represent fish groups fed with the algae-enriched diet (A). Fish were reared for 30 days with the two different diets. At that time, fish were intraperitoneally injected with cyclophosphamide (CP; as subscript in groups' abbreviation) or with a saline solution (no subscript text in groups' abbreviation). Then, fish were sampled at 3 and 10 days post-injection (p.i.), keeping the diet unaltered. In addition, fish fed with algae-enriched diet, were submitted to a diet alteration at 3 days p.i., being then fed with the standard diet for 7 days (sampled at 10 days p.i.). This diet reversion was applied to both CP injected (A_{CP}/S) and not injected (A/S) groups. Vertical lines correspond to sampling moments. t_0 corresponds to sampling just before the experiment beginning.

2.5. Evaluation of genetic damage

2.5.1. Exogenous genotoxic insult

Cyclophosphamide was the model genotoxicant selected to induce an acute challenge to genome integrity of *S. aurata*. The target for CP action in the cells is predominantly DNA, where cross-linkages occur, leading to DNA strand breaks and, ultimately, to inability to synthesize DNA and inhibition of mitotic division (Mazur and Czyzewska, 1994). This drug is an alkylating agent that causes alkylation of the purine ring and, as a result, there is miscoding and blockade of DNA replication, being thus the mutagenic usually adopted as positive control in *in vivo* tests of short duration (Ali et al., 2008). It appeared to be a pure clastogen (agent giving rise to or inducing disruption or breakages of chromosomes, leading to sections of the chromosome being deleted, added, or rearranged), also showing a weak aneugenic activity (effect on mitotic spindle apparatus, resulting in the loss or gain of total chromosomes) (Vanparys et al., 1990).

2.5.2. Comet assay

The assay was conducted according to the technique described by Collins (2004) as adapted by Guilherme et al. (2010), with the proper adjustments to assay procedure with an extra step of digesting the nucleoids with endonucleases. All slides were freshly prepared. A system of eight mini-gels per slide was adopted, based on a model created by Shaposhnikov et al. (2010), in order to increase the assay output. Each individual is represented by 2 replicate mini-gels, being 4 different individuals represented in each slide. Briefly, 0.02 mL of cell suspension were mixed with 0.07 mL of 1% low melting point agarose, in PBS, and eight drops with 0.007 mL of cell suspension were placed onto the pre coated slide (with 1% normal melting point agarose) as two rows of 4, without coverslips. The mini-gels were left for ±5 min at 4 ˚C in order to solidify agarose, and then immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 1% Triton X-100, pH 10) at 4 ˚C, and kept overnight. After lysis of agarose-embedded cells, slides were washed 3 times with buffer (0.1 M KCl, 0.0005 M EDTA, 0.04 M HEPES, 200 mg L^{-1} bovine serum albumin, pH 8) at 4 ˚C and three sets of slides were prepared: two sets were incubated with endonucleases FPG or EndoIII, which convert oxidized purines and pyrimidines into extra DNA single strand breaks, respectively (Azqueta et al., 2009), and a third set was incubated only with buffer. Hence, 0.03 mL of each enzyme (diluted in buffer) were applied in each mini-gel, together with a coverslip, prior to incubation at 37 ˚C for 30 min, in a humidified atmosphere. The slides were then placed in the electrophoresis tank, immersed in electrophoresis solution (20 min) for alkaline treatment. DNA was allowed to migrate at a fixed voltage of 25 V, with 1.04V/cm and a current of 300 mA (achieved by adjusting the solution volume in the electrophoresis tank), during 15 min. Later, the slides were stained with ethidium bromide (0.035 mL for each 4 mini-gels) and fifty nucleoids were observed per mini-gel, using a Leica DMLS fluorescence microscope (400× magnification). Slides were coded and scored blind. The DNA damage was quantified by visual classification of nucleoids into five comet classes, according to the tail intensity and length, from 0 (no tail) to 4 (almost all DNA in tail) (Collins, 2004).

The total score expressed as a genetic damage indicator (GDI) was calculated multiplying the percentage of nucleoids in each class by the corresponding factor, according to the following formula:

GDI = [(% nucleoids class 0)×0] + [(% nucleoids class 1)×1] + [(% nucleoids class 2) × 2] + [(% nucleoids class 3) × 3] + [(% nucleoids class 4) × 4]

GDI values were expressed as arbitrary units in a scale of 0 to 400 per 100 scored nucleoids (as average value for the 2 gels observed per fish). When the comet assay was performed with additional FPG and EndoIII steps, GDI values were calculated in the same way but the parameter designated GDI_{FPG} and $GDI_{EndoIII}$, respectively. Additional DNA breaks corresponding to net enzyme-sensitive sites alone (NSS_{FPG} or $NSS_{EndoIII}$) were also expressed. Moreover, the frequency of nucleoids observed in each comet class considering GDI_{FPG} and $GDI_{EndoIII}$ was also expressed, as recommended by Azqueta et al. (2009) (see attachment).

2.5.3. ENA assay

The assay was carried out in mature peripheral erythrocytes, according to the procedure of Pacheco and Santos (1996). Previously, one blood smear per animal was fixed with methanol during 10 min and stained with Giemsa (5%) during 30 min. Slides were coded and scored blind. From each smear, 1000 erythrocytes were scored, under 1000x magnification (microscope Olympus BX50), to evaluate the relative frequency of the following nuclear lesions: kidney shaped nuclei (K), lobed nuclei (L), segmented nuclei (S), vacuolated nuclei (V) and micronuclei (MN). Results were expressed as the sum of frequencies for all the categories observed (K + L + S + V + MN).

2.6. Evaluation of antioxidant system status

2.6.1. Tissue preparation and fractionation

Whole blood samples (stored at -80 ˚C) were lysed through homogenization in a 1:6 ratio (blood volume:buffer volume), using a Potter-Elvehjem homogenizer, in chilled phosphate buffer (0.1 M; pH 7.4). This lysate was then centrifuged (Eppendorf 5415R centrifuge) at 12 000 rpm for 20 min, at 4 °C, to obtain PMS (Post-Mitochondrial Supernatant) fraction. All the aliquots were stored in microtubes at -80 ˚C until analyses.

2.6.2. Measurement of enzymatic and non-enzymatic antioxidants

All measurements were carried out in a SpectraMax 190 microplate reader, at 25 ˚C.

Catalase (CAT) activity was assayed in PMS by the method of Claiborne (1996), with slight modifications. Briefly, the assay mixture consisted of 0.190 mL phosphate buffer (0.05 M, pH 7.0) with hydrogen peroxide (H_2O_2; 0.010 M) and 0.010 mL of PMS, in a final volume of 0.2 mL. Change in absorbance was measured in appropriated UV-transparent microplates (UV-Star® flat-bottom microplates, Greiner Bio-One GmbH, Germany), recorded at 240 nm and CAT activity was calculated in terms of µmol H_2O_2 consumed min^{-1} mg^{-1} protein using a molar extinction coefficient (ε) of 43.5 M^{-1} cm^{-1}.

Superoxide dismutase (SOD) was assayed in PMS with a Ransod kit (Randox Laboratories Ltd., UK). The method employs xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5- phenyltetrazolium chloride (INT) to form a red formazan dye determined at 505 nm. SOD activity is then measured by the degree of inhibition of this reaction, considering that one unit of SOD causes a 50% inhibition of the rate of reduction of INT, under the conditions of the assay. Results were expressed as SOD units mg $protein^{-1}$.

Glutathione peroxidase (GPx) activity was determined in PMS according to the method of Mohandas et al. (1984) and modified by Athar and Iqbal (1998). The assay mixture consisted of 0.09 mL phosphate buffer (0.05 M, pH 7.0), 0.03 mL ethylenediaminetetraacetic acid (EDTA; 0.010 M), 0.03 mL sodium azide (0.010 M), 0.03 mL glutathione reductase (GR; 2.4 U mL^{-1}), 0.03 mL reduced glutathione (GSH; 0.010 M), 0.03 mL nicotinamide adenine dinucleotide phosphate-oxidase (NADPH; 0.0015 M), 0.03 mL H_2O_2 (0.0025 M) and 0.03 mL of PMS in a total volume of 0.3 mL. Oxidation of NADPH to $NADP^+$ was recorded at 340 nm and GPx activity was calculated in terms of nmol NADPH oxidized min^{-1} mg $protein^{-1}$ ($\varepsilon = 6.22 \times 10^3$ M^{-1} cm^{-1}).

Glutathione reductase (GR) activity was assayed in PMS by the method of Cribb et al. (1989), with some modifications. The assay determines indirectly the GR activity by measuring the NADPH disappearance associated with reduction of oxidized glutathione (GSSG) catalyzed by GR. Briefly, the assay mixture contained 0.050 mL of PMS fraction and 0.250 mL of reaction medium consisted of phosphate buffer (0.05 M, pH 7.0), NADPH (0.0002 M), glutathione disulfide (GSSG; 0.001 M) and diethylenetriaminepentaacetic acid (DTPA; 0.0005 M). The enzyme activity was determined by measuring the oxidation of NADPH at 340 nm and calculated as nmol NADPH oxidized min^{-1} mg $protein^{-1}$ ($\varepsilon = 6.22 \times 10^3$ M^{-1} cm^{-1}).

Glutathione-S-transferase (GST) activity was determined in PMS with CDNB (1-chloro-2,4-dinitrobenzene) as a substrate, according to the method of Habig et al. (1974). The assay mixture consisted in 0.1 mL of PMS and 0.17 mL of phosphate buffer (0.2 M, pH 7.9) and GSH (0.0018 M). The reaction was initiated by addition of 0.03 mL of CDNB (0.01 M), and the increase in absorbance was recorded at 340 nm. The enzyme activity was calculated as nmol CDNB conjugate formed min^{-1} mg^{-1} protein (ε = 9.6 mM^{-1} cm^{-1}).

For total glutathione (GSHt) content determination, PMS was precipitated with trichloroacetic acid (TCA 12%) for 1 h and then centrifuged at 12 000 g for 5 min at 4 ◦C. GSHt was determined (in deproteinated PMS) adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB) (Tietze 1969; Baker et al. 1990). The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the GSH concentration in the sample. The assay mixture consisted in 0.2 mL sodium phosphate buffer (0.143 M, pH 8), EDTA (0.0063 M), DTNB (0.001 M) and NADPH (0.00034 M), added to 0.04 mL of deproteinated PMS. The reaction was initiated with 0.04 mL of GR (8.5 U mL^{-1}). Formation of TNB was measured at 415 nm. It should be noted that GSSG is converted to GSH by GR in this system, which consequently measures total GSH. The results were expressed as nmol TNB formed min^{-1} mg $protein^{-1}$ (ε = 14.1 x 10^3 M^{-1} cm^{-1}).

Total protein content was determined according to the Biuret method (Gornall et al., 1949), using bovine serum albumin (E. Merck-Darmstadt, Germany) as a standard.

2.7. Statistical analysis

Statistica 8.0 software was used for statistical analysis. All data were first tested for normality and homogeneity of variance to meet statistical demands.

For the pre-injection sampling moment, one-way Analyses of Variance (ANOVA) was applied. The remaining data were analyzed through a two-way ANOVA, followed by a post hoc Tukey test, in order to compare the different treatment groups between each other. Temporal variations were tested using the independent samples t-test by groups. In all the analyses, differences between means were considered significant when $p < 0.05$ (Zar, 1996).

3. Results

No fish mortality was observed during the experiment. Though feeding was not strictly monitored, no relevant alterations on fish feeding response were perceptible in dependence to the diet profile, since fish were very active and come to feed immediately in both diets; however, a visual evaluation of feeding response, mainly during the first 30-day period, suggested that fish fed with algae-enriched diet displayed a slightly higher appetite.

Fish condition was assessed along the experiment through the Fulton's condition factor (K), showing, in general, no significant differences, with the exception of a K decrease observed at 10 days after injection in A_{CP}/S group when compared to both S_{CP} and A/S groups (table 2).

Table 2: Mean values (± standard error) of weight, total length and condition factor (K) measured in *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning). Statistically significant differences in K data ($p < 0.05$) are: (*) within the same sampling moment. S = standard feed; A = algae-enriched feed; CP = cyclophosphamide; p.i. = post-injection.

Exposure Conditions		Weight (g)	Lenght (cm)	K	
	t_0	72.95±27.90	17.69±2.17	1.26±0.06	
30 days	S	116.92±27.59	19.12±1.93	1.67±0.20	
	A	131.58±26.47	20.53±1.25	1.50±0.08	
3 days p.i.	S	92.60±43.10	17.74±2.67	1.55±0.11	
	S_{CP}	112.11±39.29	18.74±1.93	1.63±0.16	
	A	122.64±23.88	19.67±1.33	1.60±0.18	
	A_{CP}	113.63±33.35	19.12±2.03	1.58±0.10	
10 days p.i.	S	95.45±33.40	17.97±2.29	1.59±0.11	
	S_{CP}	85.61±27.84	17.82±2.34	1.49±0.20	*
	A	110.94±35.56	19.43±2.04	1.46±0.09	
	A_{CP}	87.81±31.57	18.68±1.91	1.29±0.25	*
	A/S	124.13±34.26	19.86±1.85	1.55±0.13	*
	A_{CP}/S	85.50±29.72	19.46±2.24	1.11±0.21	*

3.1. DNA damage as comet assay

Concerning the first thirty days, where fish were fed with the different diets (S vs. A), without any genotoxic challenge, no statistically significant differences were found on GDI values (Figure 6). Three days after injection, both groups treated with cyclophosphamide (S_{CP} and A_{CP}) revealed a significantly higher DNA damage, measured as GDI, in comparison with the corresponding untreated diet-group. No significant differences were observed between both CP treated groups at this moment. In relation to the last sampling moment (ten days after injection), a similar pattern was revealed when CP treated and untreated groups were compared, depicting significant GDI increases. However, CP treated groups previously fed with algae-enriched diet (A_{CP} and A_{CP}/S) displayed GDI values significantly lower than the CP treated group feed with standard diet (S_{CP}). Paralleling the samplings after injection, all groups with the exception of S_{CP} showed a significant decrease in GDI values over time.

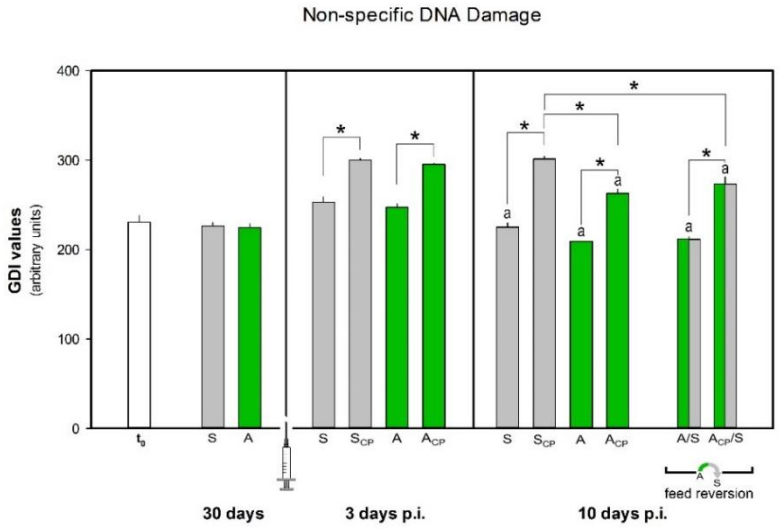

Figure 6: Mean values of genetic damage indicator (GDI), expressed as arbitrary units, measured by comet assay in blood cells of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning). Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) between groups within the same sampling moment; (a) between groups at 10 days p.i. and the corresponding groups at 3 days p.i. S = standard feed (grey columns); A = algae-enriched feed (green columns); A/S corresponds to a diet reversion from algae-enriched to standard feed (half green-grey columns); CP = cyclophosphamide; p.i. = post-injection.

Taking into account the comet assay improved with the extra-step, involving the DNA lesion-specific endonucleases EndoIII and FPG, the same pattern as previously described for GDI was observed for

both $GDI_{EndoIII}$ and GDI_{FPG} parameters (Figs. 7A and 8A), with the exception of S group at 10 days p.i. that was no longer significantly lower than the corresponding group in the previous sampling moment.

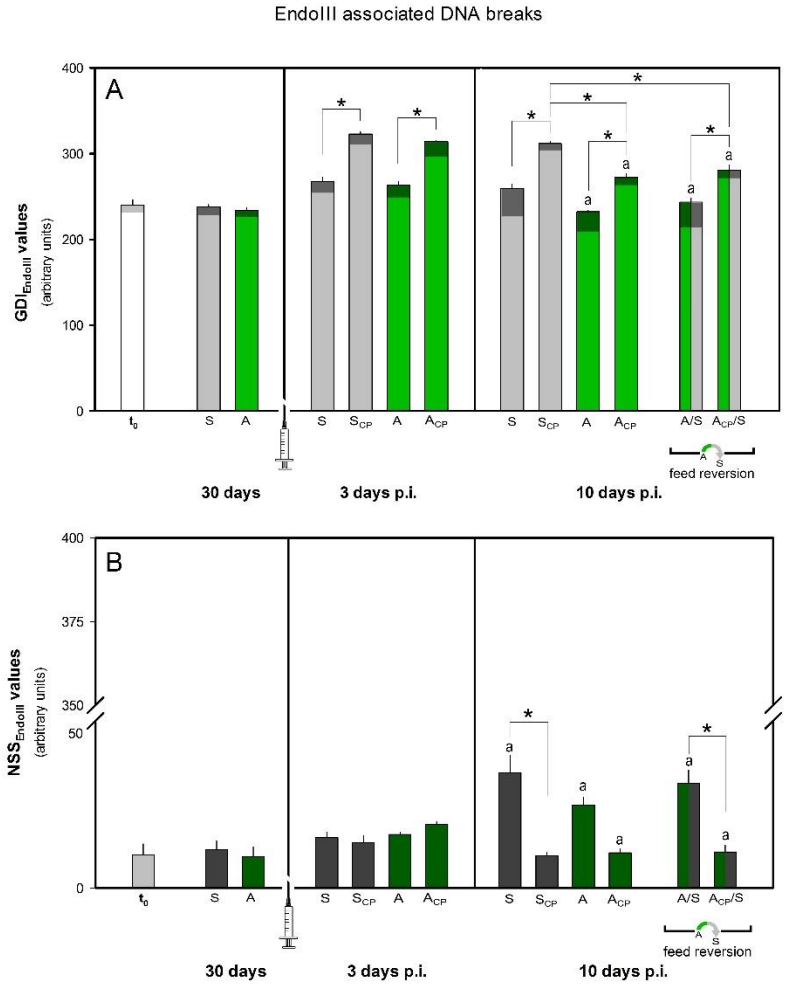

Figure 7: Mean values of DNA damage, measured by the comet assay in blood cells of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning). Values resulted from the assay with an extra step of digestion with endonuclease III (EndoIII) to detect oxidized pyrimidine bases: **(A)** overall damage ($GDI_{EndoIII}$) and partial scores, i.e. genetic damage indicator (GDI) after the standard comet assay and additional DNA breaks corresponding to net EndoIII-sensitive sites ($NSS_{EndoIII}$; dark grey/green); **(B)** $NSS_{EndoIII}$ alone. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) between groups within the same sampling moment; (a) between groups at 10 days p.i. and the corresponding groups at 3 days p.i. S = standard feed (grey columns); A = algae-enriched feed (green columns); A/S corresponds to a diet reversion from algae-enriched to standard feed (half green-grey columns); CP = cyclophosphamide; p.i. = post-injection.

Bearing in mind specifically the DNA breaks corresponding to net endonuclease-sensitive sites (Fig. 7B), differences were only observed ten days after injection, when groups S_{CP} and A_{CP}/S were

significantly lower than the corresponding untreated diet-groups. In addition, at that time all groups (except S_{CP}) were significantly different from the previous sampling moment, being notorious a time-related increase in untreated groups (S, A and A/S) while the opposite variation was observed in CP treated groups (A_{CP} and A_{CP}/S).

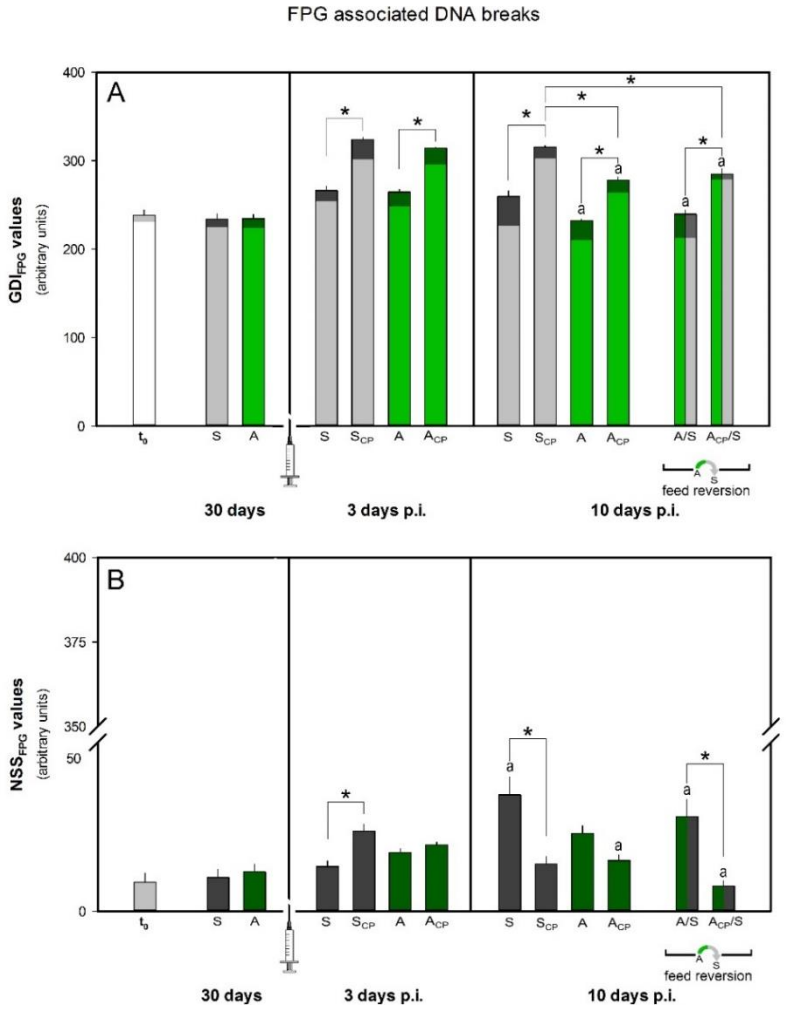

Figure 8: Mean values of DNA damage, measured by the comet assay in blood cells of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning). Values resulted from the assay with an extra step of digestion with formamidopyrimidine DNA glycosylase (FPG) to detect oxidized purine bases: **(A)** overall damage (GDI_{FPG}) and partial scores, i.e. genetic damage indicator (GDI) after the standard comet assay and additional DNA breaks corresponding to net FPG-sensitive sites (NSS_{FPG}; dark grey/green); **(B)** NSS_{FPG} alone. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) between groups within the same sampling moment; (a) between groups at 10 days p.i. and the corresponding groups at 3 days p.i. S = standard feed (grey columns); A = algae-enriched feed (green columns); A/S corresponds to a diet reversion from algae-enriched to standard feed (half green-grey columns); CP = cyclophosphamide; p.i. = post-injection.

Taking into account the NSS_{FPG} values (Fig. 8B), at three days after injection it was observed a significant increase in S_{CP} when compared to S group. In what concerns ten days after injection, it was observed the same pattern as described for $NSS_{EndoIII}$ parameter, with the exception that A group was no longer different over time.

3.2. Chromosomal damage as ENA frequency

Concerning the first thirty days of dietary trial, no significant alterations were found in total ENA frequency (Fig. 9). However, similarly to comet assay results, three days after injection, both groups submitted to cyclophosphamide injection presented a significant ENA increase in comparison with the corresponding untreated groups. No significant differences were observed between both CP treated groups at this moment.

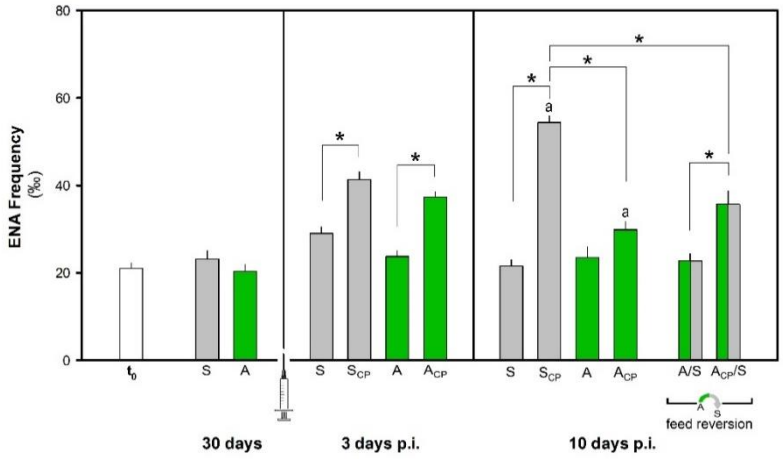

Figure 9: Mean values of erythrocytic nuclear abnormalities (ENA) frequency (‰) in peripheral erythrocytes of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning). Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) between groups within the same sampling moment; (a) between groups at 10 days p.i. and the corresponding groups at 3 days p.i. S = standard feed (grey columns); A = algae-enriched feed (green columns); A/S corresponds to a diet reversion from algae-enriched to standard feed (half green-grey columns); CP = cyclophosphamide; p.i. = post-injection.

In what concerns to ten days p.i., groups previously injected with CP had a significantly higher total ENA frequency (when compared to the respective untreated groups), with the exception of A_{CP} group. Furthermore, groups previously injected with CP and fed with algae-enriched diet, with (A_{CP}/S) or without (A_{CP}) feed reversion, showed a significantly lower ENA frequency than the CP treated group fed with standard diet (S_{CP}). Paralleling the samplings after injection, S_{CP} and A_{CP} showed significant differences, translated in time-related increase and decrease, respectively.

3.3. Antioxidant responses

Concerning the evaluation of *S. aurata* antioxidant system (Figs. 10, 11 and 12), with the exception of GST activity and GSHt content, no significant alterations were observed.

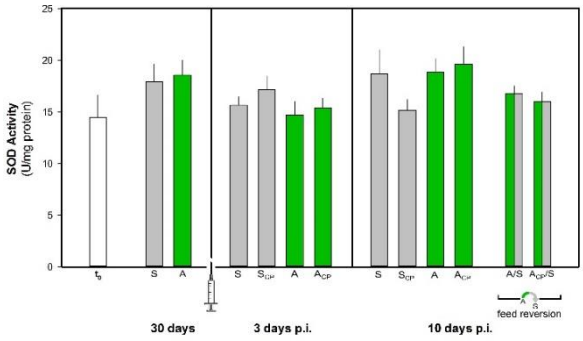

Figure 10: Mean superoxide dismutase (SOD) activity in peripheral blood of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning). Bars represent the standard error. S = standard feed (grey columns); A = algae-enriched feed (green columns); A/S corresponds to a diet reversion from algae-enriched to standard feed (half green-grey columns); CP = cyclophosphamide; p.i. = post-injection.

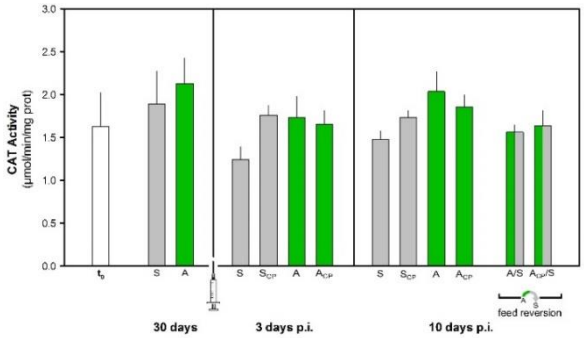

Figure 11: Mean catalase (CAT) activity in peripheral blood of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning). Bars represent the standard error. S = standard feed (grey columns); A = algae-enriched feed (green columns); A/S corresponds to a diet reversion from algae-enriched to standard feed (half green-grey columns); CP = cyclophosphamide; p.i. = post-injection.

Taking into account GST activity, ten days after injection, S_{CP} and A_{CP}/S groups were significantly higher than non-injected groups; however, the most interesting result is the comparison between CP treated groups, i.e. A_{CP} group was significantly lower than groups fed with standard feed (S_{CP}) or submitted to feed reversion (A_{CP}/S).

Temporal variations of GST activity were observed at three days after injection in A group, showing a reduction in relation to the precedent sampling moment, as well as in all groups at ten days p.i., which were also significantly lower than the corresponding groups at 3 days p.i., with the exception of A_{CP}/S group.

Considering GSHt content, at three days p.i. A group was significantly lower than S, having these two groups also decreased comparing to the previous sampling. In relation to ten days p.i., A_{CP}/S was significantly higher than corresponding untreated group, while S and A/S groups displayed a time-related decrease.

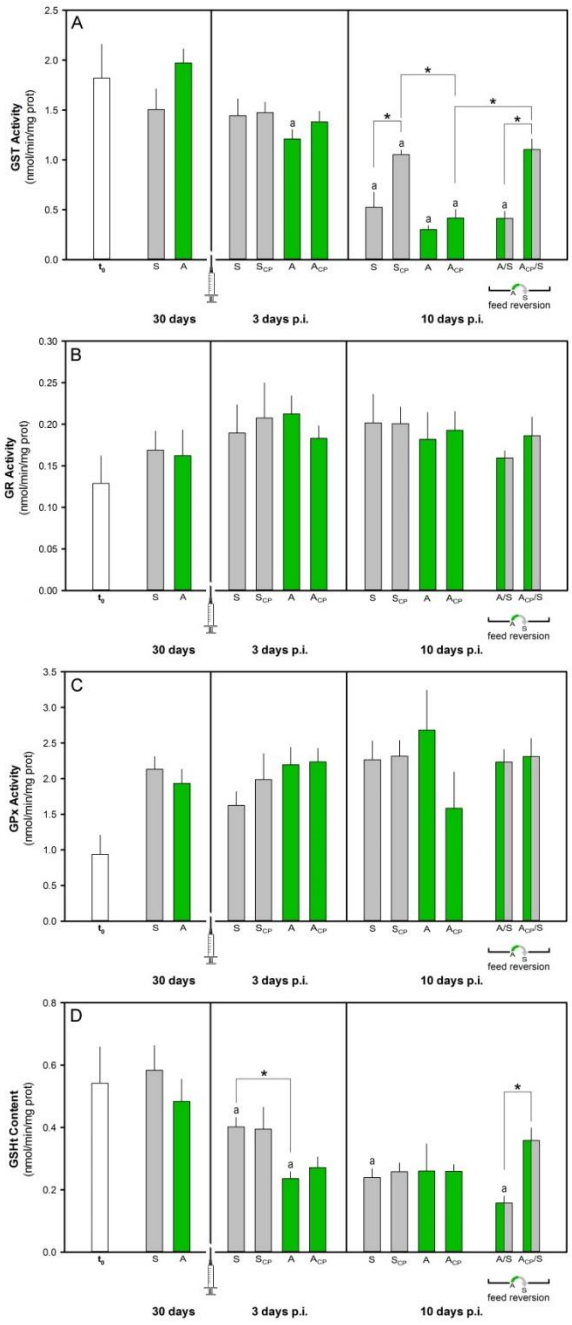

Figure 12: Glutathione related parameters in peripheral blood of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning): mean values of **(A)** glutathione-S-transferase (GST), **(B)** glutathione reductase (GR) and **(C)** glutathione peroxidase (GPx) activities, as well as **(D)** total glutathione (GSHt) content. Bars represent the standard error. Statistically significant differences ($p < 0.05$) are: (*) between groups within the same sampling moment; (a) concerning the preceding sampling moment and the corresponding group. S = standard feed (grey columns); A = algae-enriched feed (green columns); A/S corresponds to a diet reversion from algae-enriched to standard feed (half green-grey columns); CP = cyclophosphamide; p.i. = post-injection.

4. Discussion

The genome integrity of fish raised in aquaculture systems can be affected through several pathways, associated either to endogenous (e.g. ROS produced under conditions of stress and accelerated growth) or exogenous (e.g. waterborne contaminants, therapeutants, disinfectants and anaesthetics) insults (Alonso-Álvarez et al., 2007; Silva et al., 2011; Rodrigues et al., 2016). In both cases, the structural integrity of the DNA molecule may be protected by some mechanisms among which stands out the antioxidant systems. Notwithstanding the previous statements, the assessment of the balance between pro-genotoxic and anti-genotoxic processes in farmed fish has been disregarded in aquaculture research, overlooking the medium to long-term consequences for the organism health and the potential (direct or indirect) impact on productivity.

Despite the recent developments in fish nutrition towards a functional and environmentally oriented aquafeeds (e.g. Li et al., 2009) and the assumption that a nutritional-genetic combined approach has the potential to provide critical knowledge, this area of research has been mainly focused on the impact on the physiology of muscle growth (e.g. Kwasek et al., 2012). To the best of our knowledge, no studies have been done on the diet manipulation towards an improved capacity of farmed fish to protect the structural integrity of their DNA, highlighting the novelty of the current approach exploring the potential of marine macroalgae in that regard.

Many studies have been using the comet assay to evaluate DNA damage (Collins, 2004). However, this assay measures DNA lesions that can be repaired (Azqueta et al., 2009) and so, may have limited value in the evaluation of long-term consequences of exposure to genotoxicants. In order to understand a particular damaging action, namely DNA oxidation, the comet assay was improved with an extra-step with two DNA lesion-specific repair enzymes. This additional step also improves the possibility to identify a damaging action that could have been masked by the breaks score as GDI only.

Since the comet and the ENA assays allow the detection of different types of genetic damage, DNA strand-breaks and chromosomal abnormalities, respectively, both are able to provide independent and significant data and should be considered and adopted as complementary genotoxic endpoints (Wirzinger et al., 2007). Therefore, the following discussion will focus first on each type of genetic damage separately, *viz*. primary DNA damage (measured as comet assay) or potentially more persistent

alterations (chromosomal damage measured as ENA assay), and then on the interplay mechanisms with antioxidants system.

Moreover, fish erythrocytes were selected as target cells (besides practical/technical reasons such as ease of sampling and availability of dissociated cells) because they are considered as a major site for ROS production (due to their role in the oxygen transport and utilization), as well as because they are among the first cells to suffer the toxic effects of chemicals absorbed and then distributed through the bloodstream (Ruas et al., 2008).

The current determination of biometric parameters intended, primarily, to discard the possibility that the macroalgae contain anti-nutritional factors, rather than to evaluate growth (experiment was not designed to fulfil the requirements of a growth trial, namely because fish were not fed *ad libitum*). Indeed, the condition factor (K) did not show significant differences between diets (the only significant changes in K are attributable to CP). This result is in accordance with previous findings (e.g. Wassef et al., 2005) indicating that feeding *S. aurata* with 10% of *Pterocladia* or 5% of *Ulva* meal didn't affect negatively the growth and even produced the best performance, feed utilization, nutrient retention and survival. In the same direction, Batista (2008) demonstrated that the increasing incorporation of *Ulva rigida* and *Gracilaria* sp. in the diet of *S. aurata* did not significantly affect the growth, while Peixoto et al. (2016) showed that diets supplemented either with *Gracilaria* spp., *Ulva* spp. or *Fucus* spp. didn't compromise growth performance in European seabass (*Dicentrarchus labrax*).

4.1. Protective effects of algae against primary DNA damage

Analyzing the present results after thirty days of fish rearing with the two tested diets, it was perceptible that algae supplementation didn't improve the basal DNA integrity, as depicted by the parameters signaling both non-specific and oxidative damage. This was not a surprising result as the main hypothesis of the present study concerned the possibility of algae play a protective action in the presence of an exogenous genotoxic insult.

Accordingly, the confirmation that the challenging agent – cyclophosphamide – increased DNA strand breaks appears as a precondition to analyze the potential protective effects of the macroalgae-enriched diet. Hence, at 3 days post-injection, CP induced DNA damage in blood cells of *S. aurata*, in both diet-groups, measured as GDI as well as both $GDI_{EndoIII}$ and GDI_{FPG}. This is in agreement with

previous studies describing CP genotoxic potential to fish (*Prochilodus lineatus*), measured through comet assay (Cavalcante et al., 2008; Monteiro et al., 2011). In addition, when DNA strand breaks resulting specifically from FPG activity (NSS_{FPG}) are under analysis, a CP capacity to affect purine rings was perceptible (in S_{CP} group at 3 days p.i.), as previously stated by Ali et al. (2008).

Taking into account three days post-injection data, and in what concerns the overall DNA damage (either non-specific - GDI, or updated with oxidative lesions - $GDI_{EndoIII}$ and GDI_{FPG}), the algae-enriched diet didn´t display a protective action against CP genotoxic insult. Differently, when considering specifically the indicator of purine oxidation (NSS_{FPG}), it was clear that this DNA damaging process was prevented by the algae supplementation since only occurred (as consequence of CP treatment) in fish fed with the standard diet (S_{CP}). This result is a clear evidence of an improved antioxidant defense promoted by algae supplementation. This is in agreement with previous studies reporting high antioxidant activity in *Fucus* spp. and *Ulva* spp. that presented high phenolic content and carotenoids, respectively (Pinteus et al., 2017), in *Gracilaria* spp. due to its content in sulfated polysaccharides with high content of phenolic compounds (Imjongjairak et al., 2015), as well as in all three algae species incorporated in the tested diet (Queiroz et al., 2014).

With the passage of time after injection, it was notorious that genotoxicity still occurs (at 10 days p.i.) in all groups injected with CP, considering the overall DNA damage (either non-specific or updated with oxidative lesions). Nevertheless, a positive interference of algae supplementation was perceptible since groups with an algae-enriched feed history (A_{CP} and A_{CP}/S) showed an improvement on their condition, translated in a lower extent of DNA damage, when compared to S_{CP} group. This beneficial effect is strengthened by the analysis of the temporal evolution of DNA damage from 3 to 10 days p.i., namely as $GDI_{EndoIII}$ and GDI_{FPG}, as a time related decline was observed in fish groups fed with the algae-enriched diet, though extensible to both CP treated and untreated groups. This particular result suggests an additional protection of algae in relation to the basal levels of DNA damage (i.e. in the absence of an identified exogenous insult).

It was evident that the anti-genotoxic action of algae supplementation gained preponderance in the course of time p.i., which can be explained by fish physiological adjustments and/or by a decline of CP internal levels of exposure. This can be regarded as an indication of a higher efficacy of algae components to cope with a moderate genotoxic challenge (rather than an extreme insult like that

posed by 40 mg kg^{-1} at 3 days p.i.), which is more in line with the extent of the genotoxic pressures likely to occur in rearing environment.

Analysing the DNA breaks at EndoIII- and FPG-sensitive sites ($NSS_{EndoIII}$ and NSS_{FPG}, respectively), at 10 days p.i., a surprising response profile was noticeable, since in both parameters lower levels of DNA oxidatively damaged were detected in S_{CP} and A_{CP}/S groups (comparing to the respective untreated group). Though this is not a classical pattern of response in genotoxicology, it has been often reported in the literature (e.g. Marques et al., 2014). According to Marques et al. (2014), it is plausible that under a low/moderate attack by genotoxicants, fish can trigger compensatory mechanisms able to prevent the DNA damaging effect of an external threat as well as of that caused by endogenous factors. Hence, two mechanisms can be hypothesized (alternatively or in combination): enhancement of oxidative DNA damage repair capacity and mobilization of the antioxidant system as a response to ROS over-generation, whose efficacy can be able to bring down the DNA oxidative damage below the control levels. Interestingly, the phenomenon above described was not so distinguishable in the fish group continuously fed with the algae-enriched diet (A_{CP}), suggesting that an additional protection provided by algae made unnecessary the fish adjustment hypothesized for the other CP treated groups. The increase of DNA breaks corresponding to net EndoIII- and FPG-sensitive sites observed from 3 to 10 days p.i. in unchallenged fish seems to be more pronounced in the group fed with the standard diet, which, in parallel with the opposite time-related variation displayed by CP challenged groups, seems to corroborate the previous hypothesis.

In an attempt to evaluate whether the potentially favorable effects of algae persist beyond the end of supplementation, fish fed with algae-enriched diet (for 30 + 3 days) were also submitted to a diet reversion to standard diet. Thus, the analysis of these particular results as overall DNA damage (non-specific and updated with oxidative lesions) revealed no repercussions of the feeding alteration, pointing out a persistence of algae components in fish body, or subsequent favorable biochemical milieu, for at least one week. Nevertheless, data concerning DNA breaks at EndoIII- and FPG-sensitive sites denunciated a dissimilar response profile when comparing groups always fed with algae-enriched diet and groups in which the standard diet was reset in the last 7 days. This can be an indication that algal components with oxidative DNA damage-protecting capacity substantially declined in a 7-day period without uptake. However, this temporal kinetics profile was not extensible to all the algal components involved in the anti-genotoxic action.

4.2. Protective effects of algae against chromosomal damage

Structural or numerical abnormalities of chromosomes are, by their nature, unlikely to be repaired, representing later and less transient alterations, comparing with those detected by comet assay (Guilherme et al., 2010). Consequently, an agent that causes DNA chain damage measurable through the comet assay does not necessarily causes clastogenic or aneugenic events, even though both techniques are complementary and yield similar variation among treatments (Martins and Costa, 2015). Accordingly, the present results as ENA frequency, after the first step of 30 days rearing and 3 days p.i., followed a pattern similar to that described for comet assay. Thus, after thirty days, the fish baseline condition was not affected by the diet, which was expectable, once the ENA baseline frequencies were low, comparing for instance with Teles et al. (2005) study, limiting the possibility to operate a reduction. Concerning three days p.i., it was evident the genotoxic capacity of CP, since it was observed a significant increase of ENAs frequency in both injected groups (S_{CP} and A_{CP}), regardless the diet. This finding is in agreement with Monteiro et al. (2011), who also detected a CP genotoxic effect, measured through ENA assay, in *Prochilodus lineatus*.

Taking into account ten days p.i., some noteworthy differences were perceptible in relation to the response profile described for comet assay. It was particularly relevant the observation that ENA induction by CP increased from 3 to 10 day p.i. in the group fed with standard diet (S_{CP}), pointing out a progression of the genotoxic effect, which didn't occur in the groups with algae-enriched feed history (A_{CP} and A_{CP}/S). Moreover, in A_{CP} group the ENA frequency returned to the levels corresponding to the untreated group, highlighting an effectiveness of algae supplementation on providing protection against the genotoxic pressure experimentally imposed. According to Marques et al. (2014b), and assuming morphologic nuclear anomalies as hardly reparable lesions, the recovery detected in A_{CP} group relies on the removal of erythrocytes containing abnormal nuclei and/or a dilution effect resulting from erythropoiesis (releasing new normal cells into circulation). These mechanisms were probably coupled with a strong reduction of new erythrocytes carrying abnormal nucleus.

A comparative analysis of the A_{CP}/S group with the other CP treated groups, an intermediate condition in terms of anti-genotoxic defense was perceived, since the chromosomal damage was still detectable but a significant improvement was provided compared to S_{CP} group. Besides reinforcing the protective action of algae supplementation, this analysis also indicates a clear decline of that protection as a consequence of the supplementation suppression.

Unlike the comet assay data, it may be noted that by the end of the experiment the fish basal condition (in the absence of an exogenous insult), measured as ENA frequency, was maintained overtime.

4.3. DNA and chromosomal damage *vs*. antioxidants system status

Cells evolved protective mechanisms that include some enzymatic antioxidants, *viz*. superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPx), as well as non-enzymatic factors such as reduced glutathione (GSH), that should maintain an equilibrium of intracellular ROS levels (Zanchi, 2014). When this balance is affected by endogenous or exogenous factors, oxidative stress emerges, affecting DNA and other key biomolecules and disturbing the physiological state of fish (Valon et al., 2013).

Taking into account that CP-induced DNA oxidative damage was signalized by the enzyme-modified comet assay, namely as purine oxidation at 3 days p.i., it would be expectable a correspondent reaction of the antioxidant system. However, blood antioxidants were completely irresponsive to CP exposure at that time. This seems to be in accordance with Ahmad et al. (2006), who stated by that the oxidative damage cannot be predicted only on the basis of antioxidant variations.

The unaltered levels of enzymatic antioxidant activities following algae supplementation is in line with previous studies involving the same algae species as dietary supplement in *S. aurata* (Queiroz et al., 2014) and *D. labrax* (Peixoto et al., 2016).

GSH is an important antioxidant involved in numerous cellular activities, including detoxification, antioxidant defense and maintenance of cellular redox status (Basu et al., 2015). Identically to enzymatic antioxidants, the studies above cited (Queiroz et al., 2014; Peixoto et al., 2016) reported unaltered GSHt content in association with algae supplementation.

Moreover, at 3 days p.i., fish reared with algae-enriched diet displayed lower levels of GSHt, comparing with the standard diet and irrespectively of CP treatment. A possible explanation for this alteration concerns a reduction of GSH synthesis by fish, as a counterbalance reaction to the exogenous source of other non-enzymatic antioxidants provided by algae supplementation, namely vitamins. Supporting this hypothesis, *Gracilaria changgi* showed to contain high levels of vitamin C (Norziah and

Ching, 2000), vitamins A and E were abundant in *Fucus spiralis* (Paiva et al., 2014) and *Ulva rigida* was rich in vitamin E (Yildiz et al., 2012).

Nevertheless, this hypothesis was not confirmed at 10 days p.i., since a significant increase of GSHt content was observed in A_{CP}/S group comparing to A/S.

Concerning the enzymatic activities modulation at 10 days p.i., it is noteworthy that only GST revealed significant alterations. GSTs are an evolutionarily conserved enzyme important in the detoxification of numerous xenobiotic compounds. These enzymes catalyze the conjugation of GSH to electrophilic substrates, thus producing compounds that are generally less reactive and more soluble. This facilitates the removal of these compounds from the cell via membrane-based GSH conjugate pumps. The broad substrate specificity of GST allows them to protect cells against a wide range of toxic chemicals (Gumulec et al., 2013). Apart from their essential functions in intracellular transport and the biosynthesis, GSTs have a critical role in defense against oxidative damage and peroxidative products of DNA and lipids (George, 1994).

Hence, a marked GST induction was currently observed in response to CP treatment, but only perceptible in S_{CP} and A_{CP}/S groups. This is a clear evidence of lower oxidative pressure occurring in the fish group continuously fed with algae-enriched diet. The supplementation suspension for 7 days showed again to have a negative impact on the fish condition, here expressed as a need to keep GST activity upregulated. This GST response pattern fits well on the genotoxicity endpoints profile, being particularly in line with the DNA oxidative damage ($NSS_{EndoIII}$ and NSS_{FPG}). Therefore, lower levels of DNA bases oxidation coincided with higher levels of GST activity, as an expression of defense processes mobilization (augmented biotransformation/detoxification activity and/or increased antioxidant protection), also corroborating the hypothesis raised in point 4.1.

5. Conclusions

Providing an answer to the central goal of the present thesis, it was demonstrated that a macroalgae-enriched diet supplemented with *Ulva* spp. (Chlorophyta), *Gracilaria* spp. (Rhodophyta) and *Fucus* spp. (Phaeophyta), in a total percentage of 5 %, exhibits anti-genotoxic properties in gilthead seabream (*Sparus aurata*) blood cells.

This protective action of macroalgae was apparent in relation to a primary and reparable damage (DNA strand breaks) and to a potentially more permanent damage (chromosomal lesions), though it appeared more pronounced in the latter type of genotoxicity expression. Unsurprisingly, benefits were mostly expressed in relation to an exogenous genotoxic challenge (cyclophosphamide - CP), rather than in relation to a baseline genomic integrity.

A clear oxidative DNA damage-protecting activity was displayed, particularly in the presence of a strong genotoxic insult occurring three days after CP injection, when purine oxidation was prevented by algae supplementation. Nonetheless, blood antioxidants were not altered by the supplemented diet, with the exception of GST activity that was induced as response to CP treatment, but only in S_{CP} and A_{CP}/S groups. This was a clear evidence of a lower oxidative pressure occurring in the fish group continuously fed with algae-enriched diet.

Clarifying if the favourable effects of algae persist beyond the end of supplementation, it was demonstrated that 7 days without uptake was enough to partially reduce the protection efficacy, namely in what concerns the algal components with oxidative DNA damage–protecting capacity.

Overall, these results seem to be promising towards the benefits of macroalgae inclusion in fish diet, offering a potential strategy to strengthen fish fitness, and thus, to invigorate aquaculture activity (both algae and fish cultivation), also providing new insights on the mechanisms of DNA protection in fish.

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7. Attachments

Table 3: Mean frequencies (%) of each DNA damage class and sub-total of damaged nucleoids (± standard error), measured by comet assay, in peripheral blood cells of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning). Statistically significant differences ($p < 0.05$) are: (*) between groups within the same sampling moment; (a) concerning the preceding sampling moment and the corresponding group. S = standard feed; A = algae-enriched feed; A/S corresponds to a diet reversion from standard to algae-enriched feed; CP = cyclophosphamide; p.i. = post-injection.

		DNA Damage Classes					
	Exposure Conditions	0	1	2	3	4	Sub-total (2+3+4)
	t_0	0.00±0.00	20.63±2.36	39.13±2.31	29.25±2.42	11.00±2.14	79.38±2.36
30 days	S	0.00±0.00	24.00±1.62	35.67±1.52	29.83±1.34	10.50±1.63	76.00±1.62
30 days	A	0.00±0.00	26.67±1.76	33.67±1.74	28.00±2.47	11.67±1.02	73.33±1.76
3 days p.i.	S	0.00±0.00	15.10±1.42[a] *	34.60±2.59	32.40±1.87	17.90±2.64 *	84.90±1.42[a] *
3 days p.i.	S_{CP}	0.00±0.00	0.00±0.00	29.67±1.19	40.44±1.63	29.89±1.75	100.00±0.00
3 days p.i.	A	0.00±0.00	15.54±1.09[a] *	36.46±1.73	33.00±1.09 *	15.00±1.79 *	84.46±1.09[a] *
3 days p.i.	A_{CP}	0.00±0.00	0.00±0.00	28.71±1.54	45.14±1.69	26.14±1.64	100.00±0.00
10 days p.i.	S	2.30±0.80[a] *	17.10±2.20 *	44.20±1.14 *	26.00±1.43 *	10.40±1.62 *	80.60±2.03 *
10 days p.i.	S_{CP}	0.00±0.00	0.00±0.00	27.44±2.19	43.00±1.47	29.56±2.62	100.00±0.00
10 days p.i.	A	2.63±0.58[a] *	25.25±1.18 * *	42.38±1.52	19.88±1.57[a] *	9.88±0.78 *	72.13±0.69[a] * *
10 days p.i.	A_{CP}	0.00±0.00	7.75±1.51[a]	37.88±1.32	38.13±2.10	16.25±2.71[a]	92.25±1.51[a]
10 days p.i.	A/S	1.88±0.67[a] *	22.75±1.53 *	44.50±2.62	23.50±2.03[a] *	7.38±1.31 *	75.38±1.45[a] *
10 days p.i.	A_{CP}/S	0.00±0.00	6.50±2.17[a]	34.00±3.48	38.88±3.28	20.63±2.67	93.50±2.17[a]

Table 4: Mean frequencies (%)of each DNA damage class and sub-total of damaged nucleoids (± standard error), measured by the comet assay including the incubation with the FPG enzyme, in peripheral blood cells of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning). Statistically significant differences ($p < 0.05$) are: (*) between groups within the same sampling moment; (a) concerning the preceding sampling moment and the corresponding group. S = standard feed; A = algae-enriched feed; A/S corresponds to a diet reversion from standard to algae-enriched feed; CP = cyclophosphamide; p.i. = post-injection.

		GDI_{FPG} - DNA Damage Classes					
	Exposure Conditions	**0**	**1**	**2**	**3**	**4**	**Sub-total (2+3+4)**
	t_0	0.00±0.00	20.75±2.11	35.50±1.90	28.38±2.36	15.38±1.59	79.25±2.11
30 days	S	0.00±0.00	19.00±1.75	38.33±2.65	31.50±2.82	11.17±1.95	81.00±1.75
	A	0.00±0.00	20.17±1.28	38.33±2.75	28.33±2.43	13.17±1.62	79.83±1.28
3 days p.i.	S	0.00±0.00	15.20±1.44 *	25.40±1.80[a] *	37.30±1.98	22.10±2.10[a] *	84.80±1.44 *
	S_{CP}	0.00±0.00	0.00±0.00	15.67±1.04	44.78±2.14	39.56±2.93	100.00±0.00
	A	0.00±0.00	11.62±1.23[a] *	32.77±1.60 *	34.92±1.36	20.69±1.09[a] *	88.38±1.23[a] *
	A_{CP}	0.00±0.00	0.00±0.00	22.79±1.16	41.29±1.46	35.93±1.46	100.00±0.00
10 days p.i.	S	0.00±0.00	11.60±1.83 *	36.60±2.41[a] *	32.50±2.19 *	19.30±1.90 *	88.40±1.83 *
	S_{CP}	0.00±0.00	0.00±0.00	20.56±1.26	43.56±1.69	35.89±1.63	100.00±0.00
	A	0.00±0.00	17.13±1.65 *	44.25±1.88 *	28.00±1.36	10.63±0.97[a] *	82.88±1.65 *
	A_{CP}	0.00±0.00	10.38±1.85[a] *	28.50±1.21 *	34.25±2.50 *	26.88±2.20	89.63±1.85[a] *
	A/S	0.00±0.00	17.50±2.15 *	39.50±2.28	29.00±2.15	14.00±1.50 *	82.50±2.15 *
	A_{CP}/S	0.00±0.00	9.63±2.27[a] *	30.50±3.44[a]	29.50±2.05[a]	30.38±2.98	90.38±2.27[a] *

Table 5: Mean frequencies (%)of each DNA damage class and sub-total of damaged nucleoids (± standard error), measured by the comet assay including the incubation with the EndoIII enzyme, in blood cells of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning). Statistically significant differences ($p < 0.05$) are: (*) between groups within the same sampling moment; (a) concerning the preceding sampling moment and the corresponding group. S = standard feed; A = algae-enriched feed; A/S corresponds to a diet reversion from standard to algae-enriched feed; CP = cyclophosphamide; p.i. = post-injection.

		$GDI_{EndoIII}$ - DNA Damage Classes					
Exposure Conditions		0	1	2	3	4	Sub-total (2+3+4)
	t_0	0.00±0.00	21.13±2.27	33.63±2.31	29.38±2.69	15.88±1.41	78.88±2.27
30 days	S	0.00±0.00	16.83±0.96	40.33±2.06	31.00±1.53	11.83±0.98	83.17±0.96
	A	0.00±0.00	18.50±1.33	39.67±2.41	29.67±2.02	11.83±1.19	81.17±1.12
3 days p.i.	S	0.00±0.00	13.10±1.28 *	28.50±2.16[a] *	35.80±1.95	22.60±2.25[a] *	86.90±1.28 *
	S_{CP}	0.00±0.00	0.00±0.00	16.33±1.50	43.89±1.25	39.78±2.16	100.00±0.00
	A	0.00±0.00	12.31±1.11[a] *	32.54±1.89 *	34.62±1.50 *	20.54±1.84[a] *	87.69±1.11[a] *
	A_{CP}	0.00±0.00	0.00±0.00	22.00±0.97	41.93±0.75	36.07±0.99	100.00±0.00
10 days p.i.	S	0.00±0.00	10.00±1.81 *	38.00±2.12 *	34.50±1.54	17.50±1.99 *	90.00±1.81 *
	S_{CP}	0.00±0.00	0.00±0.00	23.11±1.26[a]	42.00±1.90	34.89±2.52	100.00±0.00
	A	0.00±0.00	15.75±1.18 *	46.25±1.72[a] *	27.88±1.28 *	10.13±1.45[a] * *	84.25±1.18 *
	A_{CP}	0.00±0.00	9.63±1.56[a]	29.75±1.79	38.88±3.33	21.75±2.12[a]	90.38±1.56[a]
	A/S	0.00±0.00	13.25±1.39 *	41.88±1.67[a] *	32.88±1.91	12.00±2.31 *	86.75±1.39 *
	A_{CP}/S	0.00±0.00	5.63±1.98	28.38±1.95	41.25±1.84	24.75±2.46[a]	94.38±1.98

Table 6: Mean frequency (‰) of each nuclear abnormality category (± standard deviation) in peripheral erythrocytes of *S. aurata* at the different sampling moments (t_0 corresponds to sampling just before the experiment beginning). Statistically significant differences ($p < 0.05$) are: (*) between groups within the same sampling moment; (a) concerning the preceding sampling moment and the corresponding group. S = standard feed; A = algae-enriched feed; A/S corresponds to a diet reversion from standard to algae-enriched feed; CP = cyclophosphamide; p.i. = post-injection.

		Nuclear Abnormalities Categories					
Exposure Conditions		Kidney Shaped (K)	Segmented (S)	Lobed (L)	Vacuolated (V)	Sub-total (K+S+L+V)	Micronuclei (MN)
	t_0	10.17±0.91	1.83±0.17	9.00±1.48	0.00±0.00	21.00±1.53	0.00±0.00
30 days	S	9.83±0.98	3.17±1.25	10.17±1.58	0.00±0.00	23.17±2.26	0.00±0.00
	A	8.00±1.24	3.00±0.68	9.33±0.67	0.00±0.00	20.33±1.93	0.00±0.00
3 days p.i.	S	12.63±0.68	3.75±0.37	12.63±1.38	0.00±0.00	29.00±1.80 *	0.00±0.00
	S_{CP}	16.25±1.45	7.88±1.33	17.13±1.52	0.13±0.13	41.38±2.07	0.00±0.00
	A	8.36±0.63	3.86±0.48	11.50±0.84 *	0.00±0.00	23.71±1.58 *	0.00±0.00
	A_{CP}	13.38±0.60	6.77±0.70	16.92±1.03	0.00±0.00	37.08±1.46	0.23±0.12
10 days p.i.	S	8.63±0.94 *	3.75±0.49 *	9.00±0.68 *	0.00±0.00	21.38±1.83 *	0.13±0.13 *
	S_{CP}	18.38±1.97	9.88±1.06	24.50±1.21[a] *	0.13±0.13	52.88±1.91[a] *	1.50±0.46 *
	A	8.38±1.08 *	4.75±1.18	9.88±1.53 *	0.00±0.00	23.00±2.57 *	0.50±0.27 *
	A_{CP}	11.29±1.19	6.57±0.90	11.71±1.61 *	0.00±0.00	29.57±2.13 *	0.29±0.18 *
	A/S	7.25±0.56 *	4.00±0.65	11.50±1.60	0.00±0.00	22.75±1.85 *	0.00±0.00
	A_{CP}/S	13.57±1.13	7.86±1.64	13.86±1.90	0.00±0.00	35.29±3.28	0.43±0.43