Universidade de Aveiro Departamento de Biologia
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Ana Margarida Lourenço Silva Marques

Macroalgas marinhas como alimentos funcionais e potencial biotecnológico - promoção da integridade do ADN e mecanismos subjacentes

Marine macroalgae as functional food and biotechnological potential - DNA integrity promotion and underlying mechanisms

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Mário Guilherme Garcês Pacheco, Professor Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e co-orientação científica da Doutora Isabel O’Neill de Mascarenhas Gaivão, Professora Auxiliar do Departamento de Genética e Biotecnologia da Universidade de Trás-os-Montes e Alto Douro.

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“Ninguém é tão grande que não possa aprender,
nem tão pequeno que não possa ensinar.”

Esopo

o júri

presidente

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palavras-chave

Macroalgas marinhas, *Ulva rigida*, *Fucus vesiculosus*, *Gracilaria*, Aquacultura, Alimento funcional, Potencial genoprotetor

resumo

Atualmente, assiste-se a uma crescente procura por alimentos ou compostos bioativos com potencial para melhorar a saúde humana, evidenciando a importância de construir, a esse respeito, um conhecimento de vanguarda e cientificamente suportado. Neste contexto, as macroalgas marinhas constituem um dos mais promissores recursos naturais, tendo em conta, especialmente, a sua vasta biodiversidade e rico perfil fitoquímico. De facto, as macroalgas marinhas são, atualmente, classificadas como alimentos funcionais, tendo demonstrado possuir uma multiplicidade de propriedades benéficas, nomeadamente antiviral, antifúngica, antibacteriana, anti-hipertensiva, antidiabética, neuroprotetora, anti-inflamatória, imunomoduladora, entre outras. Além destas propriedades promotoras da saúde, as macroalgas marinhas são um elemento importante das dietas de vários países orientais, alguns deles revelando as mais elevadas taxas de esperança média de vida e baixa incidência de doenças crónicas relacionadas com a dieta (ex: doenças cardiovasculares e cancro), como o Japão e a Coreia do Sul. Apesar disto, apenas alguns estudos abordaram a sua capacidade protetora do genoma, fornecendo algumas pistas sobre as propriedades antioxidante, antigenotóxica e antimutagénica das macroalgas. Tendo em mente a importância da integridade e da estabilidade do genoma, assim como a ligação causal entre o dano no ADN e múltiplas doenças, esta tese foi alicerçada na premissa de que o consumo de macroalgas marinhas, como alimento integral, pode traduzir-se numa ação genoprotetora. Assim, o principal objetivo foi avaliar as propriedades das macroalgas marinhas *Ulva rigida* (alface-do-mar) *Fucus vesiculosus* (bodelha) e *Gracilaria* (cabelo-de-velha) em relação à promoção da integridade do ADN e os mecanismos subjacentes. Além disso, como objetivo secundário, pretendeu-se avaliar a genoproteção promovida por aquelas macroalgas marinhas quando incluídas nas dietas de peixes de aquacultura e de organismos-modelo de investigação focada no Homem, validando a sua assunção como ração/alimento funcional, especialmente, no contexto de aquacultura e de nutrição humana. Deste modo, foram delineados quatro ensaios experimentais e, em todos eles, a dieta dos respetivos organismos foi suplementada com as espécies de macroalgas marinhas descritas, ou individualmente ou em mistura das três espécies em partes iguais.

No primeiro ensaio, a dieta de dourada (*Sparus aurata*) foi suplementada com *U. rigida*, *F. vesiculosus* e *G. gracilis* de aquacultura (5% de suplementação) durante 60 dias, a que se seguiu o tratamento dos peixes com doses/intervalos realistas de dois medicamentos frequentemente adotados em aquacultura para prevenir e/ou tratar doenças, oxitetraciclina (OTC) e formalina (FOR). O dano genético foi avaliado pelo ensaio das anomalias nucleares eritrocíticas (ANE) e para clarificar a modulação da dinâmica da população eritrocítica, o índice de maturidade eritrocítica (IME) foi avaliado. A dieta suplementada com macroalgas evidenciou uma sólida genoproteção contra a genotoxicidade induzida pelos medicamentos OTC e FOR, e pelo genotóxico modelo, ciclofosfamida (CP). Além disso, OTC e FOR induziram uma instabilidade na população eritrocítica (traduzida num efeito de envelhecimento), a qual foi neutralizada pela suplementação das algas.

Por seu lado, *U. rigida*, *F. vesiculosus* e duas espécies de *Gracilaria*, *G. vermiculophylla* e *G. gracilis*, foram individualmente avaliadas através da sua incorporação na dieta da mosca-da-fruta (*Drosophila melanogaster*) a 2.5 e 5%, 1.25 e 5% e 1.25 e 10% de suplementação, respetivamente. Neste ensaio, as macroalgas incorporadas foram de aquacultura ou de colheita selvagem, de forma a avaliar a influência das condições de crescimento no potencial protetor do genoma. O potencial antigenotóxico das macroalgas contra o dano genético basal e induzido pela estreptonigrina foi avaliado através do teste de mutação somática e recombinação (SMART). As macroalgas marinhas mostraram aumentar a proteção do genoma, especialmente contra o dano genético induzido pela estreptonigrina. Enquanto a *U. rigida* revelou diferenças no potencial antigenotóxico associadas às condições de crescimento (maior ação genoprotetora promovida pela alga de aquacultura), *F. vesiculosus* mostrou respostas semelhantes entre as origens. Por seu lado, a *Gracilaria* mostrou indicações bastante contraditórias, uma vez que o nível mais baixo de suplementação aumentou a integridade do genoma, enquanto o mais alto mostrou sinais de toxicidade, especialmente para a espécie de colheita selvagem.

Além disso, no segundo ensaio com *D. melanogaster*, os dois lotes de *U. rigida*, de aquacultura e de origem selvagem (suplementação de 2.5 e 5%), confirmaram diferenças no potencial protetor do genoma, avaliado através do ensaio do cometa melhorado com a adoção das endonucleases glicosilase formamidopirimidina (FPG) e endonuclease III (EndoIII). Embora a *U. rigida* das duas origens tenha mostrado uma ação protetora do ADN, especialmente contra o dano genético induzido pela estreptonigrina, uma capacidade genoprotetora dependente da origem foi evidente, sendo que a alga de aquacultura demonstrou maior potencial. Isto pode ser atribuído ao perfil fitoquímico, determinado por análises de cromatografia de gás e cromatografia líquida de ultra e alta performance acopladas a espectrometria de massa, revelando diferenças na quantidade relativa de alguns fitocompostos, nomeadamente álcoois alifáticos, esteróis, sesquiterpenos e ésteres do glicerol.

No ensaio com murganhos (*Mus musculus*), o potencial antigenotóxico das macroalgas de aquacultura *U. rigida*, *F. vesiculosus* e *G. gracilis* foi investigado, através dos ensaios do cometa e dos micronúcleos, considerando um consumo direto (5% de suplementação) ou uma ingestão indireta via peixe (*S. aurata*) (10% de suplementação) que foi previamente alimentado com uma dieta suplementada com algas. O consumo direto da mistura das macroalgas promoveu a genoproteção contra o dano genotóxico induzido pelo metanossulfonato de metilo, além de um estado pró-oxidante mais reduzido, possivelmente envolvendo substâncias desmutagénicas. Por outro lado, a hipótese relativa à transferência das propriedades genoprotetoras das macroalgas via consumo de peixe alimentado com ração suplementada com algas não se confirmou. Ainda assim, a transferência de fitocompostos das macroalgas é plausível, particularmente considerando a promoção de uma menor condição pró-oxidante.

Esta tese aporta novas perspetivas acerca das propriedades benéficas das macroalgas relativamente à integridade do genoma. De facto, *U. rigida*, *F. vesiculosus* e espécies de *Gracilaria* demonstraram aumentar a proteção do genoma em peixe de aquacultura e em organismos-modelo para o Homem. No geral, as presentes conclusões transmitem novas evidências que poderão contribuir para o desenvolvimento das indústrias de algacultura e piscicultura, assim como para a redefinição dos hábitos nutricionais humanos, reforçando o conceito de alimento/ração funcional e subsequentes benefícios para a saúde.

keywords

Marine macroalgae, *Ulva rigida*, *Fucus vesiculosus*, *Gracilaria*, Aquaculture, Functional food, Genoprotective potential

abstract

Nowadays, there is an increasing demand for food products or bioactive compounds with potential to enhance human health, highlighting the importance of building a cutting edge and scientifically supported knowledge on that regard. In this context, marine macroalgae comprise one of the most promising natural resources, especially considering their large biodiversity and rich phytochemical profile. In fact, marine macroalgae are currently labelled as functional food, since they have demonstrated to hold a multiplicity of beneficial properties, namely antiviral, antifungal, antibacterial, antihypertensive, antidiabetic, neuroprotective, anti-inflammatory, immunomodulatory, among other effects. Besides these health-promoting properties, marine macroalgae are an important element of the diets of several Eastern countries, some of them depicting the highest life expectancy rates and low incidence of diet-related chronic diseases *(e.g.* cardiovascular diseases and cancer), namely Japan or South Korea. Despite that, there are only a few studies addressing their genome protective capacity, providing some clues concerning macroalgae antioxidant, antigenotoxic and antimutagenic properties. Bearing in mind the importance of the genome integrity and stability, as well as the causal linkage between the DNA damage and multiple diseases, this thesis was built up on the hypothesis that marine macroalgae consumption, as whole food, could be translated on a genoprotective action. Hence, the main goal was to address the properties of the marine macroalgae *Ulva rigida* (sea lettuce), *Fucus vesiculosus* (bladderwrack) and *Graciliaria* (ogonori) towards the DNA integrity promotion and underlying mechanisms. Moreover, as a secondary objective, it was intended to assess the genoprotection afforded by those marine macroalgae included in the diets of farmed fish and human driven models, validating their concept as functional feed/food, especially, in the context of aquaculture and human nutrition. Therefore, four experimental trials were delineated and, in all of them, the respective organisms' diet was supplemented with the described marine macroalgae species, either individually or in a mix with equal parts of the three species.

In the first trial, the gilthead seabream (*Sparus aurata*) diet was supplemented with aquacultured *U. rigida*, *F. vesiculosus* and *G. gracilis* (5% of supplementation) for 60 days, followed by the treatment of fish with realistic doses/intervals of two aqua-medicines frequently adopted in aquaculture to prevent and/or treat diseases, oxytetracycline (OTC) and formalin (FOR). The genetic damage was assessed by the erythrocytic nuclear abnormalities (ENA) assay and, to shed light on the modulation of the erythrocytic population dynamics, the erythrocyte maturity index (EMI) was also assessed. The macroalgae-supplemented aquafeed evidenced a solid genoprotection against the toxicity induced by the aqua-medicines OTC and FOR, and model genotoxicant, cyclophosphamide (CP). Moreover, OTC and FOR induced an erythrocyte population instability (translated into an aging effect), which was counteracted by the algae supplementation.

In turn, *U. rigida*, *F. vesiculosus* and two species of *Gracilaria*, *G. vermiculophylla* and *G. gracilis*, were individually tested through their incorporation on the fruit fly (*Drosophila melanogaster*) diet, at 2.5 and 5%, 1.25 and 5% and 1.25 and 10% of supplementation, respectively. In this trial, the macroalgae incorporated were from aquaculture or wild origin, to evaluate the influence of the growing conditions on the genome protective potential. The macroalgae antigenotoxic potential against the basal and streptonigrin-induced genetic damage was evaluated through the somatic mutation and recombination test (SMART). Marine macroalgae showed to enhance genome protection, especially against the genetic damage induced by streptonigrin. While *U. rigida* revealed differences on the antigenotoxic potential associated with the growing conditions (higher genoprotective action afforded by the aquacultured alga), *F. vesiculosus* depicted similar responses between origins. In turn, *Gracilaria* showed rather contradictory indications, since the lowest supplementation level enhanced the genome integrity, while the highest showed toxicity signals, especially the wild-harvested species.

Furthermore, in the second trial with *D. melanogaster*, the two *U. rigida* batches from aquaculture and wild origin (2.5 and 5% supplementation) confirmed to hold differences on the genome protective potential, evaluated through the comet assay improved with the adoption of the endonucleases formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (EndoIII). Although *U. rigida* from both origins showed a DNA-protective action, especially against the streptonigrin-induced genetic damage, an origin-based genoprotection capacity was evident, since aquacultured alga demonstrated higher antigenotoxic potential. This may be attributed to the phytochemical profile, determined through gas chromatography- and ultra-high-performance liquid chromatography-mass spectrometry analyses, depicting differences on the relative quantity of some phytocompounds, namely fatty alcohols, sterols, sesquiterpenoids and glycerol esters.

In the mice (*Mus musculus*) trial, the antigenotoxic potential of aquacultured macroalgae *U. rigida*, *F. vesiculosus* and *G. gracilis* was investigated, through the comet and the micronucleus assays, considering either a direct consumption (5% supplementation) or an indirect intake *via* fish (*S. aurata*) (10% supplementation) that was previously fed with an algae-supplemented diet. The direct consumption of the macroalgae mixture allowed the genoprotection against the methyl methanesulfonate-induced genotoxic damage, besides the accomplishment of a more favourable oxidant status, thus possibly involving desmutagens substances. In turn, the hypothesis concerning the transference of macroalgae genoprotective properties *via* consumption of fish fed with an algae-supplemented feed was not confirmed. Nevertheless, the transference of the macroalgae phytocompounds is plausible, particularly considering the achievement of a lower pro-oxidant challenging condition.

This thesis carries new perspectives regarding the marine macroalgae beneficial properties towards the genome integrity. In fact, *U. rigida*, *F. vesiculosus* and *Gracilaria* species revealed to enhance the genome protection both in farmed fish and human driven models. Overall, the present findings convey new evidences likely to contribute to the development of algaculture and pisciculture industries, as well as to the redefinition of human nutritional habits, reinforcing and validating the concept of marine macroalgae as functional food/feed and subsequent health benefits.

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Chapter 1

General introduction

1. General introduction

1.1. Marine macroalgae ecology, diversity and biology

The oceans cover more than 70% of the earth surface and are the cradle of life, being the habitat of multiple and diversified groups of organisms (Tunnicliffe and Scheersoi 2014). Marine macroalgae, also known as seaweeds, are a vast group of multicellular, eukaryotic, photosynthetic organisms, which are an important source of food and/or shelter for diverse species of fish, shellfish, and other invertebrate species (Millar 2011; Florez et al. 2017). Macroalgae are important primary producers, work as nurseries for juvenile species and are, to some extent, major reef formers, thus playing additional and crucial ecological roles (Diaz-Pulido and McCook 2008; Millar 2011). For example, kelp (order Laminariales) forests are one of the richest, most productive, dynamic and ecologically significant ecosystems on Earth (Mann 1973; Science Direct 2019).

Algae classification has experienced great changes over the last thirty years and still no general scheme is accepted by all phycologists (Pereira and Neto 2014). Nevertheless, even though marine macroalgae share many ecological characteristics, they are primarily distinguished in three evolutionary clades (Graham et al. 2009; Keith et al. 2013), which are based on the colour of their thalli: phyla Chlorophyta (green algae), Rhodophyta (red algae) and Ochrophyta (brown algae) (Guiry and Guiry 2019). Among the great diversity of marine macroalgae, about 11,000 species are described, the most wider group being the Rhodophyta, with over 7,200 species, while there are about 2,000 of brown species (*e.g.* Figure 1.1) and more than 1,800 species of green macroalgae (Guiry and Guiry 2019).

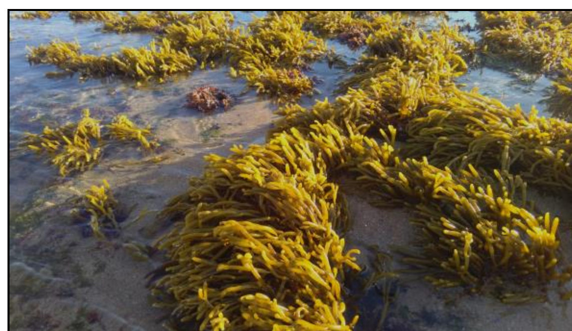

Figure 1.1. An example of Ochrophyta (*Bifurcaria bifurcata*) located at the seashore in a Portuguese beach.

Marine macroalgae, which size can range from a few millimetres to several metres, can be found in coastal areas all over the world, in different climatic zones, from the tropics to the polar regions (Mouritsen et al. 2013). In fact, macroalgae distribution is

highly dependent upon several physical (substrate, temperature, light quality and quantity, dynamic tidal activity, winds and storms), chemical (salinity, pH, nutrients, gases and pollution level), as well as biological (herbivores, microbes, epiphytes, endophytes, symbionts, parasites and diseases) factors (Fleurence and Levine 2016). These organisms obtain the nutrients from the surrounding water, relying on the continual water motion for their uptake (Fleurence and Levine 2016) and usually possess strong holdfasts, by which they are anchored to the seabed or strong substratum, such as reef, rocks, shells, among other objects, generally on the intertidal and subtidal zones (Millar 2011).

Regardless of their name and physical resemblance, seaweeds strongly differ from plants, particularly concerning their functional structures: they do not have plant-like roots (the previously mentioned holdfasts are only for physical anchoring), leaves or stems, nor do they bloom, produce seeds or fruits (Mouritsen et al. 2013).

Some macroalgae present a simple vascular system to transport nutrients and photosynthesis products, although the majority are undifferentiated and, in this case, each cell is responsible for producing what is essential (Mouritsen et al. 2013). Their cells are surrounded by a wall produced by the Golgi apparatus, that generally has a fibrillate appearance, consisting of cellulose and often containing polysaccharides, forming an amorphous mucilage. These cells present numerous organelles, among which the mitochondria, chloroplasts and nucleus present a double membrane. Photosynthetic pigments are in the thylakoids, inside the chloroplasts, where carbon dioxide fixation occurs, similarly to the land plants. In addition to chlorophylls, macroalgae present photosynthesis auxiliary pigments, the carotenoids, which can be of two different types: free oxygen or hydrocarbon carotenes (*e.g.* β-carotene) and their oxygenated derivatives called xanthophylls (*e.g.* fucoxanthin). Besides those, in Rhodophyta, thylakoids are also associated with phycobilisomes, where phycobilins (mainly phycoerythrin and phycocyanin) are contained, working also as photosynthesis auxiliary pigments (Pereira and Neto 2014).

Furthermore, when compared to vascular plants, macroalgae have rather complex life cycles and a wide variety of reproduction modes (Diaz-Pulido and McCook 2008). Their reproduction can involve either exclusively sexual or asexual phases, while some species display an alternation of generations, involving both in succession (Mouritsen et al. 2013). Sexually reproductive macroalgae produce gametes (egg and sperm cells) with a single set of chromosomes, while, in the asexual phase, spores (propagules) are produced containing two sets of chromosomes (Mouritsen et al. 2013). Moreover, some species can also reproduce asexually by vegetative spread and/or fragmentation, when the algae shed

small pieces that develop into completely independent new organisms (Diaz-Pulido and McCook 2008; Mouritsen et al. 2013). While the asexual reproduction allows a faster propagation, it brings a limitation regarding the genetic variability. On the other hand, sexual reproduction ensures the continuously genetic variability, yet with the comprehensible match-making problem, since the egg and sperm cells need to find each other in an environment habitually turbulent (Mouritsen et al. 2013). To overcome this limitation, some species developed reproductive cells with light-sensitive eyespots or with flagella so they can swim, while others make use of pheromones, secreted and released by egg cells, which serve to attract the sperm (Mouritsen et al. 2013). There are also some species (*e.g.* the large seaweed masses in the Sargasso Sea) that secrete enormous quantities of slime, ensuring that egg and sperm cells remain close to each other and do not disperse (Mouritsen et al. 2013).

1.2. Historical concepts, production and general applications of marine macroalgae

The growing of the social interest on the so-called terms of “blue economy” and “blue growth” placed the oceans and their inhabiting organisms under the spotlight for bioprospecting activities and among the numerous marine organisms disclosing a great potential, macroalgae are one of the most versatile resources. Currently, at least 221 species are of commercial interest and about 10 species are intensively cultivated, encompassing brown, red and green macroalgae (FAO 2018). Global marine macroalgae industry is worth more than USD 6 billion per annum and by 2015, the total production achieved more than 30 million tonnes, from which only 3% are wild harvested, while the majority are produced by aquaculture (FAO 2018). The leading producer countries are Chile, China and Norway for wild species (dominant species is *Lessonia nigrescens* or Chilean kelp), and China, Indonesia, South Korea and Philippines for cultured species (dominant genus is *Eucheuma* or gusô) (FAO 2018).

Macroalgae applications can be grossly categorized into human and animal nutrition field, agricultural and bioremediation purposes, industry, and biofuel production (Fleurence and Levine 2016) (Figure 1.2).

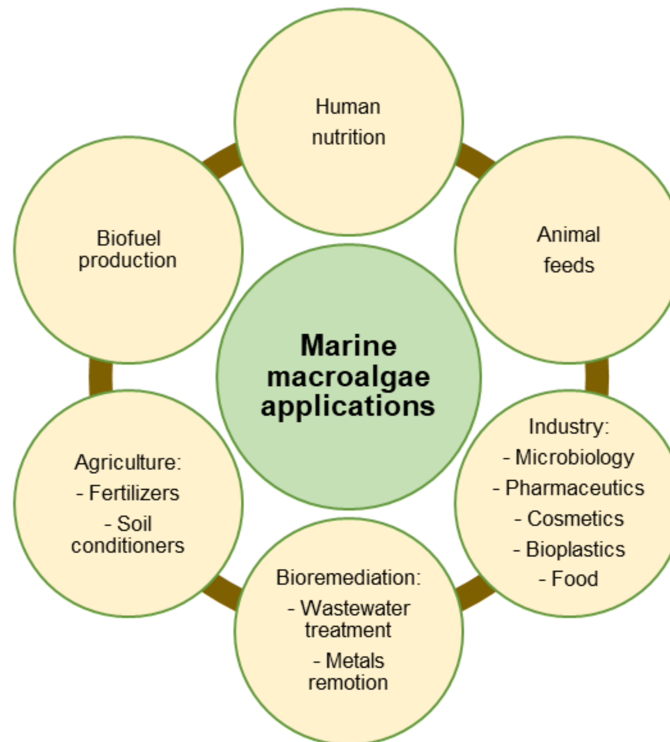

Figure 1.2. General applications of the marine macroalgae.

However, the utilization of marine macroalgae for a multiplicity of purposes is not recent. For instance, they have been used to feed livestock for thousands of years and have been mentioned as such in Ancient Greece and Icelandic sagas (Heuzé et al. 2017). In Iceland, marine macroalgae were often given to sheep, horses, and cattle, particularly when fodder was scarce for long periods (Fleurence and Levine 2016). Moreover, in the 19th and early 20th centuries, numerous reports refer to the occasional or systematic use of marine macroalgae to feed livestock in France (Brittany), in the Scottish islands (Lewis) and Scandinavia (Gotland, Norway, Finland), mostly to ruminants (including calves) and pigs (Heuzé et al. 2017). In fact, animals naturally grazed marine macroalgae on the shores on islands and other places with limited agriculture. Until today, the Orkney sheep in the North Ronaldsay Islands (Northern Scotland) are still grazing a diet almost exclusively based on marine macroalgae (Heuzé et al. 2017) (Figure 1.3). Moreover, marine macroalgae have been globally used as soil fertilizer and conditioner, especially brown algae as *Ascophyllum*, *Ecklonia* and *Fucus*. In Portugal, the use of *sargaço* or *moliço* as fertilizer for mainland agriculture has centuries of tradition (Pereira 2019). Macroalgae present a suitable content of nitrogen and potassium and their carbohydrates act as soil conditioners, improving aeration and soil structure (McHugh 2003), being

sometimes responsible to enable smallholders to produce quantities of subsistence crops beyond the normal capacities of their lands (Fleurence and Levine 2016).

Figure 1.3. Orkney sheep eating marine macroalgae in the North Ronaldsay Islands (Photo copyright: © Orkney.com/Colin Keldie).

The application of marine macroalgae on industry purposes have also an old tradition. The first recorded commercial use of marine macroalgae in Europe dates from the 17th century, when they were used to produce glass (*e.g.* France and Norway). Marine macroalgae could be burned to produce ash that would be applied as an ingredient in glass and soap manufacture. Also, in some areas of Europe, marine algae were used in housing construction (*e.g.* house roofing or furniture stuffing). In turn, the production of iodine from marine macroalgae constituted an important application until World War II, when chemical materials replaced algae, which lead to their harvesters and the processing industry to look for new uses for their products and the extraction of alginate emerged as a solution (Fleurence and Levine 2016). Alginate, along with agar and carrageenan, are water-soluble hydrocolloids (carbohydrates) extracted from various red and brown macroalgae used to thicken aqueous solutions, to form gels of varying degrees of firmness, and to stabilize some products. The gelling properties of agar were first discovered in Japan around 1658, but only in the 1930s, marine macroalgae started to have commercial importance as a source of hydrocolloids. Nowadays, they are used in a great diversity of applications from human and pet food to pharmaceutical and microbiological industries, textile and personal care products manufacturing, among others (McHugh 2003).

Furthermore, the utilization of marine macroalgae as medicine is an old tradition, dating back to as early as Greek and Roman eras (*e.g.* red algae used as anti-helminthic) or in China, where *Sargassum* was used as a treatment for goitre in the 16th century (due to its high content in iodine), or *Gelidium* for intestinal afflictions, and *Laminaria* for the

dilation of the cervix in difficult child births (Dawson 1966; Fleurence and Levine 2016). Nowadays, marine macroalgae have been targeted by pharmaceutical and medical industries, and researchers have been searching for new molecules of interest (Almeida et al. 2011). In fact, a multiplicity of algae bioactive compounds was already associated with several health assuring properties against several diseases (*e.g.* cancer, acquired immune-deficiency syndrome, inflammation, pain, arthritis, as well as viral, bacterial, and fungal infections) (Almeida et al. 2011), placing marine macroalgae under the nutraceutical and functional food categories, concepts further explored (see point 1.3).

In parallel with pharmaceutical industry, cosmetics is another demanding area of molecules with bioactive properties. Concomitantly, marine macroalgae are rich in bioactive compounds that could be exploited as functional ingredients for cosmetic applications. Macroalgae extracts have been used as active ingredients in slimming, moisturizing, antiaging, topical photoprotection and skin whitening cosmetical products, besides their utilization as a source of excipients (*e.g.* thickening agents), preservatives, dyes (*e.g.* carotenoids) and fragrances (Fleurence and Levine 2016).

Additionally, marine macroalgae have been found to possess advantages to face some ecological challenges. In the last decade, they are emerging as one of the most promising potential sources for biofuel production, application that is mostly driven by the necessity to find alternative sources to fossil fuels (Fleurence and Levine 2016). The fact that marine macroalgae are one important and abundant aquatic marine biomass, rich in carbohydrates, and which production is considered sustainable (with no need of agricultural inputs, such as pesticides, fertilizers, land or fresh water), makes them one of the most attractive renewable sources for a sustainable energy (Wargacki et al. 2012; Fleurence and Levine 2016).

Moreover, marine macroalgae can also be used on bioremediation activities. They can potentially be adopted in two general fields of wastewater treatment. First, they can be used on the treatment of sewage and some agricultural wastes to achieve the reduction of nitrogen- and phosphorus-based compounds, before the release of the treated waters into the rivers or oceans. On the other hand, marine macroalgae have the ability to remove toxic metals from industrial wastewaters, since they can accumulate copper, nickel, lead, zinc and cadmium (McHugh 2003).

Besides that, integrated multi-trophic aquaculture (Figure 1.4) has proven to be a valuable strategy to solve multiple issues at once. In this farming concept, marine macroalgae convert dissolved inorganic and suspended organic nutrients produced by aquaculture into additional crops (Abreu et al. 2009). Basically, it allows the treatment of

the aquaculture effluents potentially threatening to the environment (*e.g.* large amount of fish-excreted ammonia), being used by other species to their advantage, with a significant reduction in pollution (McHugh 2003).

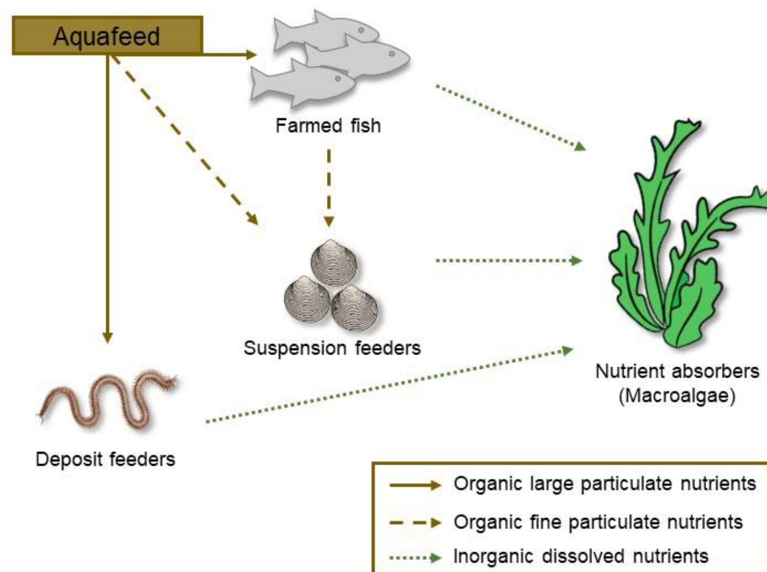

Figure 1.4. Conceptual diagram of the integrated multi-trophic aquaculture (IMTA).

Regardless these applications, the most common usage of the marine macroalgae is still the human consumption as food. The first record of macroalgae usage by man for consumption dates back 14,000 years in Monte Verde, Chile (Dillehay et al. 2008). In certain areas of Europe, mainly in the north, coastal populations were consuming marine macroalgae far back into history, particularly littoral communities suffering from famine. Furthermore, in Norway, *Palmaria palmata* has been used for human food since the Viking Era and in Ireland, *P. palmata*, *Chondrus crispus*, *Mastocarpus stallatus* and *Porphyra umbilicalis* were consumed by the coastal communities. Moreover, records confirm the consumption of macroalgae in Scotland and Wales around the 19th and 20th centuries. Paradoxically, the generalization of the non-native potato cultivation in all European countries decreased the risk of famine for those populations, but as a result, macroalgae use as vegetables in human nutrition became marginal. On the contrary, this is not the case in East Asian countries, where marine macroalgae consumption have been part of their diet since prehistoric times and remains extremely high (Fleurence and Levine 2016). For example, in Japan, remains of marine algae were found mixed with shellfish and fish in relics of aborigines on prehistoric archaeological sites and, at AD 701, the Law of Taiho confirmed the right of the Japanese to pay their annual taxes to the Emperor in

the form of marine macroalgae as *Laminaria*, *Undaria*, *Porphyra*, among other marine organisms (Nisizawa et al. 1987). In South Korea, fragments of brown macroalgae have been found in fossilized meals dating back 10,000 years (Pérez 1997). Nowadays, a great variety of macroalgae is consumed daily by South Korean populations and, in addition to their gastronomic qualities, they also hold a cultural importance. For example, dry sea mustard (*U. pinnatifida*) is offered to the goddess of childbirth and parturient woman along with prayers for the baby and mother health. South Korean newly mothers also consume sea mustard soup after childbirth, since it is believed that its content in calcium and iodine improves mothers' milk (Ii 1999; Fleurence and Levine 2016). The history of the utilization of marine macroalgae in China is one of the longest and most extensive of any country. Although the actual time they began to be consumed in China may not be identified, there are written records estimating their consumption for over 2,000 years (Xia and Abbott 1987). Along with Japan and South Korea, China completes the top 3 countries of the largest consumers of marine macroalgae as food (McHugh 2003). Globally, human consumption of marine macroalgae [about 150 species (Tiwari and Troy 2015)] includes raw products, such as in salads, soups, and main dishes, including sushi, as well as in processed form, namely food additives and flavourings, such as in chips and snacks (Fleurence and Levine 2016).

1.3. Marine macroalgae as functional foods within the context of human and animal nutrition and health

The concept behind functional foods is not new: "Let food be thy medicine and medicine be thy food" was affirmed by Hippocrates, considered the father of medicine, nearly 2,500 years ago (Hasler 1998). Yet, the term "functional foods" was first mentioned in the 1980's, in Japan, referring to some food products that could promote health benefits beyond the provision of basic nutrition (Stanton et al. 2005; Siró et al. 2008). Indeed, this is the simplest definition of functional foods. However, there have been several redefinitions throughout the years (Roberfroid 2002) and it is, actually, a quite controversial topic, adding to the differences on the legislation (or total absence) regarding this subject between countries (Siró et al. 2008). For instance, the Japanese government introduced, in 1991, rules for approval of a specific health-related food category called FOSHU (FOod for Specified Health Uses), including the establishment of specific health claims for this type of food (Kwak and Jukes 2001; Burdock et al. 2006; Siró et al. 2008). In the European Union, the definition of functional foods was established within the framework of the European research project "Functional Food Science in Europe"

(FUFOSE), but there are currently no regulations on that regard (Grochowicz et al. 2018). Moreover, functional foods may have two descriptions: (i) foods with proven and naturally-present health-promoting properties and (ii) altered food products that could be fortified or enriched with health-promoting ingredients (*e.g.* fruit juices fortified with vitamin C) or from which deleterious components were removed, reduced or replaced with other substances with beneficial effects (Grochowicz et al. 2018). In the context of this thesis, the term "functional foods" will be closer to the first description.

The historical utilization of functional food by the Eastern countries raised awareness for such products in places as Europe and the United States (Siró et al. 2008). Western contemporary generations are less willing to give in to the aging process and are actively engaged in preventive means through healthier lifestyles and dietary options, in an attempt to delay the inevitable onset of age-related diseases (Burdock et al. 2006). Also, experts in these countries realized that, besides being able to decrease the quite elevated cost of healthcare of the aging population (through improvement of the general conditions of the body, decrease of the risk of some diseases and even cure of some illnesses), functional food may also give a commercial potential for the food (Siró et al. 2008), as well as the nutraceuticals' industries (Burdock et al. 2006). In addition, the concept of nutrigenomics, as a comprehensive method to investigate the effect of nutrients on the body, gained great value, mainly in Japan, where it has been applied within the context of functional food science (Nakai et al. 2011). Indeed, nutrigenomics and related areas (*e.g.* nutriepigenetics) represent promising fields at the intersection of nutrition, medicine and individual genetics, contributing to the hopeful goal of achieving a personalized nutritional and medical approach.

In turn, despite the historical usage and ample research regarding marine macroalgae beneficial properties (*e.g.* Noda 1993; Patarra et al. 2011; Liu et al. 2012; Yende et al. 2014; Cornish et al. 2015; Fleurence and Levine 2016), their definition as functional food, defended by several authors, is relatively recent (Plaza et al. 2008; Holdt and Kraan 2011; Mohamed et al. 2012; Wells et al. 2017). Furthermore, diverse epidemiological studies highlighted an association between marine macroalgae consumption and lower incidence of certain illnesses, namely frequently diet related diseases as osteoporosis, diabetes, metabolic syndrome (*e.g.* dyslipidaemia, hypertension) and related cardiovascular disorders and cancer (Fleurence and Levine 2016). For instance, Japanese populations, whose diets include marine macroalgae as a significant element – 10 to 25% of food intake (Teas 1981; Skibola 2004), comparing to marginal consumption in Europe (Fleurence and Levine 2016) – show one of the longest average life expectancies in the

world (World Population Review 2019) and have shown low rates of cardiovascular diseases and cancer (Yamori et al. 2001; Yuan and Walsh 2006; Teas et al. 2013). Moreover, Japanese who have migrated to other regions of the globe, for example Brazil or USA, have depicted higher mortality rate from cardiovascular diseases and cancer (*e.g.* prostate, colon and breast cancers) and lower life expectancies than their counterparts living in Japan (Marmot and Syme 1976; Weisburger et al. 1980; Moriguchi et al. 2004). This fact suggests that these differences may be attributed to environmental factors, such as the healthier lifestyle and, particularly, to their diet, rather than to these populations' genetics (Shimizu et al. 1991; Yamori et al. 2001; Yuan and Walsh 2006). From Japanese, Okinawan people have shown similar or even higher average life expectancies and lower rates of the previously mentioned diseases (Yamori et al. 2001; Miyagi et al. 2003), highlighting the traditional "Okinawan diet" (which includes marine macroalgae as a significant element, alongside fish, sweet potato, spices and green leafy vegetables and is reduced in meat, refined grains, saturated fat, sugar, salt, and full-fat dairy products) as a role model diet with potential health-enhancing properties (Willcox et al. 2009).

Backing up the epidemiologic data, recent studies, through *in vivo* and *in vitro* trials, have confirmed those and disclosed additional beneficial properties of marine macroalgae (*e.g.* Mohamed et al. 2012; Fleurence and Levine 2016), assembling an innovative and scientifically supported knowledge regarding the concept of macroalgae as functional food and health promoter. Accordingly, marine macroalgae extracts or isolated compounds have also shown great neuroprotective activity against neuropsychiatric disorders (*e.g.* depression, anxiety and insomnia), as well as neurodegenerative diseases (*e.g.* Alzheimer's and Parkinson's), neuroinflammation, epilepsy or general pain (Mohamed et al. 2012; Fleurence and Levine 2016). Additionally, several studies have reported the macroalgae regeneration, repair and protection properties on organ or tissue regeneration (*e.g.* hepatoprotection or skin wound healing abilities) (Mohamed et al. 2012). Moreover, marine macroalgae have shown immunomodulatory properties, namely through antiallergic, anti-inflammatory and immunostimulant actions, as well as antimicrobial (*viz.* antiviral, antibacterial and antifungal) and antiparasitic activities (Mohamed et al. 2012; Fleurence and Levine 2016). In addition to those properties, these marine organisms can play a crucial role on modulating endocrine system, namely the thyroid activity, due to their high iodine content, which may have repercussions on different endocrine functions, besides being an important source of micro and macronutrients, minerals and vitamins, essential for growth and well-being (*e.g.* dietary fibre and enhanced gut health) (Mohamed et al. 2012; Fleurence and Levine 2016).

Similarly, although the concept of functional food in the context of animal feed is relatively underapplied and undervalued, it seems reasonable that marine macroalgae could be applied on the livestock, aquaculture and pet nutrition fields, to enhance the animals' general health status. As addressed before, marine algae have been used to feed animals for centuries, but during those times the primary reason was the survival of the horses, sheep and cattle, since the algae were used mainly when the fodder was scarce (Evans and Critchley 2014; Fleurence and Levine 2016). Nowadays, the producers' attention is focused on the animals' growth and feed utilization (*e.g.* weight gain and feed conversion rate), as well as the general health parameters that can, ultimately, affect production. In fact, there is a growing interest in the market for functional feeds, based on the principle of added-value linked to the health benefits of livestock (Tiwari and Troy 2015). Recently, research findings showed that animals' diet supplementation with macroalgae, as a source of macronutrients (carbohydrates, protein and fat) and micronutrients (as minerals and vitamins) may bring many benefits apart from the nutritional improvement (Tiwari and Troy 2015). For example, the supplementation of cattle diets with marine macroalgae or extracts improved their rumen fermentation and digestion, their immune and stress responses and influenced the carcass characteristics and the meat and milk quality (Braden et al. 2007; Bendary et al. 2013; Hwang et al. 2014). Additionally, the inclusion of macroalgae extracts on pigs' diet have shown to improve animals' feed intake, gut health and immune response (Turner et al. 2002; O'Doherty et al. 2010). Moreover, marine algae seem to help goats and sheep enhancing their immune system and stress response (Saker et al. 2004; de Lima et al. 2019) and to influence advantageously eggs' nutritional profile and laying hens performance in poultry farming (Michalak et al. 2011; Carrillo et al. 2012). In turn, pets can also benefit from algae or extracts supplementation on their diets, since algae can have not only advantageous effects on their health, but also on their external appearance (*e.g.* shiny hair) (Pulz and Gross 2011; McCusker et al. 2014; Tiwari and Troy 2015).

Still, one of the most promising areas involving marine macroalgae as animal feed is the aquaculture. Indeed, some of the aquatic animals raised in aquaculture naturally embrace macroalgae on their diets (Tiwari and Troy 2015). Being transversal to all animal industries, any feed supplement must attain certain parameters, namely respecting the nutritional composition and the presence of toxic elements. In the case of aquafeeds, protein and lipid contents are the most important factors, influencing the feasibility of a specific feed supplement (Tiwari and Troy 2015). Particularly, protein is considered one of the most expensive but important feed ingredients required for the animals' growth and its

content can reach 47% of the dry weight in macroalgae, while despite the lower amount in lipids (2-5% of the dry weight), much of this content is polyunsaturated fatty acids (PUFAs), essential elements for the growth of several marine animals (MacArtain et al. 2007; Tiwari and Troy 2015). Other macroalgae elements, such as polysaccharides, pigments, minerals and vitamins play also an important role on improving the nutritional status and growth, but also the overall animals' health (Tiwari and Troy 2015). Hence, several studies have demonstrated that the incorporation of marine macroalgae on fish diet may improve immune defence, tolerance against diverse stress factors and resistance to diseases. For example, the red sea bream (*Pagrus major*) diet supplementation with the macroalgae *A. nodosum*, *P. yezoensis*, or *Ulva pertusa* at 5% of the fish meal resulted in increased body weight, feed utilization, and muscle protein deposition in all three groups (Mustafa et al. 1995). Moreover, 5% incorporation of *Ulva* meal improved growth performance, feed efficiency, nutrient utilization, and body composition of Nile tilapia (*Oreochromis niloticus*) (Ergün et al. 2008). In addition, recent studies showed that the supplementation of European seabass (*Dicentrarchus labrax*) with *Ulva* spp., *Fucus* spp. and *Gracilaria* spp. up to 7.5% have no impact on growth performance, but generally improved the immune system and antioxidant responses (Araújo et al. 2016; Peixoto et al. 2016). These authors also demonstrated that *O. niloticus* can accept *Ulva* spp. in its diet up to 10% without influencing the growth performance or flesh organoleptic properties, but enhancing the innate immune response of the fish (Valente et al. 2016). Also, another study showed that feeding *Sparus aurata* with a supplementation of 10% of *Pterocladia capillacea* or 5% of *U. lactuca* enhanced fish growth performance, feed utilization, nutrient retention and survival, and 10% of *P. capillacea* improved fish stress response after a 5-minutes anoxia (Wassef et al. 2005).

On the other hand, the macroalga *P. purpurea*, included at 16 and 33% in the diets of mullet (*Chelon labrosus*), was found to suppress growth performance and feed utilization efficiency (Davies et al. 1997). Moreover, *S. aurata* fed with 7.5% of *Gracilaria* sp. supplementation displayed an elevation in lipid peroxidation (Queiroz et al. 2014) and *G. arcuata* and *G. vermiculophylla* also showed adverse effects at 20 and 30% on *Clarias gariepinus* diet (tolerating up to 10% of the alga) (Al-Asgah et al. 2016) and 10% on *O. niloticus* diet (Silva et al. 2015), respectively. Nevertheless, in these studies, the eventual adverse effects were attributed to antinutritional factors, as polysaccharides with low digestibility, highlighting that it is crucial to increase studies on this context, as well as to bear in mind to evaluate favourable but realistic algae concentrations to include in the animals' diets.

1.3.1. Linking marine macroalgae properties and phytochemical composition

The current attention devoted to marine macroalgae due to their beneficial properties is directly linked with their unique phytochemical composition. In fact, those organisms must face highly diverse and harsh environments, coping with rapid environmental changes, which contributes significantly to the variety and quantity of the bio-compounds and, even, the potential of their bio-activities (Tiwari and Troy 2015). For instance, the most abundant terrestrial bioactive compounds have three interconnected rings. In turn, algae phenolic compounds phlorotannins may have up to eight interconnected rings, making them between 10 and 100 times more powerful and more stable as free radical scavengers than other polyphenols (*e.g.* green tea catechins, have only four rings) (Mohamed et al. 2012). Furthermore, macroalgae phytochemical compounds vary with the alga species, the harvesting period, the geographic habitat, and environmental factors (*e.g.* water temperature, light intensity and salinity), as well as nutrients and minerals availability (Mabeau and Fleurence 1993; Marinho-Soriano et al. 2006; Marsham et al. 2007; Abreu et al. 2009; Tiwari and Troy 2015). Hence, bearing in mind the great taxonomic diversity of marine macroalgae, as well as their vast habitats, their phytochemical profile and possible applications may be considered an almost unlimited field regarding the exploration of functional ingredients (Tiwari and Troy 2015).

From a basic nutritional point of view, marine macroalgae contain a high concentration of proteins, polysaccharides (including dietary fibres), lipids, minerals, vitamins, pigments and polyphenols (Holdt and Kraan 2011). In this context, the phytocompounds must be available to exert their bioactivity following the algae ingestion, *i.e.*, they have to be susceptible to the digestion process to be bioavailable to consumers (Wells et al. 2017). Most marine macroalgae have a protein content as high as soybean, leguminous plants, or eggs (Fleurence and Levine 2016). In addition, marine algae have proved their value on human health because of their high content of essential amino acids (approximately 40% of total amino acids, whatever the phylum) (Fleurence and Levine 2016). Polysaccharides are a source of energy to humans and other animals, but some more complex polysaccharides are not digestible by the gut. This brings rather beneficial effects, since those soluble dietary fibres help slow down digestion and calorie absorption, decrease blood cholesterol levels and maintain stable glucose levels, contributing to lower risk of obesity, diabetes, metabolic syndrome and cardiovascular diseases, as well as lower risk of developing colon cancer (Patarra et al. 2011; Mohamed et al. 2012). Moreover, marine algae are rich in PUFAs of the *n*-3 and *n*-6 series (in higher amounts than land vegetables), which are considered essential fatty acids, since they are not

synthesized by mammals and must be taken *via* food chains (Fleurence and Levine 2016). The benefits of PUFAs, particularly eicosapentaenoic, arachidonic and docosahexaenoic acids (EPA, ARA and DHA, respectively) are vast and well-studied, including cardiovascular effects, decrease of blood pressure and improvement of heart and liver function (Fleurence and Levine 2016). In line, they have also shown anti-inflammatory, antithrombotic, immunomodulatory and antioxidant effects, besides their role in the prevention of several types of cancer, pre and postnatal development of the brain and the retina, regulation of membrane fluidity, electron and oxygen transport (Fleurence and Levine 2016). In addition, they also are precursors of the biosynthesis of several bioregulators, exerting important functions on many cellular processes, mitochondrial function, inflammation processes, immune reactions and skin growth and protection (Fleurence and Levine 2016). Sterols are a different class of lipids and, specifically, phytosterols are present in plants and marine algae. Phytosterols have shown to decrease blood cholesterol levels and to hold antifungal, antibacterial, anti-inflammatory, antitumor, antioxidant, and anti-ulcerative properties (Tiwari and Troy 2015). Marine algae are also rich in pigments, namely chlorophylls, fucoxanthin and phycoerythrin, which may vary according with the phylum, but generally display antioxidant, antiproliferative and cancer-preventive capacities (Holdt and Kraan 2011; Tiwari and Troy 2015; Fleurence and Levine 2016). Macroalgae have also a high content of minerals, some presenting larger amounts than terrestrial vegetables. For instance, marine algae are a natural and safe source of iodine, an important nutrient in metabolic and hormonal regulation (MacArtain et al. 2007). In turn, vitamins, essential micronutrients that an organism cannot synthesize and which must be obtained through the diet, are also present in favourable amounts in marine algae (MacArtain et al. 2007; Wells et al. 2017). These have crucial cellular functions, serve as precursors of essential enzyme cofactors and are needed for essential metabolic pathways. Vitamins deficiencies have been connected to several human diseases (*e.g.* scurvy and anaemia) and their inclusion have direct beneficial effects, such as antioxidant potential and reduction of cardiovascular diseases and cancer incidence, as well as ophthalmologic disorders (Mohamed et al. 2012; Wells et al. 2017). Phenolic compounds, as phlorotannins, present a strong reducing power, showing a significant radical scavenging activity (Holdt and Kraan 2011). They can work as preventive medicines for cardiovascular diseases, cancer, arthritis and autoimmune disorders by helping to protect tissues against oxidative stress (Holdt and Kraan 2011). In addition, polyphenols were found to be anti-inflammatory and have anti-allergic effect and antibacterial activity (Holdt and Kraan 2011).

Regardless of the apparent plethora of beneficial properties afforded by marine macroalgae, it is important to consider, as with any food or feed, some aspects concerning the safety about their consumption. In particular, the origin and/or growing conditions of the macroalgae should be contemplated. Indeed, an important issue regarding wild harvested macroalgae or from unknown origin relies on the possible absorption and contamination with toxic elements, namely metals, dioxins, pesticides, ammonia, radioactive isotopes or microplastics (van der Spiegel et al. 2013; Tiwari and Troy 2015; Gutow et al. 2016; Desideri et al. 2016). Additionally, some macroalgae hold a great iodine content and, if consumed in excess, this compound can be deleterious and contribute to thyroid-related disorders (Fleurence and Levine 2016). Besides that, some species may also possess antinutritional factors. For instance, there are reports suggesting some health complications came from direct consumption of species of the genera *Caulerpa*, *Gracilaria* and *Acanthophora*, which were associated to intrinsic compounds, as caulerpin and caulerpinic acid (genus *Caulerpa*) or prostaglandins and polycavernosides (genus *Gracilaria*) (Fleurence and Levine 2016). Moreover, carrageenan, a polysaccharide produced by red macroalgae, which is probably the most used macroalgae isolated product, has been in the centre of some controversy. Used for decades in the food and pharmaceutical industries as thickener, emulsifier or stabilizer, some studies have suggested it may contribute to harmful gastrointestinal disorders (*e.g.* Tobacman 2001), while recent review articles have been defended this food additive to be safe (*e.g.* McKim 2014; Weiner 2014).

Nonetheless, the number of harmful effects reports is small and occurring sporadically and, in most cases, the source of toxins involved is questionable and may not even be produced by the macroalgae, but instead produced by epiphytic cyanobacteria contaminants (Fleurence and Levine 2016), which toxicity is well documented (*e.g.* Carmichael 2001; Navarro et al. 2015). Besides, the treatment given to macroalgae before their consumption (*e.g.* washing, cooking method) should be also taken into consideration on these singular events (Fleurence and Levine 2016).

1.3.2. DNA integrity promoting actions

The importance of the genome integrity and stability is undeniable, since the DNA molecule carries the genetic information of each living cell and, thus, its fidelity plays a major key role in the balance between health and disease. However, the DNA molecule is under constant attack from both endogenous and exogenous (or environmental) sources of damage (Figure 1.5). Besides the direct effect on the DNA perpetrated by some agents,

reactive oxygen species (ROS) are formed as by-products of the action of those genotoxic compounds and due to endogenous cellular metabolism (Clancy 2008). At the DNA level, different genetic lesions may occur, namely base modifications (*e.g.* alkylation, oxidation or deamination), adducts or single and double strand breaks (Geacintov and Broyde 2010). Some of those DNA lesions occur through covalent binding of the genotoxicant to the DNA molecule. This susceptibility is explained by the fact that DNA has nucleophilic sites (negatively charged) easily attacked by electrophilic (positively charged) substances (Tretyakova et al. 2012). Moreover, substances can exert their genotoxicity through clastogenic and/or aneugenic mechanisms, *i.e.*, through the induction of structural breaks, resulting in fragments or entire chromosomes lost and effects at the mitotic spindle apparatus, respectively (Fenech 2000; Stoiber et al. 2004). In addition, some genotoxic compounds exert their effects also after metabolic activation (Pacheco and Santos 1998; Russo and Russo 2004). Any resulting damage, if not repaired, may lead to mutations or other permanent structural alterations, possibly developing into diseases (Clancy 2008). This could be due to the consequences of the DNA damage at the cellular level, namely protein dysfunction (leading to metabolic impairments, changes in biological specificity or protein turnover), oncogene activation or chromosomal aberrations (Shugart and Theodorakis 1996).

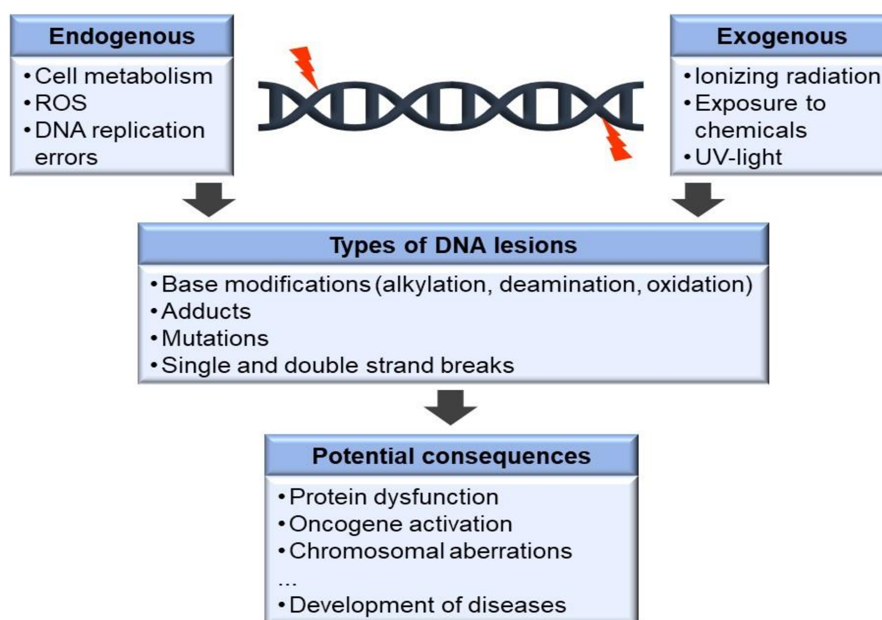

Figure 1.5. Potential sources of DNA lesions and consequences.

Furthermore, DNA damage results from a sensible balance between pro-genotoxic actions and DNA repair mechanisms. However, this is such a susceptible equilibrium,

since even DNA replication and DNA repair processes may lead to the induction of genetic damage *per se* (Negritto 2010). In fact, DNA single strand breaks may result from the DNA repair machinery attempting to repair an oxidized base, for instance, but can be repaired through DNA ligases action (Weaver 2008). In turn, DNA double strand breaks are considered a more severe and hard to repair type of damage, by which chromosomal parts or entire chromosomes could be disconnected from the remaining genetic material, possibly resulting in cell death or in the development of cancer cells (Weaver 2008). Hence, cells respond to DNA damage through reliable DNA damage response pathways, allowing lesion-specific DNA repair pathways to physically remove the damage in a substrate-dependent manner. At least five major DNA repair pathways – base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ) – may occur throughout different stages of the cell cycle, allowing the cells to repair the DNA damage and preventing the onset of diseases (Chatterjee and Walker 2017).

On the other hand, in the context of animal production, the commercial factor depends on the health, fitness and survival of the animals. In fact, the genetic damage can have a negative impact on the fish fitness, with subsequent repercussion on the productivity (Silva et al. 2011), mainly due to a higher energy expenditure related with DNA repair processes (Olson and Mitchell 2006) and homeostasis threatening (Pacheco and Santos 2002).

Interestingly, nutrition may simultaneously be a source of genotoxic substances (O'Brien et al. 2006), as well as antigenotoxic capable compounds (Izquierdo-Vega et al. 2017). Those chemicals, which may reduce the mutagenicity of physical and chemical mutagens are referred to as antimutagens. Yet, considering all mutagens are genotoxic, but not all genotoxic substances are mutagenic, those substances that reduce the DNA damage caused by genotoxic agents are also called antigenotoxic agents (Ferguson et al. 2004; De Flora and Ferguson 2005; Bhattacharya 2011; Izquierdo-Vega et al. 2017). Moreover, the antimutagenic substances have been classified either as desmutagens and bio-antimutagens (Izquierdo-Vega et al. 2017); the first group refers to substances that promote the elimination of the genotoxic compound from the organism, as well as agents that inactivate the mutagens partially or fully by enzymatic or chemical interaction before the mutagen attacks the genes (also considered as apparent antimutagens). In turn, the bio-antimutagens (or true antimutagens) can suppress the process of mutation after genes are damaged by mutagens, acting on the repair and replication processes of the

mutagen-damaged DNA, resulting in a mutation frequency decline (Ferguson et al. 2004; De Flora and Ferguson 2005).

In this context, marine macroalgae have been described as a potential source of genoprotection promoting substances. In fact, they have shown to possess antioxidant, antigenotoxic and antimutagenic properties in various studies (*e.g.* Athukorala et al. 2006; Yuan and Walsh 2006; Celikler et al. 2008; Celikler et al. 2009b; Zubia et al. 2009; Valentão et al. 2010). An enzymatic extract of *E. cava*, as well as its crude polysaccharide and crude polyphenolic fractions showed antioxidant and antiproliferative activity on various cancer cell lines (Athukorala et al. 2006). Moreover, a crude extract of *U. rigida* showed antigenotoxic potential against chromosome aberration, sister chromatid exchange and micronuclei induced by the mutagenic agent mitomycin-C (Celikler et al. 2008). The same research group demonstrated that an ethanolic extract of the same alga enhanced anti-hyperglycaemic and antigenotoxic capacities in diabetic rats (Celikler et al. 2009a), as well as the antigenotoxic ability on hypothyroid rats (Celikler et al. 2014). In addition, these authors also demonstrated that a crude ethanolic extract of *Codium tomentosum* efficiently reduced the genotoxic effects of mitomycin-C, ethyl methanesulfonate and H_2O_2 (chromosome aberrations, sister chromatid exchange and micronuclei) in human lymphocytes *in vitro* (Celikler et al. 2009b). A dried powder of *F. vesiculosus* depicted strong antioxidant activity on different *in vitro* antioxidant assays (Díaz-Rubio et al. 2009) and the pre-treatment of cultured human lymphocytes with an extract of *F. vesiculosus* revealed genoprotection properties against chromosome aberrations and DNA fragmentation induced by doxorubicin (Leite-Silva et al. 2007). Additionally, aqueous and ethanolic extracts of 16 marine macroalgae collected along the Danish coast globally revealed antioxidant activity on several *in vitro* antioxidant assays (Farvin and Jacobsen 2013). Moreover, extracts of *S. dentifolium* showed protective effects against cyclophosphamide-induced genotoxicity (chromosome aberrations, micronuclei and DNA fragmentation) induced in mice (Gamal-Eldeen et al. 2013). In turn, an aqueous-ethanolic extract of *U. fasciata* showed antioxidant and antigenotoxic effects against benzo[a]pyrene-induced micronuclei in mice (Rodeiro et al. 2015). Also, aqueous extracts of the edible *G. tenuistipitata* revealed *in vitro* protective capacity against H_2O_2-induced oxidative DNA damage (Yang et al. 2012). In turn, Yuan and Walsh (2006) determined the antioxidant and antiproliferative properties of *P. palmata*, *L. setchellii*, *Macrocystis integrifolia* and *Nereocystis leutkeana* methanolic extracts on HeLa cells. Crude extracts of some Ochrophyta collected in the Brittany coasts also revealed antioxidant and antiproliferative effects on three different cancer cell lines (Zubia et al.

2009). Despite some *in vivo* studies, the majority addresses *in vitro* effects and only with isolated compounds or extracts.

Likewise, marine macroalgae have been demonstrating antioxidant and antigenotoxic properties when incorporated on farmed fish diets. Particularly, a mixture of *Ulva*, *Fucus* and *Gracilaria* genera representative species enhanced the antioxidant capacity of *D. labrax* when incorporated in its feed (Peixoto et al. 2016). Moreover, a recent study highlighted the genoprotection enhancement of the same macroalgae mixture on *S. aurata*, particularly against erythrocytic nuclear abnormalities and DNA fragmentation induced by cyclophosphamide (Pereira et al. 2019).

1.3.3. Biotechnological potential

This thesis also encompasses facets of significant biotechnological potential. First, the possibility of modulating the marine macroalgae phytochemical composition through different growing conditions, namely in aquaculture context. As addressed before, it is clear the macroalgae phytochemical profile vary with several factors, namely the harvesting season, the geographic habitat, and environmental aspects, as the water temperature and salinity, light intensity and nutrients and minerals availability (Mabeau and Fleurence 1993; Marinho-Soriano et al. 2006; Marsham et al. 2007; Abreu et al. 2009; Tiwari and Troy 2015). Hence, it is undeniable that the algaculture industry can develop their technological and commercial potential, considering, for example, the eventual production of macroalgae with higher content of certain desired phytocompounds.

Furthermore, the manipulation of fish diet under farming conditions has been suggested as a mean to increase their nutritional value for human consumption (Bourre 2005). In fact, marine macroalgae may act as nutrients source in fish, which could ultimately function as a vehicle of the algae phytocompounds for humans (Valente et al. 2015). For instance, the inclusion of between 3% and 6% of the macroalga *M. pyrifera* significantly increased PUFAs levels in the muscle of rainbow trout (*Oncorhynchus mykiss*) and, for that reason, the final product was considered to be more beneficial for human health (Dantagnan et al. 2009). Moreover, the incorporation of *P. dioica* at 10% of the diet fed to this fish showed no negative effects on growth performance and was found to increase flesh pigmentation (Soler-Vila et al. 2009). Additionally, the incorporation of *Gracilaria* sp. at 5% in the same fish species diet lead to the fish nutritional value increase, namely their organoleptic characteristics, as well as iodine content, without compromising the animals' growth (Valente et al. 2015).

Besides that, the integrated multi-trophic aquaculture (as described before) presents itself as a valuable biotechnological and sustainable tool, promoting both algaculture, as well as fish farming industries.

1.4. Objectives, structure and research components of the thesis

The main goal of this thesis was to address the marine macroalgae properties towards the DNA integrity promotion and underlying mechanisms. In addition, as a secondary objective, it was intended to assess the genoprotection afforded by marine macroalgae included in the diets of farmed fish and human driven models, validating their concept as functional food and, specifically, in the context of aquaculture and human nutrition.

To achieve these general goals, the following specific objectives were considered:

i. To evaluate the potential of a mix of *U. rigida*, *F. vesiculosus* and *G. gracilis*, incorporated on the diet of fish (*S. aurata*), against chromosomal damage and erythrocytes population instability induced by aqua-medicines under an aquaculture context;
ii. To address the antigenotoxic potential of individually incorporated *U. rigida*, *F. vesiculosus* and *G. gracilis*, from two distinct origins, on *Drosophila melanogaster* diet against endogenous and exogenously induced genetic damage;
iii. To assess the antigenotoxic potential of *U. rigida*, from two distinct origins, relating its genome protection capacity with the respective phytochemical profile;
iv. To evaluate the genome protection promoted by a macroalgae mix (*U. rigida*, *F. vesiculosus* and *G. gracilis*) incorporated on mice (*Mus musculus*) diet, directly and indirectly (using *S. aurata* as a vehicle of algae phytocompounds), inferring about a possible transference of the antigenotoxic capacity;
v. To infer about general physiological status of *M. musculus* after the differential dietary background and eventual contribution of the antioxidant system modulation as a mechanism to accomplish genome protection.

Consequently, besides the present one, this thesis comprises 4 additional chapters (2-5), corresponding to articles published or submitted to publication:

Chapter 2 – Macroalgae-enriched diet protects gilthead seabream (*Sparus aurata*) against erythrocyte population instability and chromosomal damage induced by aqua-medicines;

Chapter 3 – Searching for antigenotoxic properties of marine macroalgae dietary supplementation against endogenous and exogenous challenges;

Chapter 4 – Comparative genoprotection ability of naturally growing (wild) and aquacultured *Ulva rigida* coupled with phytochemical profiling;

Chapter 5 – Marine macroalgae as a dietary factor promoting DNA integrity – Assessing potential benefits of direct consumption and indirect intake of algae-borne phytocomponents using fish as a vehicle;

as well as a general discussion (Chapter 6), integrating the previous chapters and presenting the final remarks.

The selection of the three marine macroalgae species adopted in this study, *U. rigida* C.Agardh, *F. vesiculosus* Linnaeus and *G. gracilis* (Stackhouse) Steentoft, L.M.Irvine & Farnham (common names – sea lettuce, bladderwrack and slender wart weed, or *alface-do-mar*, *bodelha* and *cabelo-de-velha*, in Portuguese, respectively), relied on: (i) the representativeness of each taxonomic group *i.e.*, Chlorophyta, Rhodophyta and Ochrophyta; (ii) the wide geographical distribution of these species, including their presence on the Atlantic coast; (iii) the easiness to cultivate them in aquaculture, particularly *U. rigida* and *G. gracilis*; (iv) being considered edible species and currently consumed; (v) having demonstrated several beneficial properties linked to their rich phytochemical composition. In two of the trials performed in the context of this thesis, wild-harvested and aquacultured algae specimens were evaluated in order to address the influence of the growing conditions on the genome protection ability, considering the knowledge about the modulation of the phytochemical profile of macroalgae according to the growing conditions (Marinho-Soriano et al. 2006; Abreu et al. 2009).

Gilthead seabream (*S. aurata* Linnaeus) was adopted as fish model species (Chapter 2). This species was chosen mainly because, though primarily carnivorous, it can be accessorily herbivorous, thereby accepting well macroalgae in its diet, besides being a highly and increasingly important commercial species in fisheries and aquaculture, mainly in Europe. For that reason, this species has been frequently adopted in diverse studies, namely on nutritional, stress response and general performance trials (*e.g.* Fountoulaki et al. 2009; Yildiz and Ergonul 2010; Queiroz et al. 2014).

D. melanogaster Meigen and *M. musculus* Linnaeus were also adopted in this thesis, as human driven models, on Chapters 3 and 4, and 5, respectively. The fruit fly is an intensively studied organism, adopted in diverse research areas, mostly due to a high conservancy, relatively to humans, of the molecular pathways required for the development of a complex animal (Mirzoyan et al. 2019). Thus, it serves as a model system for the investigation of several developmental and cellular processes, common to higher eukaryotes, including humans, being also adopted in genotoxicity/antigenotoxicity studies (*e.g.* Gaivão and Comendador 1996; Romero-Jiménez et al. 2005; Morais et al. 2016). Likewise, mouse has been used in multiple research areas for decades and the undeniable similarity of genome and molecular pathways with humans turns it into an excellent model organism to study complex human diseases or physiological responses to pharmaceuticals or diets (Perlman 2016).

To fulfil the goals previously mentioned, four experimental trials were designed and, in all of them, the respective organisms' diet was supplemented with the described marine macroalgae species, either individually or in a mix with equal parts of the 3 species. Furthermore, considering the thesis main goal regarding the conceptualization and investigation of marine macroalgae as functional food, all trials involved the algae as a whole food, not isolated compounds or extracts, as defended by Holdt and Kraan (2011). In those trials, specific genetic damage evaluation endpoints were used, namely the single cell gel electrophoresis (comet) assay, the somatic mutation and recombination test (SMART), the erythrocytic nuclear abnormalities (ENA) assay and the micronucleus (MN) test, allowing the evaluation of different types of genetic damage.

The alkaline version of the comet assay (adopted in both Chapters 4 and 5) allows the measurement of DNA single and double strand breaks, and, through the inclusion of specific-lesions repair endonucleases, enables the detection of other types of DNA lesions, such as pyrimidine dimers, oxidized bases and alkylation damage. This technique relies on the principle that a lised cell embedded in agarose on a microscope slide with electrophoresis at high pH, reveals nucleoids with comet shape. The comet tail intensity relatively to head reflects the level of DNA strand breaks (Collins 2004) (Figure 1.6).

Figure 1.6. Images of comets stained with ethidium bromide, elucidating the visual scoring of the nucleoids and classification from 0 (no tail) to 4 (almost all DNA in tail) (Adapted from: Kleinsasser et al. 2004).

The SMART (only performed in *D. melanogaster*, on Chapter 3) allows the assessment of genetic damage in somatic cells of adult flies after larval stage. Briefly, it consists on the observation of the adult female flies' red eyes, searching for white spots or clones, translating genetic damage events occurring during the larval phase in the eye precursor structures (Graf et al. 1984; Graf and Würgler 1996) (Figure 1.7).

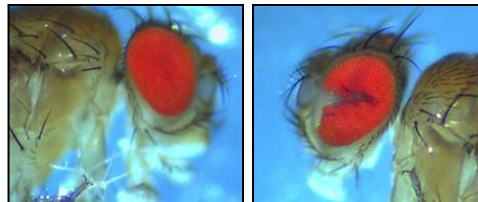

Figure 1.7. *D. melanogaster* individuals depicting a normal eye and an eye with mutant spots (Photo credit: João Ferreira).

The ENA assay (only performed in organisms with nucleated-erythrocytes, thus, in this thesis, in the fish model, on the Chapter 2) allows the assessment of the cytogenetic damage through the induction of structural chromosomal damage, that can be provoked by cleavage (clastogenicity) or the total loss of the chromosome and by mitotic spindle apparatus dysfunction (aneugenicity) mechanisms (Fenech 2000; Stoiber et al. 2004). Thus, blood smears are observed, and nuclear abnormalities detected in contrast with normal nuclear erythrocytes (Pacheco and Santos 1997). Despite some controversy regarding the establishment of the abnormality categories, five nuclear lesions can be considered: kidney-shaped nuclei (K), lobed nuclei (L), binucleate or segmented nuclei (S), vacuolated nuclei (V) and micronuclei (MN) (Pacheco et al. 2005; Guilherme et al. 2008; Marques et al. 2014) (Figure 1.8).

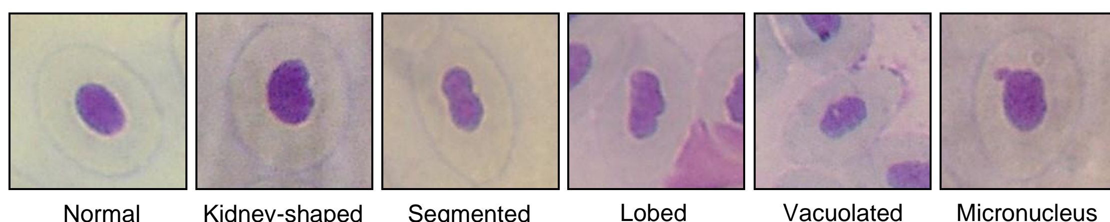

Figure 1.8. *S. aurata* erythrocytes showing a normal nucleus and several nuclear abnormalities.

In turn, the micronucleus test, here performed in mammal erythrocytes (Chapter 5), is also used to detect cytogenetic damage, through the induction of damage to chromosomes or the mitotic apparatus of erythroblasts. Briefly, when a bone marrow

erythroblast develops into a polychromatic (mature) erythrocyte, the main nucleus is extruded. Any micronucleus that has been formed, containing lagging chromosome fragments or whole chromosomes, may remain inside of the otherwise anucleated cytoplasm (OECD 1997) (Figure 1.9).

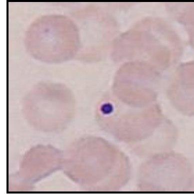

Figure 1.9. Example of a micronucleus observed in a *M. musculus* mature erythrocyte.

Additional methodologies were applied to complement the information obtained with the genetic damage evaluation biomarkers. Hence, the erythrocytic maturation index (EMI) was applied in fish erythrocytes as a nucleo-cytoplasmatic ratio to assess eventual alterations on erythrocytes populations dynamics induced by the different factors tested (Chapter 2). Briefly, through measurement of the minor axis of the nuclei and the major axis of the cells, followed by a nucleo-cytoplasmatic ratio determination, it is possible to categorize the erythrocytes on different maturation classes (Maceda-Veiga et al. 2010; Castro et al. 2018). Moreover, when comparing the genome protection ability of *U. rigida*, from wild-harvested and aquacultured origins, it seemed pertinent to complement the information obtained regarding the antigenotoxic potential with a semi-quantitative analysis of the respective phytochemical profiles. For that purpose, gas chromatography- and ultra-high-performance liquid chromatography-mass spectrometry analyses were applied (Chapter 4). Furthermore, and considering the involvement of the oxidative stress and antioxidant defences as an important equilibrium for the genotoxicity and antigenotoxicity mechanisms (Dusinská and Collins 1996), several antioxidant system related biomarkers were evaluated during the mice trial (Chapter 5). Besides that, to address the general physiologic status of mice following the different dietary backgrounds tested, a few parameters were tested, namely haematocrit (allowing to evaluate alterations on the volume percentage of red blood cells in blood), alanine transaminase activity (ALT; a commonly used hepatoxicity related parameter), as well as lactate dehydrogenase and isocitrate dehydrogenase (LDH and IDH, respectively; energy metabolism related parameters).

Additionally, bearing in mind that organisms are frequently under endogenous, as well as exogenous genotoxic insults, the macroalgae antigenotoxic potential was always

evaluated against both endogenously generated genetic damage, but also induced by different compounds, some of them considered genotoxicity induction models.

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Chapter 2

Macroalgae-enriched diet protects gilthead seabream (*Sparus aurata*) against erythrocyte population instability and chromosomal damage induced by aqua-medicines

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2. Macroalgae-enriched diet protects gilthead seabream (*Sparus aurata*) against erythrocyte population instability and chromosomal damage induced by aqua-medicines

Abstract

Macroalgae incorporation on aquafeeds has been suggested to increase fish growth and organoleptic characteristics. Moreover, macroalgae potential to strengthen fish capacity to cope with different stressors is a challenging topic in the field of applied phycology. The adverse side effects of aqua-medicines, combined with the hypothesis that a macroalgae-enriched diet can minimize that impact, are the starting point for the present study. Hence, a mix of *Ulva rigida*, *Fucus vesiculosus* and *Gracilaria gracilis* was incorporated (5%) in the gilthead seabream (*Sparus aurata*) diet, to evaluate a possible protection against the chromosomal damage (measured as erythrocytic nuclear abnormalities) induced by the antibiotic oxytetracycline and the antiparasitic formalin, as well as by the model genotoxicant cyclophosphamide. A 60-day differential dietary background was implemented (one fish group fed with standard diet and other with macroalgae-supplemented diet), after which, fish were challenged by oxytetracycline, formalin and cyclophosphamide, and appraisals carried out 4 and 18 days later. To shed light on the modulation of the erythrocytic population dynamics, the erythrocyte maturity index was assessed. Oxytetracycline and formalin displayed a chromosome damaging potential that remained for 18 days. The macroalgae-enriched diet evidenced a solid genoprotection against the three agents, revealing a broad-spectrum action. Oxytetracycline and formalin induced an erythrocyte population instability (translated into an aging effect), counteracted by algae supplementation, which seems to convey erythropoiesis promoting factors. Overall, the benefits given by the algae supplementation recommend its adoption within a framework of sustainable aquaculture practices, namely as a prophylactic measure to mitigate productivity losses when oxytetracycline and formalin are used.

Keywords

Cyclophosphamide; erythrocyte maturity index; formalin; gilthead seabream; marine macroalgae; oxytetracycline.

2.1. Introduction

The manipulation of rearing conditions in aquaculture aiming a fast-growing performance and maximum productivity poses recurring challenges, namely concerning the maintenance of water quality and the prevention/treatment of diseases. Hence, several chemicals and aqua-medicines are used for pond preparation, water quality control and infrastructures disinfection, as well as for manipulation of reproduction, growth promotion, and fish health management (*e.g.* anaesthetics, antiparasitic and antibiotics) (Sharker et al. 2014).

Oxytetracycline (OTC) is one of the most commonly adopted antibiotics in fish farms and hatcheries (Romero et al. 2012). It belongs to the group of tetracyclines and is a broad-spectrum antibiotic with bacteriostatic action that is produced by *Streptomyces* spp. and interferes with bacterial protein synthesis (mRNA translation) (Rigos et al. 2003). This antibiotic is adopted in the treatment of infections caused by gram-positive and gram-negative bacteria, mycoplasma and large viruses (Botelho et al. 2015). OTC is usually administered to fish in pelleted feed, although it can also be applied directly into the water (AADAPP 2010). Formalin (FOR; aqueous solution of the gas formaldehyde) is a therapeutic agent commonly used in disinfection procedures and treatment of diseases in aquaculture systems (Pedersen et al. 2010; Stuart et al. 2010). It is usually applied in short-term repetitive baths, to treat external fish parasites and fungal diseases (Pedersen et al. 2010; Wooster et al. 2011).

Considering that OTC and FOR are biologically active agents, it is important to address the eventual secondary toxic effects to fish (when used to control diseases) and develop possible strategies to mitigate them. Bearing in mind the central importance of the genome integrity and stability to organisms' health, fitness and, ultimately, survival, it is crucial to assess the genotoxic potential of these agents on farm-raised fish. Although previously hypothesized that genetic damage can have a negative impact on fish fitness, with subsequent repercussion on the aquaculture productivity (Silva et al. 2011), mainly due to a higher energy expenditure related with DNA repair processes (Olson and Mitchell 2006) and homeostasis threatening (Pacheco and Santos 2002), this matter has been frequently neglected on the aquaculture framework.

Nonetheless, some studies have been pointing out the genotoxic potential of OTC and FOR to fish. El-Sayed et al. (2013) reported that OTC added to the diet (medicated feed; 80 and 120 mg kg^{-1}), for 90 days, is genotoxic to *Oreochromis niloticus*, as shown by augmented chromosomal aberrations and micronuclei (MN) frequencies. Additionally, Botelho et al. (2015) demonstrated that OTC (at 4 µg L^{-1}, in water, for 96 h) induced

genotoxicity in the blood cells of *O. niloticus*, evaluated through the comet assay. Similarly, Rodrigues et al. (2017) found that OTC induced DNA damage, as measured by the comet assay (at 0.5, 5 and 50 mg L^{-1} for 96 h, as well as at 2.5 and 5 µg L^{-1} for 28 days), and chromosomal damage, as measured by the erythrocytic nuclear abnormalities (ENA) assay (at 50 mg L^{-1} for 96 h, as well as at 1.25, 2.5 and 5 µg L^{-1} for 28 days) in *Oncorhynchus mykiss*. In turn, Jerbi et al. (2011), whose study evaluated the genotoxic effects of OTC (40 mg L^{-1}) and FOR (200 µL L^{-1}), in individual and combined exposures, revealed that both therapeutic agents induced chromosomal damage in erythrocytes of *Dicentrarchus labrax*, following a time-dependent pattern (in a 15-day exposure trial) and with cumulative effects when the two agents were jointly applied. Another study revealed that sublethal concentrations of FOR (15, 30 and 75 mg $L^{-}1$ for 24, 48, 96 or 168 h) caused genotoxicity in *O. niloticus* (Mert et al. 2015). However, to the authors' knowledge, no study investigated the progression of the genetic damage throughout a post-treatment period.

The incorporation of marine macroalgae on fish feeds has been recommended by different authors, invoking multiple reasons: (i) as a source of protein, targeting a cost-efficient (Zinadah et al. 2013) and environmentally sustainable replacement of meal from wild-caught fish (Garcia-Vaquero and Hayes 2016); (ii) to increase the nutritional value of the aquaculture final product (Dantagnan et al. 2009; Valente et al. 2015) and (iii) to enhance general fish performance on growth, immune defence and tolerance against several challenges common on aquaculture (*e.g.* Wassef et al. 2005; Peixoto et al. 2016; Valente et al. 2016). Moreover, the integration of macroalgae in aquafeeds to strengthen fish antigenotoxic capacity has been suggested (Nagarani et al. 2012; Zinadah et al. 2013; Pereira 2016; Pereira et al. 2019), which could reinforce its election as functional feed additive within the context of fish nutrition. This possibility becomes more plausible since several studies already demonstrated the antigenotoxic and antioxidant properties of macroalgae (or extracts), both *in vitro* (Athukorala et al. 2006; Yuan and Walsh 2006; Celikler et al. 2009b; Zubia et al. 2009; Yang et al. 2012) and *in vivo* (Celikler et al. 2009a; Celikler et al. 2014; Marques et al. 2018). Nevertheless, to the authors' knowledge, this is the first study hypothesizing that the incorporation of the macroalgae *Ulva rigida*, *Fucus vesiculosus* and *Gracilaria gracilis* in the fish diet could enhance genomic protection, specifically against the potential damaging actions of OTC and FOR.

Hence, the main goals of this study were (i) to further investigate the genotoxic threat of OTC and FOR, frequently used in fish farms to prevent/treat diseases and (ii) to determine to what extent a macroalgae-enriched diet can, on one hand, alleviate the

genetic damage induced and, on the other, make the recover faster and more effective. For that purpose, two distinct dietary backgrounds (standard *vs*. algae-enriched diets) were given to gilthead seabream (*Sparus aurata*) during a 60-day period, being then challenged by OTC or FOR treatments (according to routine sanitary procedures), and evaluations carried out 4 and 18 days later. The macroalgae protective properties were evaluated by the (eventual) reduction of ENA frequency [allowing the detection of MN and other nuclear abnormalities (NA) (Pacheco and Santos 1998), signalizing *in vivo* clastogenicity and/or aneugenicity processes (Fenech 2000; Stoiber et al. 2004)] in fish blood. In parallel, to extend and consolidate the scope of the current appraisal of macroalgae potential, *S. aurata* was also challenged by the model genotoxicant cyclophosphamide (CP). Additionally, to shed some light on the erythrocytic population dynamics alterations provoked by the agents tested and/or the different dietary backgrounds adopted, the erythrocyte maturity index (EMI) was assessed. This parameter, based on the ratio between erythrocytes' nuclear and cytoplasmic volumes (Maceda-Veiga et al. 2010; Castro et al. 2018), also complements the information provided by the ENA assay, elucidating about the balance between erythropoiesis and cell removal, and thus, about the recovery pathways occurring in the post-treatment period.

2.2. Material and methods

2.2.1. Chemicals

Cyclophosphamide (CAS 6055-19-2) was obtained from Sigma-Aldrich Chemical Company (Spain) and formaldehyde 37% (w/w) aqueous solution, stabilized with methanol (CAS 50-00-0; formalin) was obtained by VWR International, LLC (USA). Veterinary antibiotic Oxykel 80% (800 mg g^{-1} of OTC) by Kela Laboratoria N.V. was purchased at a local veterinary pharmaceutical supplier. All other chemicals were obtained from Sigma-Aldrich Chemical Company.

2.2.2. Fish, rearing conditions and experimental diets

Gilthead seabream (*S. aurata*) fingerlings weighting approximately 8-10 g were purchased from a local fish farm (Nasharyba, Lda., Figueira da Foz, Portugal). Before trials, fish were acclimatized to the experimental tanks (500-L cylindric PVC tanks) for 3 weeks, and to the standard diet (as detailed in Table 2.1) for one week. The tanks were maintained under a natural photoperiod, as open systems (each one was independently supplied by a flow-through seawater system, corresponding to a water renewal rate of 6-8 times per day), with the following physical-chemical conditions: salinity 35.6 ± 1.4‰,

Table 2.1. Formulation and proximate composition of the experimental diets.

Ingredients (Provider)	Standard diet (%)	Algae-enriched diet (%)
Fishmeal Super Prime (Pesquera Diamante, Peru)	27.00	27.00
Fishmeal 60 (COFACO, Portugal)	20.00	20.00
Fish protein concentrate (Sopropêche, France)	3.00	3.00
Porcine blood meal (SONAC, Netherlands)	5.00	5.00
Soy protein concentrate (ADM, Netherlands)	8.00	8.00
Wheat gluten (Roquette, France)	10.00	10.00
Wheat meal (Casa Lanchinha, Portugal)	7.50	2.40
Pea starch (Cosucra, Belgium)	5.10	5.10
Fish oil (SAVINOR, Portugal)	13.00	13.10
Vit & Min INVIVO 1% (Premix, Portugal) [a]	1.00	1.00
Binder - guar gum (Seah, France)	0.40	0.40
U. rigida	0.00	1.67
G. gracilis	0.00	1.67
F. vesiculosus	0.00	1.67
Proximate composition (%)		
Crude protein	52.69	52.71
Crude fat	17.62	17.64
Fibre	0.55	0.44
Ash	9.51	9.42
Total P	1.38	1.37
Gross energy (MJ kg^{-1} feed)	20.84	20.43

[a] Composition:

Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate - 100 mg; sodium menadione bisulphite - 25 mg; retinyl acetate - 20000 IU; DL-cholecalciferol - 2000 IU; thiamine - 30 mg; riboflavin - 30 mg; pyridoxine - 20 mg; cyanocobalamin - 0.1 mg; nicotinic acid - 200 mg; folic acid - 15 mg; ascorbic acid - 1000 mg; inositol - 500 mg; biotin - 3 mg; calcium pantothenate - 100 mg; choline chloride - 1000 mg; betaine - 500 mg.

Minerals (g or mg kg^{-1} diet): cobalt carbonate - 0.65 mg; copper sulphate - 9 mg; ferric sulphate - 6 mg; potassium iodide - 0.5 mg; manganese oxide - 9.6 mg; sodium selenite - 0.01 mg; zinc sulphate - 7.5 mg; sodium chloride - 400 mg; calcium carbonate - 1.86 g; excipient wheat middlings.

temperature 20.9 ± 2.9 ˚C, ammonia 0.4 ± 0.5 mg L^{-1}, nitrite 0.03 ± 0.02 mg L^{-1} and dissolved oxygen 7.2 ± 0.3 mg L^{-1}.

Two experimental diets (2.0 mm pellets) were designed and produced by SPAROS I&D (Olhão, Portugal), composed by the same basic ingredients, corresponding to a standard diet (S), formulated according *S. aurata* nutritional requirements, and an algae-enriched diet (A) (as detailed in Table 2.1). The supplementation concerned a total percentage of 5% (dry weight), incorporating three different macroalgae species, *viz. U. rigida* (Chlorophyta), *F. vesiculosus* (Ochrophyta) and *G. gracilis* (Rhodophyta), in equal amounts (approx. 1.67% each). Macroalgae were reared at ALGAplus, Lda. (Ílhavo, Portugal), a certified organic and integrated multi-trophic aquaculture (IMTA) company. After the harvesting, macroalgae were washed in seawater (treated with UV and filtered to 5 µm) and then dried during 12 h in a chamber with controlled temperature (25 ºC), achieving 10–12% of humidity, after which they were preserved in multiple layer packaging (paper and plastic). Prior to the aquafeeds production, macroalgae were grinded to powder and incorporated in the respective aquafeed.

Fish were hand-fed once a day, at a daily rate of approximately 3.8, 3.5 and 3.0% [as percentage of fish total weight; in line with that described by Henriques, (1998)] on the first and second months of dietary background and in the post-treatment period, respectively. Fish were not fed on the handling (treatment/sampling) days.

2.2.3. Experimental design

At the beginning of the experimental trial (August 2017), 8 fish were sampled as t_0. Then, 216 fish were divided into 12 (2x6) groups, each placed in a 500-L tank (18 fish per tank). For 60 days, fish from 6 tanks were fed with standard feed (S) and the other 6 tanks with algae-enriched (A) feed (Figure 2.1). After this period, 8 fish (n = 8) were randomly sampled from the 6 tanks of each dietary condition and, then, three sub-trials were established testing the three agents selected, *i.e.*, CP, OTC or FOR. For each sub-trial, four groups were considered, corresponding to the two tested diets, with (S_x and A_x; x represents the challenging agent) and without (S and A) treatment.

For the CP sub-trial (total fish number = 64), fish were injected intraperitoneally with the model genotoxicant at 20 mg kg^{-1} body weight (b.w.) dissolved in saline solution, establishing S_{CP} and A_{CP} groups (control groups, S and A, were injected only with saline solution). To calculate the volume to inject, respecting the intended CP dose as a function of b.w., fish were weighted, and the volume of a CP solution (2.4 mg mL^{-1}) was proportionally defined according the ratio 500 µL per 60 g b.w. The CP dose was selected

based on previous studies with the same (Pereira et al. 2019) and other fish species (*e.g.* Grisolia, 2002).

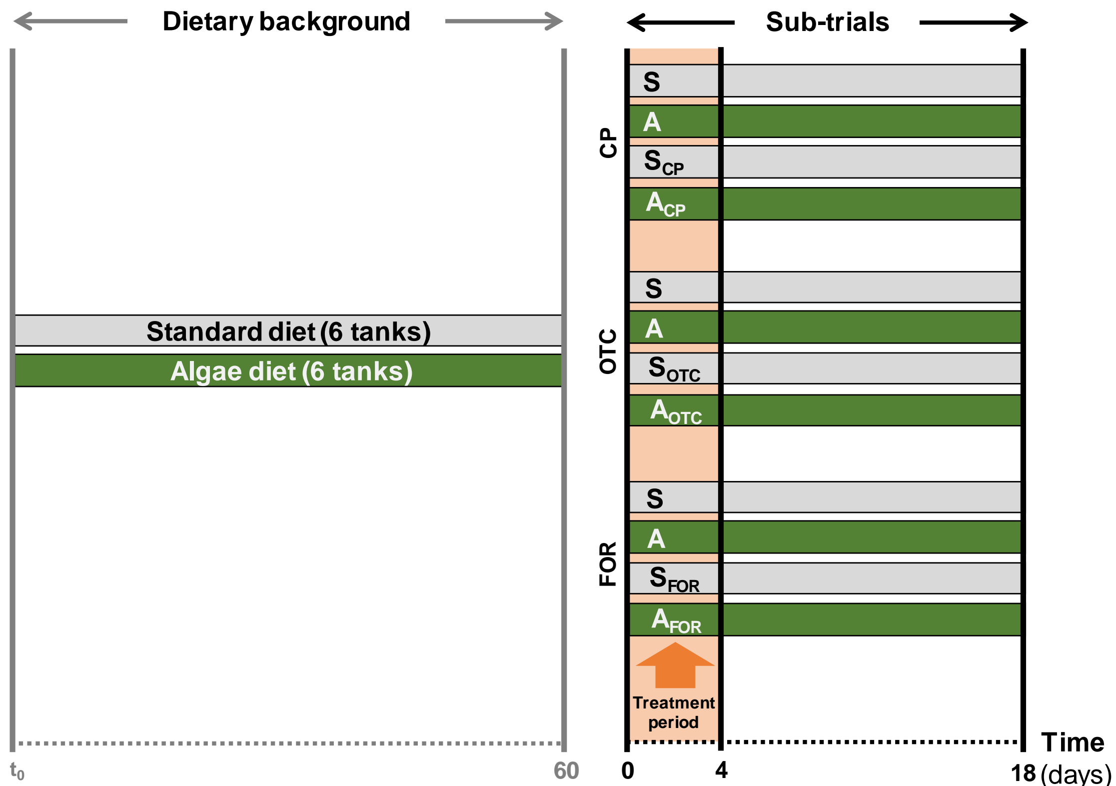

Figure 2.1. Schematic representation of the experimental design, depicting a 60-day period of dietary background [standard (S) versus algae-supplemented (A) diets)], followed by the different sub-trials (CP - cyclophosphamide, OTC - oxytetracycline and FOR - formalin). For each sub-trial, four groups were considered, corresponding to the two tested diets, with (Sx and Ax; x represents the challenging agent) and without (S and A) treatment. Sampling was carried out at the beginning (t0) and end of the dietary background, as well as on days 4 and 18 following the initiation of the respective treatment.

In the OTC and FOR sub-trials (total fish number = 64, for each), the two dietary groups were exposed, in 230-L tanks (cylindric PVC tanks), to a single bath of 300 mg L^{-1} of OTC for 4 h or to a double bath (on days 0 and 2) of 150 µL L^{-1} of FOR during 1 h, creating S_{OTC}/A_{OTC} and S_{FOR}/A_{FOR} groups, respectively. To do that, the water level was reduced to 1/4 in the 500-L tanks corresponding to OTC and FOR sub-trials, and fish were carefully transferred (with fishing nets and without anaesthesia) to the 230-L treatment tanks with similar water conditions. At the end of treatment, fish that will constitute the experimental groups on day 18 returned to the initial 500-L thanks, adopting the same procedure. The OTC and FOR concentrations as well as the treatments duration were

selected considering the recommendations of the US Fish and Wildlife Service – Aquatic Animal Drug Approval Partnership Program (AADAPP 2010). The control groups of OTC and FOR sub-trials were subjected to similar conditions/handling but without OTC/FOR treatment.

Four and 18 days after the CP injections, the OTC bath and the first FOR bath, fish were sampled (n = 8 per condition) (Figure 2.1). Fish were anesthetized with 0.2 mg L^{-1} tricaine methanesulfonate (MS-222; buffered with $NaHCO_3$) for approximately 10 min, after which they were weighed (to nearest 0.1 g) and measured (total length; to the nearest 0.1 cm). Then, fish blood was drawn from the posterior cardinal vein, using heparinized (27 mg mL^{-1} heparin) glass Pasteur pipettes, and blood smears were immediately prepared for ENA and EMI assays. Fish were sacrificed by cervical transection.

Fish biometric parameters such as weight (g), total length (cm), Fulton's condition factor (K) (initial and final), as well as the specific growth rate (SGR), were assessed concerning the 60-day period of dietary background.

2.2.4. ENA assay

The ENA assay was performed in *S. aurata* mature peripheral erythrocytes, according to the methodology described by Pacheco and Santos (1997). Thus, one blood smear for each animal was fixed with methanol for 10 min and stained with Giemsa (5%) for 30 min. Slides, previously coded, were subsequently blind scored. From each smear, 1000 erythrocytes were scored, under 1000x magnification (microscope Olympus BX50), to assess the relative frequency of the following nuclear lesions: kidney shaped nuclei (K), lobed nuclei (L), binucleate or segmented nuclei (S), vacuolated nuclei (V) and micronuclei (MN) (Carrasco et al. 1990; Pacheco and Santos 1996). Blebbed and lobed nuclei were considered in a single category – lobed nuclei – and not differentially scored due to some ambiguity in their distinction, as suggested by Guilherme et al. (2008). These chromosomal anomalies could be a direct outcome and manifestation of damage at the DNA level (Fenech 2000), as DNA double-strand breaks may result in chromosome breaks or DNA misrepair could lead to chromosome rearrangements (Savage 1993). These nuclear abnormalities (NA), formed during the proliferative phase of the cell cycle, could result from either clastogenic (chromosomal breaking) or aneugenic (mitotic spindle apparatus dysfunction) processes, and be a consequence of the exposure to genotoxicants (Braham et al. 2017).

Though the frequency (‰) of each nuclear abnormality category was individually reported (as supplementary material), the results of the ENA assay were expressed as the sum of frequencies for all the categories considered (Total = K + L + S + V + MN).

2.2.5. EMI determination

The EMI was determined according with Maceda-Veiga et al. (2010), with the modifications proposed by Castro et al. (2018). Briefly, 10 microscopic fields were randomly selected per slide (one slide per fish; the same slides used for the ENA assay) and photographed (Moticam 3000) under 1000x magnification (microscope Zeiss Axiolab RE). Then, in each microscopic field, 25 random cells were analysed with ImageJ software, measuring the minor axis of the nuclei and the major axis of the cells (A and B, respectively; see Figure 2.2). EMI was calculated for each cell by dividing A by B values, to a total of 250 cells. From the values of the ratio, cells were then categorized into one of the 10 maturity classes: [0.0 ≤ class 1 < 0.1]; [0.1 ≤ class 2 < 0.2]; [0.2 ≤ class 3 < 0.3]; [0.3 ≤ class 4 < 0.4]; [0.4 ≤ class 5 < 0.5]; [0.5 ≤ class 6 < 0.6]; [0.6 ≤ class 7 < 0.7]; [0.7 ≤ class 8 < 0.8]; [0.8 ≤ class 9 < 0.9]; [0.9 ≤ class 10 ≤ 1], where the class 1 represents erythrocytes with the higher maturity level and class 10 corresponds to cells with lower maturity status. Finally, average values for the frequency (%) of cells observed in each maturity class were represented for each experimental group.

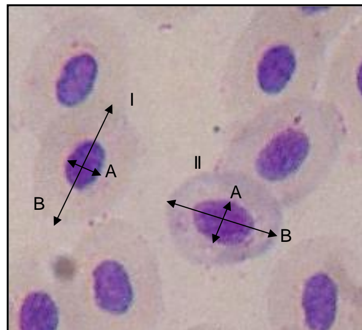

Figure 2.2. *S. aurata* peripheral erythrocytes (with nuclear normal shape), elucidating the measurements performed for the calculation of the erythrocyte maturity index (EMI) (Giemsa stain). Erythrocytes in later (I) and earlier (II) maturation stages are represented. A - minor axis of the nucleus; B - major axis of the cell.

This parameter has been incorporated in studies assessing genotoxicity to complement the information obtained, since organisms' response to the contaminants may also be translated into alterations in the erythrocyte population dynamics (Maceda-Veiga et al. 2010; Castro et al. 2018).

2.2.6. Statistical analysis

Statistica 8.0 software (StatSoft, Inc., USA) was used for the statistical analysis. First, all data were tested for normality (Shapiro–Wilk test and graphical analysis) and homogeneity of variances (Levene's test) and, when necessary, transformed to meet these statistical assumptions. A t-test was used to compare the 2 groups (S *vs.* A) at the end of the 60-day period and a two-way ANOVA (factors: dietary background and treatment with agents) was applied at each sampling moment in the post-treatments period, followed by a post-hoc Tukey HSD test for all pairwise comparisons (Zar 1996). A two-way ANOVA on ranks was applied when the normality assumption failed.

2.3. Results

No fish mortality was registered during the experimental trials. In addition, no markedly or measurably alterations on fish behaviour were noticeable relatively to the different diets, since all fish were generally very responsive at the feeding times. Nevertheless, a slightly higher responsiveness to the macroalgae-enriched diet was perceptible based on the visual evaluation of feeding habits throughout the experiment.

Though an evaluation of growth performance was not within the goals of the current investigation, the variation of fish weight, total length and Fulton's condition factor (K), as well as SGR were recorded concerning the 60-day trial for each dietary group (Table 2.2). No significant differences were detected in any of the previous parameters.

Table 2.2. Weight (g), total length (cm) and Fulton's factor (K) of the fish at the beginning (t_0) and the end (60 days) of the dietary background (mean value ± standard error), as well as specific growth rate (SGR; % of body weight gain/day).

		Weight (g)	**Length (cm)**	**K factor** [a]	**SGR (%)** [b]
Dietary background	**t_0**	21.46 ± 0.90	11.06 ± 0.19	1.58 ± 0.04	-
	S	72.60 ± 3.88	16.70 ± 0.31	1.55 ± 0.05	2.03129
	A	72.75 ± 3.14	16.72 ± 0.19	1.56 ± 0.06	2.03473

[a] K factor calculated according with the formula: $K = (W \times 100) / L^3$, where W = weight (g) and L = total length (cm); [b] SGR calculated according with the formula: SGR = [(Log_n final fish weight - Log_n initial fish weight) / time interval] x 100.

2.3.1. ENA assay

After the 60-day period with the different dietary backgrounds, mean values (‰) of ENA frequency (± standard error) were 42.38 ± 2.85 for group S and 36.00 ± 4.36 for group A (t_0 = 41.38 ± 2.16), showing no significant differences. Similarly, no statistical differences were observed for the individual nuclear lesions' categories (Appendix I, Table 1). The kidney shaped nucleus was the most frequent nuclear abnormality in all groups.

2.3.1.1. Cyclophosphamide sub-trial

The CP genotoxic potential was confirmed (only) in fish fed with the standard diet, at 4 and 18 days after the injection, since S_{CP} group displayed higher damage than S group in both sampling moments (Figure 2.3). Also, at 4 days after the treatment, fish fed with A diet presented lower chromosomal damage than fish fed with S diet, both in fish injected with saline or CP. In addition, the algae-enriched diet continued to support the decrease on chromosomal damage 18 days after the treatment of fish with CP.

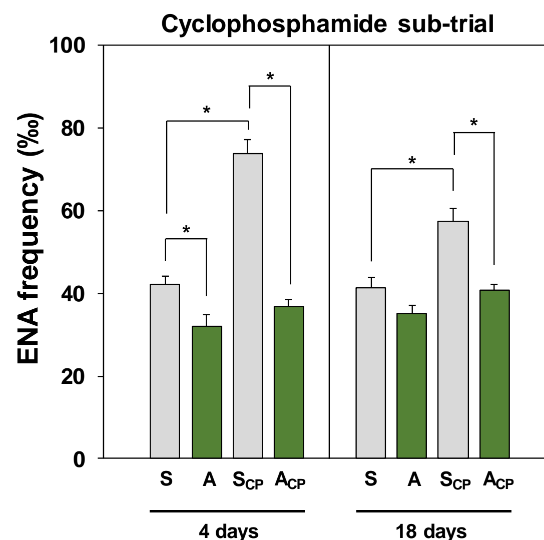

Figure 2.3. Cyclophosphamide (CP) sub-trial - Mean values of erythrocytic nuclear abnormalities (ENA) frequency (‰) evaluated in peripheral erythrocytes of *S. aurata* at days 4 and 18 following the CP injection. S = standard diet; A = algae-supplemented diet; S_{CP} = S + CP treatment; A_{CP} = A + CP treatment. Bars represent standard errors. * = statistically significant differences ($p < 0.05$; n = 8) between groups, within each time.

Considering the nuclear lesions categories individually (Appendix I, Table 2), their expression is in line with the total ENAs frequency evaluated on all the groups at both

sampling moments. The kidney shaped nuclei were the most frequent nuclear abnormality in all groups at both sampling moments.

2.3.1.2. Oxytetracycline sub-trial

Regarding the OTC sub-trial, 4 days after the treatment with the antibiotic, both S_{OTC} and A_{OTC} groups showed higher chromosomal damage than the respective controls, *i.e.*, groups S and A (Figure 2.4). Notwithstanding, the group treated with OTC and fed with algae-enriched diet (A_{OTC}) revealed lower damage than the group fed with standard diet (S_{OTC}). Eighteen days after the treatment, OTC effect was only detected in the S_{OTC} group, which also showed significantly higher levels of chromosomal damage in comparison with A_{OTC} group.

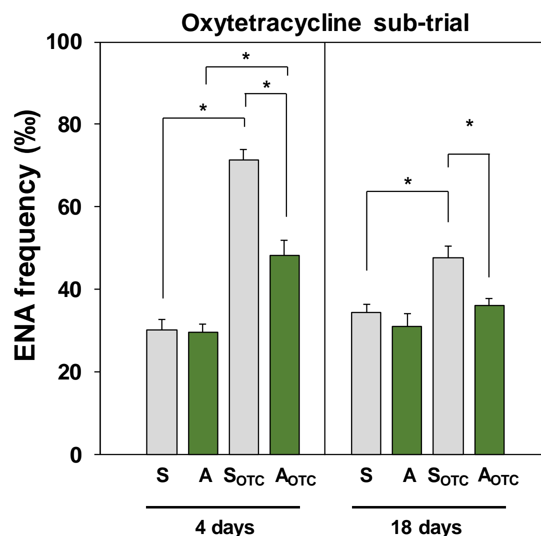

Figure 2.4. Oxytetracycline (OTC) sub-trial - Mean values of erythrocytic nuclear abnormalities (ENA) frequency (‰) evaluated in peripheral erythrocytes of *S. aurata* at days 4 and 18 following the OTC bath. S = standard diet; A = algae-supplemented diet; S_{OTC} = S + OTC treatment; A_{OTC} = A + OTC treatment. Bars represent standard errors. * = statistically significant differences (p < 0.05; n = 8) between groups, within each time.

Again, in this experimental sub-trial, kidney shaped nuclei were the most frequently found nuclear lesion, while the individual expression of the nuclear abnormalities was in line with the frequency of total ENAs for all the groups at both samplings (Appendix I, Table 3).

2.3.1.3. Formalin sub-trial

Fish fed with the standard diet and treated with FOR baths (S_{FOR}) revealed higher ENA frequency than the untreated group (S), at both sampling moments (4 and 18 days after the 1st FOR bath) (Figure 2.5). Differently, no genotoxicity was observed when fish were fed with the algae-enriched diet (A_{FOR}), at both sampling moments. In addition, S_{OTC} group displayed significantly higher ENA frequency in comparison with A_{OTC} group, at both sampling moments.

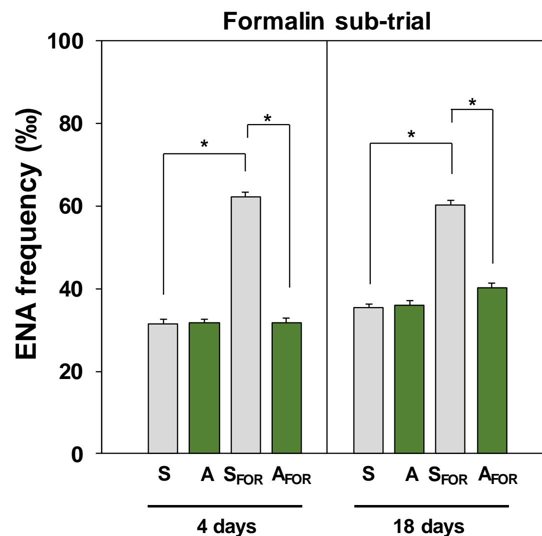

Figure 2.5. Formalin (FOR) sub-trial - Mean values of erythrocytic nuclear abnormalities (ENA) frequency (‰) evaluated in peripheral erythrocytes of *S. aurata* at days 4 and 18 following the 1st FOR bath. S = standard diet; A = algae-supplemented diet; S_{FOR} = S + FOR treatment; A_{FOR} = A + FOR treatment. Bars represent standard errors. * = statistically significant differences ($p < 0.05$; n = 8) between groups, within each time.

Once more, the most frequent nuclear abnormality was the kidney shaped nucleus. In general, the nuclear categories expression was in line with the total ENAs frequencies on the groups evaluated at both sampling moments (Appendix I, Table 4).

2.3.2. EMI determination

Since no cells with classes 8, 9 and 10 were determined in the present study, only frequencies of cells from class 1 to class 7 were depicted (Figures 2.6-2.8).

After the 60-day period with the differential dietary background, fish fed with A diet showed a higher frequency of erythrocytes on class 5 of maturity (2.45%) relatively to fish fed with S diet (0.95%). No significant differences were observed on the other maturity

classes, with S group revealing 1.3%, 64.8% and 33% of cells in classes 2, 3 and 4, respectively, while A group showed 0.8%, 56.6%, 40.1% and 0.1% of cells in classes 2, 3, 4 and 6, respectively. In both groups, class 3 was the most frequent.

2.3.2.1. Cyclophosphamide sub-trial

As a general pattern, it was observed no significant effects induced by CP while differences associated with the diet only occurred in unchallenged groups. Thus, 4 days after the injection with saline, fish fed with A diet revealed a significantly higher frequency of erythrocytes on class 5 than fish fed with S diet, having the class 4 as the most prevalent (Figure 2.6). On day 18, the group A revealed a decrease of erythrocytes of class 3 and an increase of cells on class 4, relatively to fish fed with S diet.

2.3.2.2. Oxytetracycline sub-trial

It is worth noting that significant differences associated with OTC were detected only within the context of the standard diet (Figure 2.7). Hence, at 4 days post-treatment, fish treated with OTC (S_{OTC}) depicted higher frequency of class 2 erythrocytes and lower frequencies of class 4 and 5 cells than untreated fish (S). Eighteen days after the OTC treatment, S_{OTC} group revealed higher frequency of class 2 erythrocytes than untreated fish (S) (Figure 2.7).

Additionally, comparing untreated fish (S *vs*. A) at 4 days post-treatment, A diet group showed lower frequencies of class 5 and 6 erythrocytes relatively to the S group.

2.3.2.3. Formalin sub-trial

As in the previous sub-trial, significant differences associated with FOR were noticed only within the context of standard diet (Figure 2.8). Thus, treated fish (S_{FOR}) demonstrated lower frequencies of class 4 and 5 erythrocytes, in comparison with untreated fish (S). This effect was no more detectable on day 18.

Moreover, 4 days after the 1st bath, untreated fish fed with algae-enriched diet (A group) showed higher frequency of erythrocytes of class 2, coupled with a depletion of class 4 and 5 cells, relatively to fish fed with S diet (Figure 2.8).

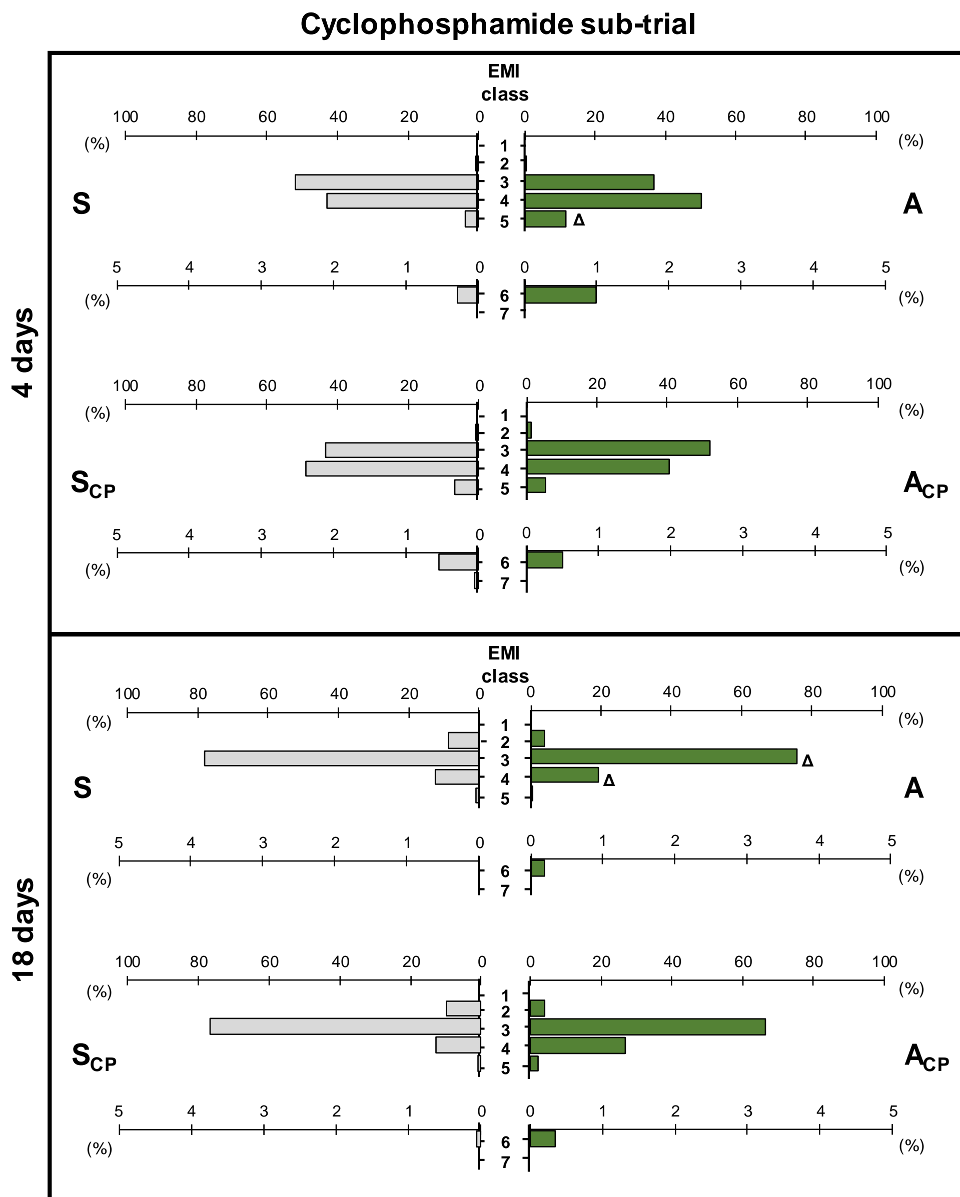

Figure 2.6. Cyclophosphamide (CP) sub-trial - Erythrocytic maturity index (EMI) evaluated in peripheral erythrocytes of *S. aurata* at days 4 and 18 following the CP injection. S = standard diet; A = algae-supplemented diet; S_{CP} = S + CP treatment; A_{CP} = A + CP treatment. For each condition, average values for the frequency (%) of cells observed in each maturity class (maturity decreases from class 1 to class 7) are depicted. Δ = statistically significant differences ($p < 0.05$; n = 8) between diets, within each time.

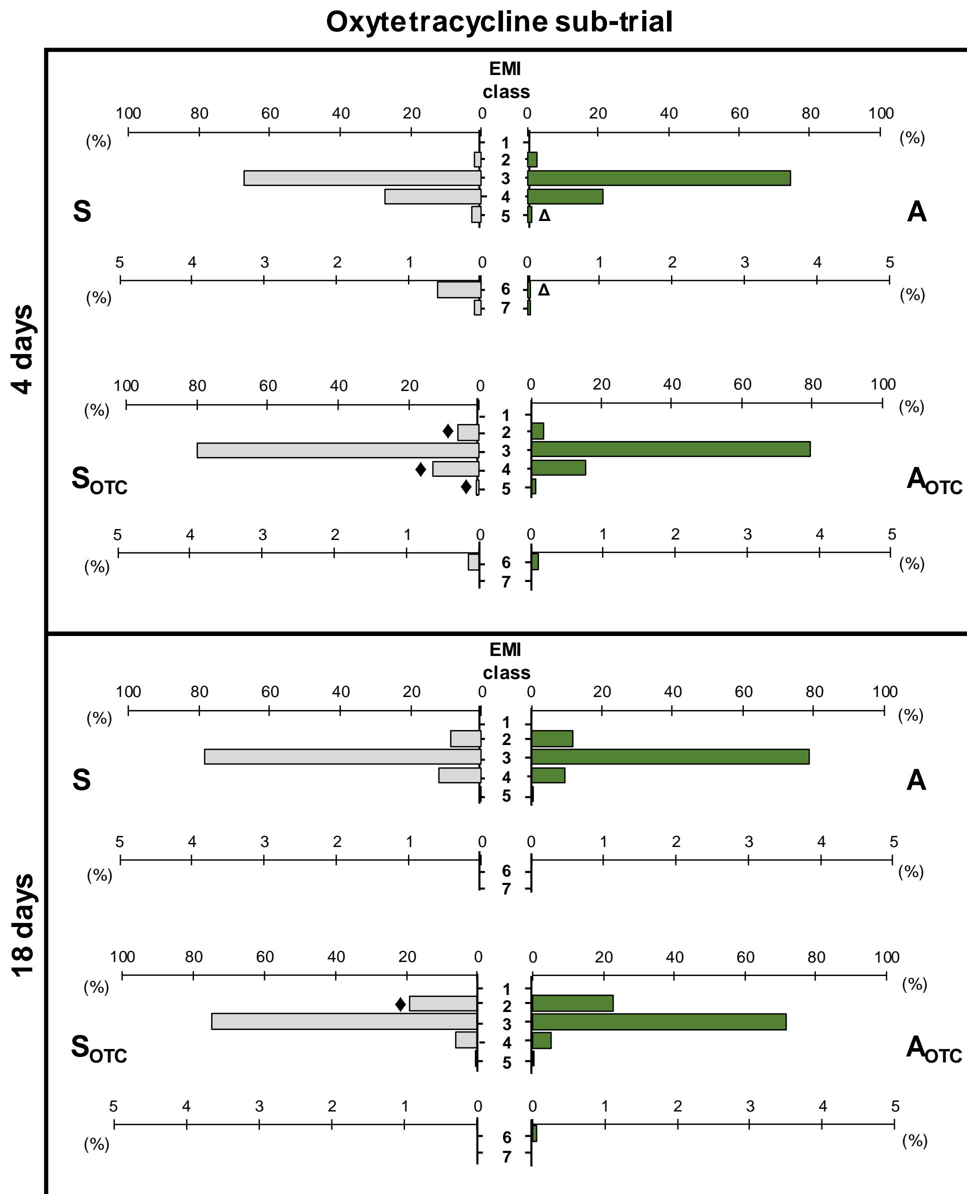

Figure 2.7. Oxytetracycline (OTC) sub-trial - Erythrocytic maturity index (EMI) evaluated in peripheral erythrocytes of *S. aurata* at days 4 and 18 following the OTC bath. S = standard diet; A = algae-supplemented diet; S_{OTC} = S + OTC treatment; A_{OTC} = A + OTC treatment. For each condition, average values for the frequency (%) of cells observed in each maturity class (maturity decreases from class 1 to class 7) are depicted. Δ = statistically significant differences ($p < 0.05$; n = 8) between diets, within each time; ♦ = statistically significant differences ($p < 0.05$; n = 8) due to OTC treatment, within the same diet.

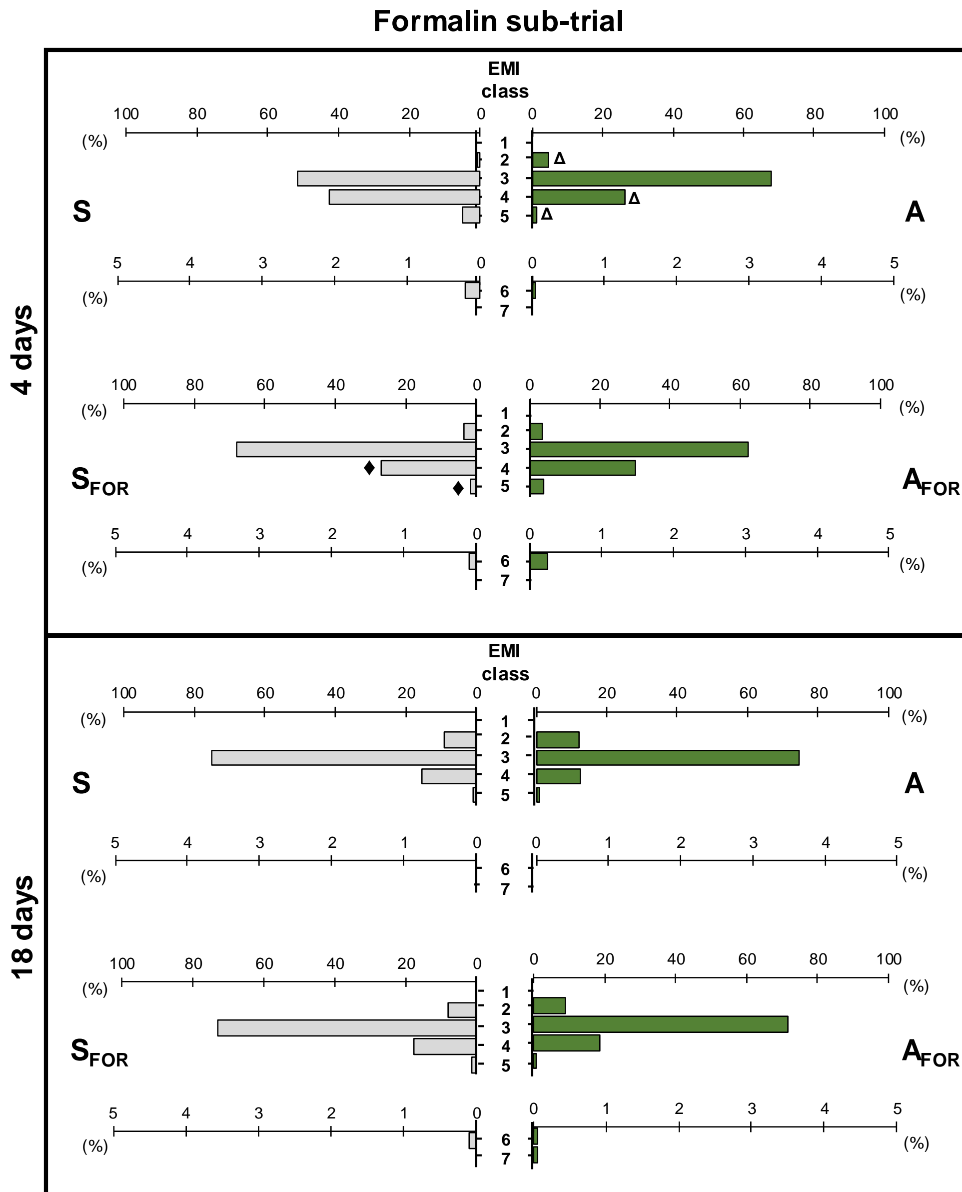

Figure 2.8. Formalin (FOR) sub-trial - Erythrocytic maturity index (EMI) evaluated in peripheral erythrocytes of *S. aurata* at days 4 and 18 following the 1st FOR bath. S = standard diet; A = algae-supplemented diet; S_{FOR} = S + FOR treatment; A_{FOR} = A + FOR treatment. For each condition, average values for the frequency (%) of cells observed in each maturity class (maturity decreases from class 1 to class 7) are depicted. Δ = statistically significant differences ($p < 0.05$; n = 8) between diets, within each time; ♦ = statistically significant differences ($p < 0.05$; n = 8) due to FOR treatment, within the same diet.

2.4. Discussion

Macroalgae have been presented as functional food on the human nutrition context (Holdt and Kraan 2011; Mohamed et al. 2012). Although lacking a direct definition and applicability of this concept to the animal nutrition field, several studies have suggested

that the incorporation of macroalgae on the animal feeds, particularly in aquaculture systems, could increase the growth, improve organoleptic characteristics and enhance fish defences against diseases and stress (*e.g.* Fleurence et al. 2012; Araújo et al. 2016; Garcia-Vaquero and Hayes 2016; Peixoto et al. 2016; Xie et al., 2018).

Blood analyses have great potential in studies of toxicology and veterinary (Maceda-Veiga et al. 2015). In line, parameters evaluating the erythrocytic nuclear morphology are regarded as sensitive, suitable and reliable tools to address toxic effects of diverse substances in fish (Cavas et al. 2005; Gomes et al. 2015; Castro et al. 2018). Hence, in the present study, a mix of *U. rigida*, *F. vesiculosus* and *G. gracilis* was added to *S. aurata* diet, evaluating to what extent does this supplementation protect fish erythrocytes against the chromosomal damage induced by the model genotoxicant CP and two therapeutic agents commonly used in aquaculture, OTC and FOR, and modulate the haematological response to those agents.

The gilthead seabream (*S. aurata*) was chosen mainly because, though primarily carnivorous, it can be accessorily herbivorous, thereby accepting well macroalgae in its diet, besides being a highly important commercial species in aquaculture. Additionally, the inclusion of macroalgae (*Pterocladia capillacea* and *U. lactuca*) in *S. aurata* diet previously proved to have favoured growth, nutrient retention and tolerance to stress (Wassef et al. 2005). The selection of the three macroalgae species relied on: (i) the representativeness of each taxonomic group, *i.e.*, Chlorophyta, Rhodophyta and Ochrophyta; (ii) the wide geographical distribution of these edible species, namely in the Atlantic coast and the easiness to grow them in aquaculture; (iii) the previous findings demonstrating their potential to enhance *S. aurata* immune and antioxidant responses (Queiroz et al. 2014), as well as its antigenotoxic potential against DNA and chromosomal damage induced by CP (Pereira et al. 2019).

Therefore, after a 60-day period with the differential dietary supplementation, a slight decrease of chromosomal damage was perceptible in the group fed with the algae-enriched diet (A), though this variation was insufficient to set up a statistically significant genoprotective action against the basal chromosomal damage. In the same period, EMI data pointed out a slightly younger erythrocyte population in fish fed with the A diet, which can be explained by a promotion of the erythropoiesis accomplished by the algae diet. In fact, the three macroalgae, presently tested on a mix, showed to individually possess at least some of the elements that play crucial roles in the erythropoiesis process (Koury and Ponka 2004), namely iron and vitamins B9 and B12 (Morel et al. 2005; Taboada et al. 2010; Rasyid et al. 2019). This effect can also be at the basis of the slight tendency of this

group to display lower damage, through a dilution effect of the cells with NA among new normal cells entering the system after enhanced erythropoiesis.

In addition, no markedly or measurably alterations on fish behaviour were noticeable relatively to the different diets, since all fish were generally very responsive at the feeding times. Nevertheless, a slightly higher responsiveness to the macroalgae-enriched diet was perceptible based on the visual evaluation of feeding habits throughout the experiment.

While the improved aquafeed formulation enriched in macroalgae demonstrated to have shielding actions (as discussed further), no adverse effects were detected on the fish behaviour and feeding responsiveness (which was slightly higher to the macroalgae-enriched diet) as well as on fish general status and growth performance (roughly evaluated as Fulton's condition factor). Therefore, fish farmers can safely adopt this improved aquafeed formulation, since it will not jeopardize the current productivity obtained with regular aquafeeds.

2.4.1. Cyclophosphamide sub-trial

The model genotoxicant CP is a strong indirect alkylating agent, *i.e.*, following biotransformation, it forms phosphoramide mustard and acrolein that alkylate DNA and proteins, respectively (DrugBank 2005). DNA alkylating agents induce damage through three different mechanisms: the attachment of alkyl groups to DNA (resulting in the DNA fragmentation, when the DNA repair machinery attempts to repair the damage), the formation of cross-links (impairing DNA synthesis or transcription) and the induction of nucleotides mispairing (leading to mutations) (DrugBank 2005). For this reason, CP is frequently used as a positive control in diverse genotoxicity assays with fish (Pacheco and Santos 1997; Ayllón and Garcia-Vazquez 2001; Grisolia 2002), while, in this study, it was adopted to ensure the occurrence of genotoxic damage (OTC and FOR genotoxicity was, *ad initium*, hypothesized but not unquestionable), allowing the identification of algae protective actions, and to promote a comparative perspective with the OTC and FOR results.

Hence, in this sub-trial, the algae-enriched feed clearly demonstrated its antigenotoxic capacity, either in the presence or in the absence of CP. This protection profile (decreasing basal damage and avoiding CP-induced damage) was evident on day 4. Besides that, the genoprotection against the genotoxic pressure of CP lasted up to 18 days post-injection. Previously, Pereira et al. (2019) had already shown that a 30-day algae supplementation, using a diet formulation equivalent to the one adopted in the present study, protected *S. aurata* blood cells against CP (40 mg kg^{-1} b.w.) genotoxicity. In

that study, the DNA and chromosomal damage was assessed 3 and 10 days after the injection, and the genoprotection conferred by the diet was only evident in the second sampling period, differently to the present study where that capacity was evident on day 4. It is important to take into consideration that, in the current study, the CP dose was half and the dietary background lasted double in relation to those adopted by Pereira et al. (2019). Nevertheless, in both studies the genoprotective action of the algae-enriched feed was kept for several days.

From the mode of action viewpoint, the natural antimutagens have been classified as desmutagens and bio-antimutagens (Bhattacharya 2011; Izquierdo-Vega et al. 2017). The former, assumed as apparent antimutagens, act before the mutagen attacks the DNA, through partial or full inactivation (by enzymatic or chemical interactions). The later, assumed as true antimutagens, suppress the mutation process after genes are damaged, improving the repair and replication processes of the mutagen-damaged DNA (Bhattacharya 2011; Izquierdo-Vega et al. 2017).

Considering the macroalgae included in this study, diverse phytochemicals were already described as genome protecting. The green alga *U. rigida* contains chlorophylls a and b, carotenoids, vitamins A, C and E, phenolic compounds, as well as sulphated polysaccharides (*e.g.* ulvan), that are the most likely phytochemicals responsible for its antioxidant and antigenotoxic properties (Lahaye and Robic 2007; Celikler et al. 2009a; Yildiz et al. 2012; Celikler et al. 2014; Mezghani et al. 2016). Regarding the brown alga species, *F. vesiculosus* is known to be particularly rich in polyphenols (*e.g.* phlorotannis), non-digestible polysaccharides (*e.g.* fucans, alginates, laminaranes and cellulose) and natural pigments, as fucoxanthin and pheophytin, phytochemicals that have been described as possessing antioxidant (Rupérez et al. 2002; Díaz-Rubio et al. 2009; Farvin and Jacobsen 2013), antigenotoxic (Okai and Higashi-Okai 1994; Gamal-Eldeen et al. 2013) and anticarcinogenic (Higashi-Okai et al. 1999) properties. In turn, the red macroalga *G. gracilis* has demonstrated as well its antioxidant potential, which is generally attributed to the rich phenolic and flavonoid content, as well as polyunsaturated fatty acids and natural pigments (Francavilla et al. 2013; Yildiz et al. 2014; Heffernan et al. 2015; Ebrahimzadeh et al. 2018).

The approach of the present study does not allow the elucidation of the predominant mechanisms associated to the macroalgae antigenotoxic effect, and thus, both desmutagen and bio-antimutagen actions should be hypothesized. However, only based on the dominant indications found in the literature, a desmutagenic effect gains

plausibility, *via* namely the macroalgae phytochemicals with high radical scavenging activity.

Alternatively or concomitantly, an increased efficiency of spleen erythrophagia promoted by algae components, operating a selective removal of erythrocytes with abnormal nuclei [these cells may as well be affected in their membranes, being more easily detected and destroyed on the spleen (Pacheco and Santos 2002)], should not be overlooked.

Regarding the fluctuation on the *S. aurata* erythrocyte dynamics resulting from the dietary backgrounds and/or the CP treatment, the analysis of the nucleo-cytoplasmatic ratios only signalised effects attributable to the diet. That is, CP treatment did not induce alterations on the frequency of erythrocytes within the different maturity stages. A previous fish (*Pimephales promelas*) study also revealed that CP induces no effect on the ratio of immature *vs*. mature erythrocytes at any dose administered (50 to 400 mg kg^{-1}) (Winter et al. 2007). On the other hand, and in line with the observations following the 60-day dietary background, the A diet (in the absence of CP) promoted a rejuvenation of the circulating erythrocyte population, depicted in the increase of younger classes (*viz*. 4 and 5, respectively on days 18 and 4) and decrease of a higher maturity stage (*viz*. class 3 on day 18). As stated before, this may result from a favouring of the erythropoiesis rate promoted by the algae supplemented feed.

The absence of alterations on the overall erythrocyte's maturity status related with CP injection points out that the expression of its genotoxic effect was not enhanced or masked by fluctuations on the erythrocyte dynamics/lifespan. On the other hand, CP inhibited the algae effect on the rejuvenation of circulating erythrocyte population, as depicted on the saline-injected groups. This can be indicative that the macroalgae components involved in the erythropoiesis promotion may have been driven to the genoprotective action against CP.

Conversely, the lower basal chromosomal damage observed after 4 days (A *vs*. S groups) cannot be dissociated from the dilution effect described before, since a promotion of a younger erythrocyte population occurred on fish fed with the A diet.

2.4.2. Oxytetracycline sub-trial

In the present study, the antibiotic OTC proved to be genotoxic, through the induction of chromosomal damage over gilthead seabream erythrocytes. This finding is in line with the results of Jerbi et al. (2011), reporting OTC chromosomal damage in erythrocytes of *D. labrax*. Likewise, it was demonstrated the induction of DNA breaks by this antibacterial

agent on blood cells of *O. niloticus* (Botelho et al. 2015). Moreover, Rodrigues et al. (2017) also reported the OTC genotoxicity (chromosomal damage and DNA breakage) on blood cells of *O. mykiss*. This agent, which mode of action on the target organisms relies on the inhibition of protein synthesis (Zounková et al. 2011), showed a great affinity to DNA, as other tetracyclines, leading to the formation of OTC-DNA binary complexes, which provoke alterations in the secondary structure of the native DNA double helix (Khan et al. 2003; Khan and Musarrat 2003). Regardless those studies confirming OTC potential to induce genetic damage on fish, the progression of OTC-induced genotoxicity on a post-treatment period was, to the authors' knowledge, never explored before.

Considering that the current antibiotic treatment consisted on a single fish immersion in the OTC solution (300 mg L^{-1}; 4 h), followed by the fish transference to OTC-free water, the uptake and metabolism rates may not be comparable to the ones regarding the antibiotic injection or oral administration. In fact, Rigos et al. (2006) studied the uptake of OTC (50 mg L^{-1}) in *S. aurata* during a 24-h bath (at 1, 3, 6 and 24 h), as well as at 1, 2, 3, 4 and 6 days following the bath, reporting detectable levels only at the end of the treatment (24 h) in muscle (0.096 µg g^{-1}) and plasma (0.047 µg mL^{-1}). In comparison with Rigos et al. (2006) conditions, the current OTC bath concentration was 6 times higher, though 6 times shorter. This suggests that OTC-induced chromosomal damage, as presently observed throughout the post-treatment period (at day 4 and 18 days) in non-supplemented groups (S_{OTC}), can be perceptible even when OTC body burdens stand below mensurable levels. In addition, considering that kidney may be the primary target organ when chemicals are taken up through the gills (Schlenk and Benson 2001), OTC may has reached preferentially this important haematopoietic organ, which can support the genotoxic damage observed on the erythrocytes during the post-treatment period.

Regarding the antigenotoxic potential of the algae-supplemented diet against OTC-induced damage, this was undeniable on day 4 (lower ENA levels, but not as low as the unexposed fish) and sharply notorious on day 18, when the OTC genotoxicity was completely barred. The putative explanations for this protective action are the same previously presented in CP sub-trial.

Through the EMI data, it was apparent a tendency towards an erythrocyte population aging 4 days after the OTC bath (translated in a concomitant increment of higher maturity cells and decrease of lower maturity cells). This propensity remained on day 18, though considerably less evident. The assessment of haematological alterations as possible toxic effects of OTC in fish was first addressed by Kreutzmann (1977), reporting a low red blood cell (RBC) count on *Anguilla anguilla*, and later confirmed by Ambili et al. (2013) on

Labeo rohita. A wider haematological disturbance was detected by Omoregie and Oyebanji (2002), who stated that this antibiotic is responsible for decreases in leukocyte, RBC, thrombocyte, haematocrit and haemoglobin values on *O. niloticus*. Although it cannot be fully demonstrated, the combination of these effects reflected in the bibliography and the present EMI data suggests a potential of OTC to tone-down haematopoiesis (and erythropoiesis in particular).

The analysis of the EMI profiles as a function of the diet provided somewhat surprising clues, considering the indications extracted from the 60-day dietary background and the CP sub-trial, since in the present sub-trial the algae supplementation (day 4) seems to limit erythrocyte renewal in the unchallenged group. This apparent divergence can only be explained by the circumstantial interference of specific fish handling factors and procedures associated with the sub-trial operationalization, which also highlights the erythrocyte dynamics as a complex net of cellular processes (cell proliferation and differentiation, maturation and removal) subject to a fine-tune. On the other hand, it should be highlighted that the algae supplementation promoted a regularization of the erythrocyte population dynamics, impairing the aging effect induced by OTC (both at day 4 and day 18). A causal relationship between this effect and the lower ENA frequency registered in OTC-exposed fish, when comparing algae supplemented and non-supplemented groups (S_{OTC} *vs.* A_{OTC}), should be considered. A higher persistence of older cells, probably including chromosomal disruptions, may potentiate the expression of genotoxicity as ENA frequency in S_{OTC} groups.

2.4.3. Formalin sub-trial

In this sub-trial, FOR demonstrated to be a strong genotoxic threat to *S. aurata* erythrocytes, since FOR baths resulted in high chromosomal damage, evident (only) in S_{FOR} groups, at both post-treatment moments. The fact that FOR (as the aqueous solution of gas formaldehyde) is genotoxic brings little novelty. For decades, several studies have demonstrated that formaldehyde is genotoxic and mutagenic to rodents and humans, as well as in cell cultures (*e.g.* Grafstrom et al. 1985; Craft et al. 1987; Ma and Harris 1988; Costa et al. 2008; Swenberg et al. 2012), being also considered carcinogenic to humans (IARC 2006). Besides that, this agent may also disrupt the balance between oxidants and antioxidants and cause oxidative stress, which, itself can cause DNA damage (Jerbi et al. 2011). Surprisingly, only a few studies addressed FOR genotoxic potential on fish, especially considering that it is a therapeutic agent frequently applied in aquaculture. To our knowledge, this is the first study assessing its genotoxicity on gilthead seabream

(simulating realistic procedures adopted in aquaculture) and evaluating the progression of the effect on a post-treatment period. The current results are in line with Jerbi et al. (2011), whose study also demonstrated that short-term repetitive FOR baths (200 µL L^{-1}; for 15 consecutive days) increased the chromosomal damage (as MN) in *D. labrax* erythrocytes. Comparatively, our study demonstrated that only two FOR baths (150 µL L^{-1}) of 1 h in alternate days are enough to induce diverse NA, that lasted up to 16 days after the last bath. Additionally, Mert et al. (2015) demonstrated the FOR genotoxicity through the general induction of MN and NA in *O. niloticus* erythrocytes, after treatment with sublethal concentrations. Furthermore, considering that treatment consisted on two fish immersions on the FOR solution for 1 h each, followed by the fish transference to FOR-free water, the uptake of the agent is limited by the time of exposure, which contrasts with longer baths. Accordingly, Jung et al. (2001) found that *Paralichthys olivaceus* and *Sebastes schlegeli* specimens treated with a 1-h FOR bath (100, 300 or 500 mg L^{-1}) displayed muscle concentrations of FOR similar to those of untreated fish just after 24 h. Hence, the long lasting of the chromosomal damage currently observed may be explained by FOR mode of action. Due to its electrophilic character, formaldehyde [as the major constituent of the aqueous solution FOR, and both presenting the same chemical reactivity (Kiernan 2000)] reacts with functional groups of several biological macromolecules (Leal et al. 2018). It is known to react with amine, thiol, hydroxyl, and amide groups to form various types of adducts (Barker et al. 2005). However, covalent DNA-protein crosslinks are the major class of DNA lesions associated with FOR, acting as bulky helix-distorting adducts, which can physically block DNA replication and transcription, ultimately, leading to the impairment of DNA metabolic machinery (Barker et al. 2005; Jerbi et al. 2011). Also, after its uptake through gills, FOR can accumulate in fish kidney, an important haematopoietic tissue, which can help to explain the generation/presence of NA upon the post-treatment period (in non-supplemented fish).

Taking FOR genotoxicity as departing point, the algae-enriched diet showed great antigenotoxic potential against it (groups A_{FOR}), in both samplings, through the maintenance of ENA level as lower as on the untreated groups (S and A).

EMI data, on day 4, pointed out a limitation of erythrocyte renewal in S_{FOR} group. Accordingly, Jerbi et al. (2011) also reported a lower frequency of immature erythrocytes, though after 1-h daily treatment to FOR during 15 days, which was regarded as a sign of cytotoxicity. These results contrast with previous studies describing an increase of immature erythrocyte frequency, as a response to FOR, on fishes *P. olivaceus* (Jung et al. 2003) and *Salmo gairdneri* (Smith and Piper 1972). Jung et al. (2003) only noticed these

effects after 3 h of FOR treatment (100, 212 and 300 μL L^{-1}) and no effects were measured after 1 h. Smith and Piper (1972) observed increases on immature erythrocytes as a response to hypoxia, resulting from damaged gill respiratory epithelium, a histopathological effect frequently associated to FOR treatment (*e.g.* Mert et al. 2015).

As described in OTC sub-trial, an older erythrocyte population was perceptible on day 4 in fish fed with A diet (in comparison with S diet). Once again, this punctual (circumscribed in time) effect shall be associated with the interference of fish handling factors related to the sub-trial implementation. More importantly, the algae supplementation favoured the readjustment of the erythrocyte population dynamics, overcoming the aging effect induced by FOR (at day 4). This regularization of erythrocyte lifespan probably played a role on the ENA frequency reduction observed in FOR-exposed fish, when comparing algae supplemented and non-supplemented groups (S_{FOR} *vs.* A_{FOR}).

Overall, the same hypotheses described for CP and OTC sub-trials should be considered as responsible for the antigenotoxicity observed on the A_{FOR} group.

2.5. Conclusions

The current study demonstrated that the aqua-medicines OTC and FOR are genotoxic to *S. aurata*, under realistic application conditions. Moreover, the chromosomal damage observed remained on a post-treatment period for 18 days.

The adopted algae-supplemented diet evidenced a solid genoprotection against the three agents tested (CP, OTC and FOR), notwithstanding the specificities of the inherent mechanisms of damage, disclosing a broad-spectrum action. This protective effect was particularly pronounced in FOR sub-trial, as the chromosome damaging potential of this therapeutic agent was completely blocked by the algae supplementation. The genoprotection under basal conditions (*i.e.*, in the absence of imposed exogenous challenges) was not categorically demonstrated but cannot be excluded.

The EMI assay pointed out an erythrocyte population instability induced by OTC and FOR, translated into an aging effect, which was counteracted by the algae-enriched feed. The underlying action of macroalgae components seems to normalize the replacement of a susceptible erythrocyte sub-population (denoting genetic damage) by less susceptible cells. In addition, algae supplementation seems to convey erythropoiesis promoting factors, with a subsequent rejuvenation of the erythrocyte population, which was especially noteworthy in unchallenged fish (dietary background) and in the CP sub-trial.

Finally, considering the genoprotection provided by the algae-enriched diet, the adoption of aquafeeds containing macroalgae such as *U. rigida*, *F. vesiculosus* and *G. gracilis* should be considered within a framework of sustainable aquaculture practices, namely as a prophylactic measure to mitigate productivity losses when the therapeutic agents OTC and FOR are used.

Ethical statement

This study was conducted in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes, with a certified operator authorized by the Portuguese Veterinary Directorate (approval no. 0421/000/000).

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Chapter 3

Searching for antigenotoxic properties of marine macroalgae dietary supplementation against endogenous and exogenous challenges

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3. Searching for antigenotoxic properties of marine macroalgae dietary supplementation against endogenous and exogenous challenges

Abstract

The functional characterization of marine macroalgae toward their potential to strength genome protection is still scarce. Hence, the aim of this study was to assess the antigenotoxic potential of *Ulva rigida*, *Fucus vesiculosus*, and *Gracilaria* species in *Drosophila melanogaster* following dietary exposure and adopting the somatic mutation and recombination test (SMART). All macroalgae displayed a genoprotection activity, namely against an exogenous challenge (streptonigrin). The action against subtler endogenous pressures was also noted indicating that supplementation level is a critical factor. *Gracilaria* species provided ambivalent indications, since 10% of *G. vermiculophylla* inhibited the egg laying and/or larvae development, while 10% of *G. gracilis* promoted spontaneous genotoxicity. The effects of *U. rigida* were modulated (in intensity) by the growing conditions, demonstrating higher genoprotection against streptonigrin-induced damage when grown in an aquaculture-controlled system, while the effectiveness against spontaneous genotoxicity was more apparent in specimens grown under wild conditions. In contrast, *F. vesiculosus* did not produce significant differences in its potential under varying growing conditions. Overall, these findings shed some light on the macroalgae ability toward genome protection, contributing to the development of algaculture industry, and reinforcing the concept of functional food and its benefits.

Keywords

Ulva; *Fucus*; *Gracilaria*; *Drosophila melanogaster*; Eye-spot test; Antigenotoxicity.

3.1. Introduction

The growing search for bioactive compounds with beneficial effects for human health promotes the challenge of building a cutting edge and scientifically supported knowledge (Moraes et al. 2011). In this context, marine macroalgae, also known as seaweeds, represent a prominent and promising resource, considering their large biological diversity and varied phytochemical composition, leading to a wide range of applications, namely in the nutritional/nutraceutical and pharmaceutical areas (Holdt and Kraan 2011; Desideri et al. 2016). Polysaccharides, lipids (*e.g.* polyunsaturated fatty acids, sterols), proteins, essential elements, and pigments appear as macroalgae constituents with recognized biological properties (Holdt and Kraan 2011; Desideri et al. 2016). These phytochemicals were reported to exhibit antiviral, antidiabetic (Gupta and Abu-Ghannam 2011), anti-hyperlipidaemic, anti-inflammatory and immunomodulatory (Mohamed et al. 2012) actions, as well as anti-tumour (Murphy et al. 2014), and neuroprotective (Wijesekara et al. 2011) properties. Furthermore, epidemiologic studies suggested a minor prevalence of diet-related chronic pathologies (*e.g.* cardiovascular diseases and cancer) in human populations, namely Asian, whose diets include macroalgae as a significant element (Yamori et al. 2001; Yuan and Walsh 2006). Consequently, the inclusion of algae in the diet as functional food has been recently defended by some authors (Holdt and Kraan 2011; Mohamed et al. 2012; Desideri et al. 2016). In this direction, macroalgae genome protective capacity is still a poorly explored subject, albeit several reports indicating antioxidant, antigenotoxic and antimutagenic properties were noted (Athukorala et al. 2006; Yuan and Walsh 2006; Celikler et al. 2008; Celikler et al. 2009b; Zubia et al. 2009; Valentão et al. 2010). Therefore, considering the importance of the genome integrity for the survival and proper functioning of biological systems, as well as the association between DNA damage and several diseases, it was of interest to examine the relationship between macroalgae properties and genome protection.

Some edible macroalgae of the genera *Ulva* (Chlorophyta), *Gracilaria* (Rhodophyta), and *Fucus* (Ochrophyta) are native species in the Atlantic coast and, in the case of *Ulva* and *Gracilaria*, relatively easy to grow in aquaculture; however, these species are not fully explored regarding potential biomedical and nutritional/nutraceutical uses. In fact, few studies were conducted on genome protective ability. Concerning green algae of the genus *Ulva*, ethanolic extracts of *U. rigida* decreased the micronuclei (MN) frequency in rats with diabetes mellitus (Celikler et al. 2009a) and hypothyroidism (Celikler et al. 2014). In addition, crude extracts of the same species alleviated clastogenic effects such as chromosome aberrations, sister chromatid exchanges and MN initiated by the

chemotherapeutic agent mitomycin-C in human lymphocytes cultured *in vitro* (Celikler et al. 2008). Rodeiro et al. (2015) found that an aqueous-ethanolic extract of *U. fasciata* inhibited the induction of micronucleated polychromatic erythrocytes by benzo[a]pyrene in rats. Although the antigenotoxic capacity of *U. lactuca* remains to be determined, an extract containing the sulphated polysaccharides fraction elevated antioxidant defences as evidenced by increased hepatic enzymatic activities of catalase (CAT), glutathione peroxidase (GP_x) and superoxide dismutase (SOD) and non-enzymatic reduced glutathione (GSH) and total thiols, while diminishing levels of lipid peroxidation in hypercholesterolemic rats (Hassan et al. 2011). Yang et al. (2012) demonstrated that an aqueous extract of the red alga *G. tenuistipitata* markedly reduced the oxidative DNA damage as measured through the comet-nuclear extract assay induced in a cell line by hydrogen peroxide (H_2O_2), while an ethanolic extract of *G. vermiculophylla* produced enhanced free radical scavenging potential (Farvin and Jacobsen 2013). Leite-Silva et al. (2007) observed that an aqueous extract of the brown alga *F. vesiculosus* was found to possess antigenotoxic potential against doxorubicin-induced DNA damage in human lymphocytes and the elevated antioxidant protection was attributed to the sulphated polysaccharides (Rupérez et al. 2002) and polyphenols (Zaragozá et al. 2008; Díaz-Rubio et al. 2009; Wang et al. 2012).

Taken together, it is recognized that most of the previously mentioned studies involved simplified approaches evaluating the influence of extracts or specific components of macroalgae through *in vitro* trials, noting a lack of knowledge regarding *in vivo* effects of the whole macroalgae ingestion on genome integrity maintenance. In this context, Holdt and Kraan (2011) mentioned legislation from European Advisory Services defending that functional foods should not be consumed as pills or capsules, but must remain as foods, demonstrating their effects in amounts that can normally be expected to be consumed in the diet (EAS, 2008).

The fruit fly *Drosophila melanogaster* is one of the most intensively studied organisms, serving as a model system for the investigation of diverse developmental and cellular processes, common to higher eukaryotes including humans (Adams et al. 2000; Alaraby et al. 2016). In fact, many molecular pathways required for the development of a complex animal have been highly conserved since the evolutionary divergence of flies and humans and, once disrupted, similar consequences are frequently observed in invertebrates and vertebrates (Reiter et al. 2001). Furthermore, this model has been adopted in several genotoxicity/antigenotoxicity studies (Siddique et al. 2005; Carmona et al. 2011; Carmona et al. 2017) and, in particular, assessing the effects of a wide range of

agents incorporated in their diet (Romero-Jiménez et al. 2005; Fernández-Bedmar et al. 2011; Amkiss et al. 2013; Alaraby et al. 2015; Alaraby et al. 2016). However, to date, the potential use of drosophila as a model to assess the genome protective capacities of macroalgae remains to be determined.

Hence, considering the previously identified knowledge gaps, the aim of the current study was to examine the beneficial effects on genome protection provided by specimens from three macroalgae genera, commonly found in the Atlantic coast: *Ulva*, *Gracilaria* and *Fucus*. Taking into account the difficulty to clearly identify the algae species on the basis of morphological analysis (Saunders 2005), the different spatial distribution of morphologically similar species and possible influence of growing conditions on the algae phytochemical composition, two sampling sites were selected for each genus, to provide a broader representativeness. The beneficial potential of the algae was postulated through involvement of an increased genome protection. Thus, the antigenotoxic potential of those macroalgae against spontaneous genotoxicity and genotoxicity induced by streptonigrin, a well-known genotoxic agent (Bolzán and Bianchi 2001), was assessed utilizing *D. melanogaster* following dietary exposure. For this purpose, the somatic mutation and recombination test (SMART) was adopted, enabling assessment of genetic damage in somatic cells of adult flies after larval feeding with macroalgae, simulating the exposure route for human consumption.

3.2. Material and methods

3.2.1. Chemicals

The instant treatment Carolina Drosophila Medium Formula 4-24® (hereinafter referred as Instant Drosophila Medium - IDM) was purchased from Carolina Biological Supply Company, Burlington, USA. Streptonigrin (CAS 3930-19-6) was obtained from Santa Cruz Biotechnology Inc., Texas, USA. All other chemicals were purchased from the Sigma-Aldrich Chemical Company (Madrid, Spain).

3.2.2. Macroalgae harvesting and preparation

For each macroalgae genus addressed, two harvesting sites were selected and designated “site 1” and “site 2”. The two macroalgae batches from the genus *Ulva* (sea lettuce) were collected at the Mindelo beach, Vila do Conde, Portugal (41°18'36.8"N, 8°44'25.9"W; site 1 – U1) and obtained from an integrated multi-trophic aquaculture (IMTA) system at ALGAplus, Lda., Ílhavo, Portugal (certified organic production) (site 2 – U2), in the same temporal period (September 2015). Similarly, *Fucus* specimens

(bladderwrack) were collected at the Mindelo beach in September 2015 (site 1 – F1) and in a Ria de Aveiro area, Portugal (Canal de Ílhavo; 40°36'45.4"N 8°40'44.6"W) surrounding the ALGAplus facilities, where it was maintained for 1 week and harvested in June 2015 (site 2 – F2). *Gracilaria* specimens (ogonori) were collected in Ria de Aveiro, at Torrão do Lameiro, Ovar, Portugal (40°49'33.1"N 8°39'58.2"W) in November 2015 (site 1 – G1) and obtained from the ALGAplus facilities in September 2015 (site 2 – G2), respectively. The areas selected to harvest the wild batches have no apparent known punctual sources of contamination and previous environmental surveys on those locations corroborates that status (Pacheco et al. 2005; Reis et al. 2014).

Following harvesting, macroalgae batches were washed in seawater (treated with UV and filtered to 5 µm) and then dried during 12 h in a chamber with controlled temperature (25 ºC), achieving 10-12% of humidity, after which they were preserved in multiple layer packaging (paper and plastic) until further experimental procedures. Prior to the experiments, macroalgae were grinded with a coffee mill, obtaining particles with < 1 mm for *Ulva* and *Fucus* specimens and < 2 mm for *Gracilaria* specimens.

3.2.3. Macroalgae identification

Macroalgae species can be difficult to identify owing to their relatively simple morphology and anatomy, convergence, phenotypic plasticity, and alternation of heteromorphic generations (Saunders 2005). Hence, identification of *Ulva* and *Gracilaria* specimens was performed based upon morphological traits, known geographical distribution and by DNA-barcoding, while *Fucus* specimens were identified based upon morphology as *F. vesiculosus* – F1 and F2.

3.2.3.1. DNA extraction, amplification and sequencing

Approximately 5 mg pieces of dried tissue of both *Ulva and Gracilaria* specimens were frozen in liquid nitrogen and homogenized using a pestle and a mortar. The DNA of homogenates was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. DNA-barcoding of algae was performed using partial *rbcL* gene (large subunit of the ribulose-bisphosphate carboxylase/oxygenase) with taxon specific primers for *Ulva* (rbcLF and rbcR, Pierce et al. 2006) and *Gracilaria* (FrbcLF and R753, Hommersand et al. 1994). Amplification reactions were performed with 0.5 µL of genomic DNA in 20 µL of total volume reaction supplied with 5.5 µL sterilized water, 2 µL Qiagen Q-Solution, 10 µL double concentrated Qiagen Multiplex PCR Master Mix and 1 µL 10 pmol/µL concentrated of each primer. The

amplification of *rbcL* was performed by an initial denaturation of 15 min at 95 °C, followed by 9 touch-down cycles at 94 °C for 45 s, 60 °C (-1 °C per cycle) for 45 s, 72 °C for 90 s, followed by 25 standard cycles (94 °C for 45 s, 51 °C for 45 s and 72 °C for 90 s) and a final extension at 72 °C for 10 min. Amplicons were purified using ExoSAP-IT (Applied Biosystems, Foster City, USA) following the manufacturer's instructions. Sequencing was performed by Macrogen (Amsterdam, The Netherlands) with the same primers used for the amplification.

3.2.3.2. Sequence analysis and species identification

Sequence quality was individually assessed using PhyDE (v. 0.9971) and forward and reverse sequences combined to a consensus sequence (no ambiguous sites were detected). Sequence identity was determined by a nucleotide blast search (Basic Local Alignment Search Tool) against the nucleotide collection database of the National Center for Biotechnology Information (NCBI). For species identification *rbcL* sequences of various *Ulva* and *Gracilaria* species (Appendix II – Table 1) were downloaded from the NCBI database and alignments (572 bp for *Ulva* and 688 bp for *Gracilaria,* respectively) created using MAFFT (v. 7, Katoh and Standley 2013) in the G-INS-i mode for each species individually. Maximum likelihood trees were calculated using IQ-TREE (v. 1.6.5, Nguyen et al. 2014) in default mode with pseudo-replicates of 1000 bootstraps using the TN+F+I model for *Ulva* and the TIM+F+G4 model for *Gracilaria*, based on the Bayesian Information Criterion scores (BIC) determined by IQTREE. Species identification was based on clustering in the maximum likelihood tree (Appendix II – Figure 1) and sequence similarity based on p-distances (Appendix II – Table 1) between taxa in the respective cluster. Based on this analysis and on known species distribution, *Ulva* 1 (U1) could be identified as *U. rigida*. *Ulva* 2 (U2) was already genetically determined by ALGAplus as *U. rigida*, as well. *Gracilaria* 1 (G1) could be identified as *G. vermiculophylla* and *Gracilaria* 2 (G2) as *G. gracilis*.

Sequences were deposited at NCBI with accession number MH682138 for *Ulva* 1 and MH682140 and MH682139 for *Gracilaria* 1 and 2, respectively.

3.2.4. Experimental design

D. melanogaster strain Oregon-K (*Ok*) was chosen since a study from Gaivão and Comendador (1996) noted that between 6 strains with different metabolic activities, it is one of the most suitable strains to be used for genotoxicological assays. The *Ok* strain presented the highest susceptibility for reactive oxygen species (ROS) formation when

exposed to toxic chemicals and the lowest activity of antioxidant enzymes, being this last one necessary to restrict the antioxidant activity to non-enzymatic antioxidants, which are mainly obtained through diet. Two distinct pairs of alleles for the sex-linked *white* (*w*) gene were used: wild-type with red eyes (w^+/w^+, *Ok-yellow*) and mutant with white eyes (*w*/*w*, *Ok-white*). The *Ok* strain with the two different phenotypes for eye colour was kindly provided by Professor E. W. Vogel (Leiden, The Netherlands).

Stock cultures were maintained in a chamber at 25 ºC, with relative humidity of approximately 60%, in culture vials containing 20 mL standard lab-made growth medium (10% sucrose; 10% yeast; 1.2% agar-agar; 0.2% NaCl; 0.5% of propionic acid in water). Since this growth medium needs to be heated to 90 ºC, to prevent denaturation of macroalgae bioactive components, the IDM (6 g of medium hydrated with 20 mL phosphate buffered saline - PBS) was used when performing the dietary trials as it needs neither cooking nor sterilizing. When macroalgae supplementation was intended, the appropriate quantity of grinded algae was mixed with the dry powder medium before hydration for a complete IDM preparation.

An independent experiment was carried out for each macroalgae genus addressed, *i.e.* *Ulva*, *Fucus* and *Gracilaria*. As represented in Figure 3.1, 20 virgin females *Ok-yellow* were mass-crossed with 20 males *Ok-white* in glass culture vials with approximately 20 mL standard growth medium for 2 days. Then, couples were transferred to other culture vials (in triplicate for each condition) containing 20 mL of IDM, and 2 major groups were formed: one unchallenged and another challenged with streptonigrin (S). The first major group was further divided into 3 subgroups (see Figure 3.1): (i) control (C; with IDM and no algae supplementation), (ii) supplementation with algae from site 1 (X1, where X is replaced in the groups' abbreviation by U, F or G, representing, respectively *Ulva*, *Fucus* or *Gracilaria*; algae incorporated in IDM) and (iii) supplementation with algae from site 2 (X2; algae incorporated in IDM). The second major group was also divided into 3 subgroups: (i) streptonigrin (S; with IDM and no algae supplementation), (ii) streptonigrin plus supplementation with algae from site 1 (S + X1; streptonigrin added to algae-supplemented IDM) and (iii) streptonigrin plus supplementation with algae from site 2 (S + X2; streptonigrin added to algae-supplemented IDM). Considering the final medium volume, streptonigrin was added to the IDM, dissolved in PBS, to attain the final concentration of 20 µM. This concentration was selected according to the literature (Gaivão et al. 1999). In contrast, as algae are taken here as functional foods, their levels of supplementation were expressed as % alga relatively to IDM (weight/weight), to enable a better perception of the ingested amount in relation to the whole food intake. This option

was previously adopted in several studies with comparable goals (Sousa et al. 2009; Rezende et al. 2013). Two levels of supplementation were investigated for each alga origin/site, as follows: 2.5 or 5.0% for *U. rigida* batches, 1.25 or 5.0% for *F. vesiculosus* batches, and 1.25 or 10.0% for *Gracilaria* batches, according to a preliminary study (Appendix II – Table 2). In those preliminary experiments, a broader range of algae concentrations (1.25 – 20.0%) was tested and the prolificacy (nº of hatched individuals) per condition was recorded. The selection of supplementation levels to test in the present study relied on identification of the two lowest algae concentrations showing the higher prolificacy.

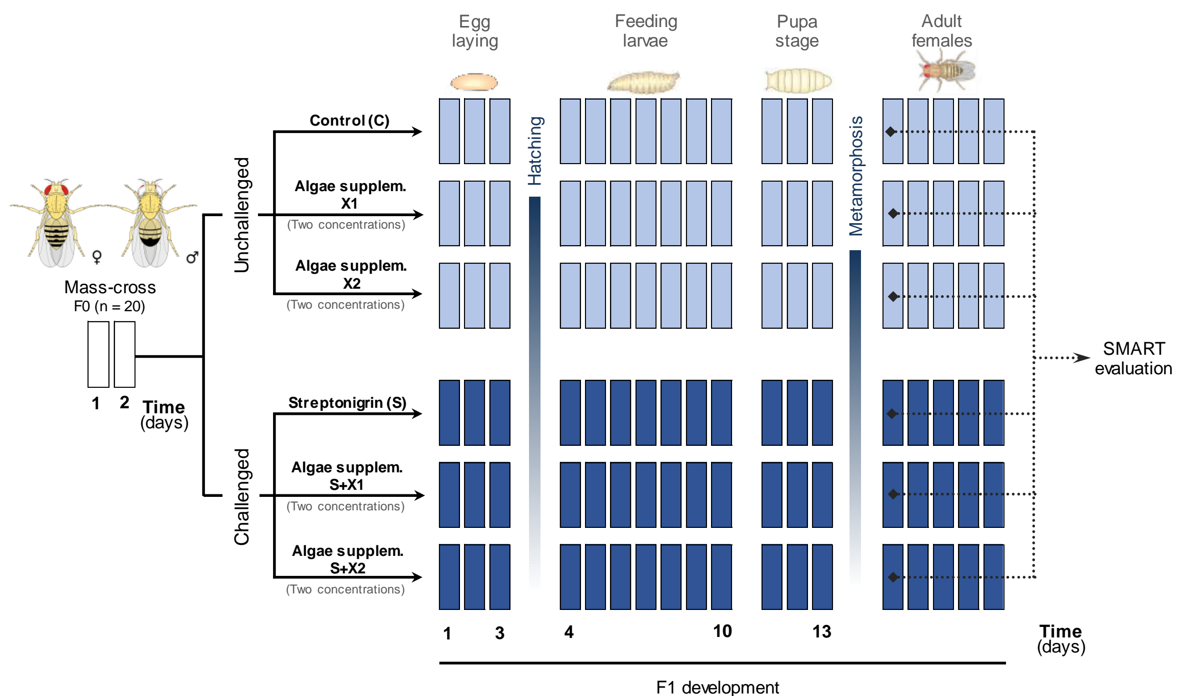

Figure 3.1. Schematic representation of the experimental design, elucidating the mass-cross of *D. melanogaster* (2 days), followed by the egg laying (3 days) and the subsequent development of F1 generation, in which SMART procedure was applied to adult females following metamorphosis. Couples were divided into two major groups: one unchallenged (light blue time scale) and another challenged (dark blue time scale) with streptonigrin (S). An independent experiment was carried out for each macroalgae genus addressed, where the previous groups were split into three subgroups, corresponding to no algae supplementation (C or S, respectively for unchallenged or streptonigrin-challenged groups), alga supplementation from sampling site 1 (X1 or S + X1; X represents the genus, *i.e. Ulva*, *Fucus* or *Gracilaria*) and site 2 (X2 or S + X2). Two levels of dietary supplementation were tested for each alga origin (not depicted).

The mated flies could lay eggs for 3 days, after which adults (F_0) were discarded. Hence, according to the *D. melanogaster* life cycle, it was expected that the feeding larvae were exposed to the algae/streptonigrin *via* ingestion for approximately 5-7 days (see Figure 3.1), after which it follows the pupa stage (approximately 3-5 days). Culture vials were maintained at 25 ºC until F_1 adults reached metamorphosis, moment from which the SMART analysis was accomplished in female flies (5-8 days).

3.2.5. Somatic mutation and recombination test

Flies were first etherized and maintained in a 10x diluted solution consisting of ethanol, milli-Q water and Triton X-100 (90:9:1; v/v/v) during scoring. Four hundred eyes per condition (equitably taken from the 3 replicates) from F_1 adult heterozygous females were analysed and inspected (two eyes per individual) under 40x magnification (Leica Wild M3Z stereo microscope, with Hund Wetzlar halogen optic fiber light source), for the presence of white spots as recommended by Vogel and Nivard (1993). The spots size (according to the nº of affected ommatidia) was recorded, which resulted in the classification of the spots as small (1-2 ommatidia affected) or large (> 2 ommatidia affected). Total spots were calculated by the sum of small and large spots. Results were expressed as the number of spots (small, large or total) per 400 eyes observed. The inhibition percentage (IP) of genotoxic events (spontaneously-generated or streptonigrin-induced) by the macroalgae was calculated for total spots as proposed by Abraham (1994) through the following formulae, respectively for unchallenged and challenged groups:

$$IP\ (\%) = [(\text{control} - \text{algae supplementation}) / \text{control})] \times 100$$

or

$$IP_S\ (\%) = [(\text{streptonigrin alone} - \text{streptonigrin plus algae supplementation}) / \text{streptonigrin alone})] \times 100.$$

3.2.6. Data analysis

Considering that eye spots data were extracted from three replicates, a first analysis focused on checking for statistical differences between triplicates was performed using a χ^2 test (Franke et al. 2012). When statistical differences were observed, the replicate inducing the deviation was eliminated. Thereafter, a double decision test of χ^2 was applied according to Frei and Würgler (1988) to detect statistical differences between groups and decide if the result is positive (+), weakly positive (w+), negative (n) or inconclusive (i) (H_0:

the number of spots is equal in the two groups; H_1: the number of spots is different *m* times between the two groups).

To test the effect of algae supplementation against the spontaneous genotoxicity, all comparable groups (with one independent variable, *i.e.*, % of supplementation or algae origin) were compared within unchallenged groups. The equivalent comparisons were carried out within challenged groups to appraise the effect of algae supplementation against the genotoxicity induced by streptonigrin. Thus, to confirm the effect of the genotoxic agent, the corresponding groups with and without streptonigrin treatments were also compared.

A multiplication factor (*m*) = 2 was applied when small and total spots were under analysis, while a *m* = 5 was applied when large spots were evaluated (Frei and Würgler 1988) as well as in the comparisons directly made between groups with and without streptonigrin (for the three parameters). The criterion for significance for data analysis was set at $p < 0.05$.

3.3. Results

3.3.1. Effects of dietary supplementation with *U. rigida*

Regarding the reduction of the spontaneous genotoxicity rate (*i.e.* without streptonigrin challenge) in *D. melanogaster*, the lowest concentration of wild-harvested *U. rigida* (2.5U1) exhibited the most promising result as this macroalga decreased the number of spots relative to control, when small and total spots were analysed (Figure 3.2). The IP values (as total spots) for spontaneously-generated genotoxicity were 38.6, 16.1 and 19.1% for 2.5U1, 5U1 and 5U2 conditions, respectively.

Considering that chronic exposure to streptonigrin increased the genotoxicity in *D. melanogaster* as observed in all the parameters and for all diet conditions (light green *vs.* dark green columns) (Figure 3.2), the antigenotoxic potential of *U. rigida* toward that genotoxic insult might be subsequently assessed. Thus, both alga concentrations, from both origins, significantly lowered the number of small and total spots compared to streptonigrin group without algae supplementation (S), as opposed to large spots that displayed no significant differences. The values for the IP_S were 19.4, 19.7, 54.1 and 55.4% for S + 2.5U1, S + 5U1, S + 2.5U2 and S + 5U2 groups, respectively. Further, in challenged subgroups, aquacultured *U. rigida* (U2), at both concentrations, presented higher antigenotoxic potential than wild-harvested *U. rigida* (U1), particularly on small and total spots (Figure 3.2). When analysing large and total spots, the 3 replicates of S + 2.5U2 group displayed significant differences between each other and, for that reason,

those values were not illustrated in the graphs nor considered in the statistical comparisons (identified in Figure 3.2 as not determined = n.d.).

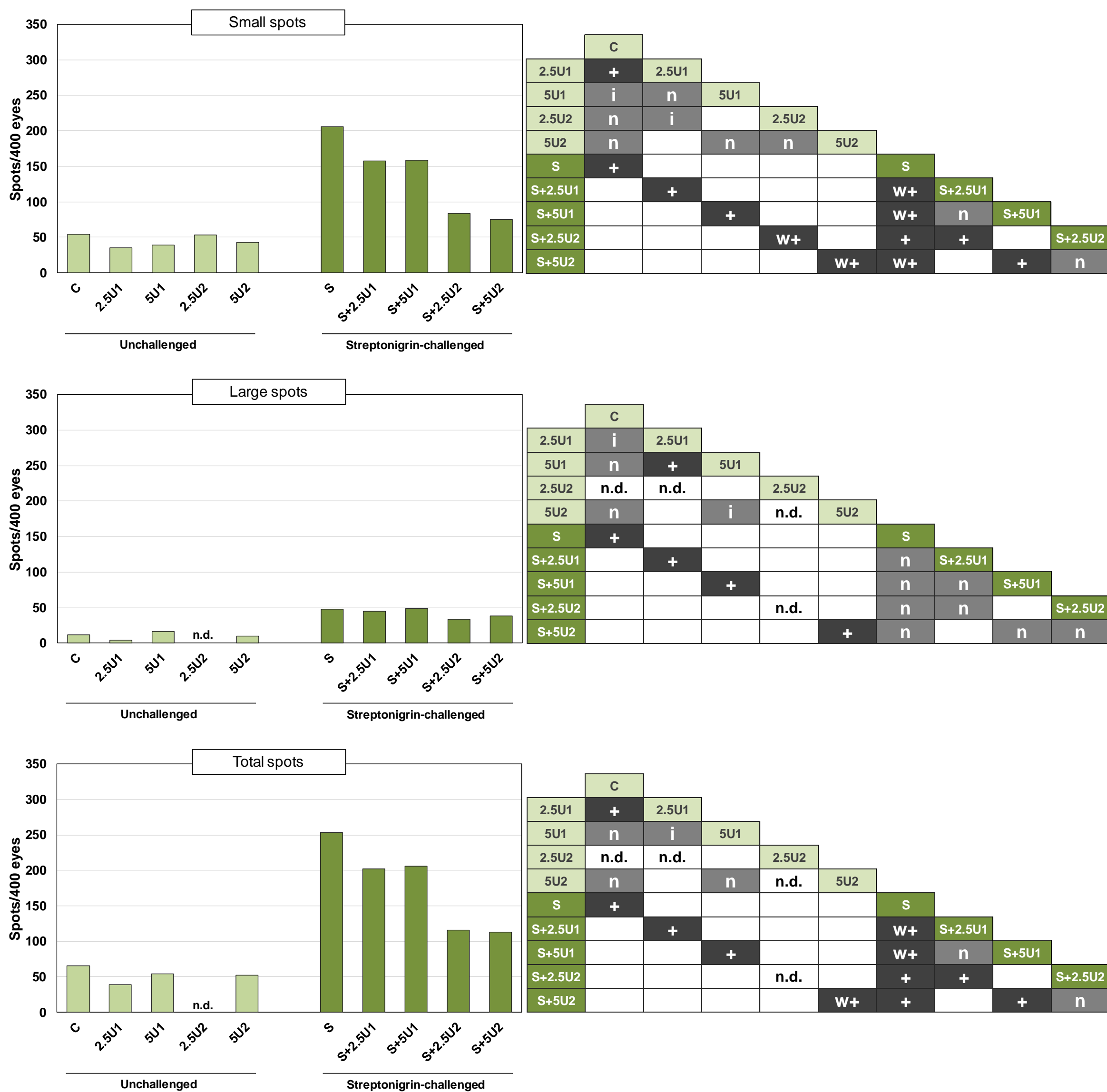

Figure 3.2. *Ulva* supplementation - number of small, large and total spots per 400 eyes observed in the *D. melanogaster* eye spot test and the respective table with statistical diagnosis (only comparable conditions were depicted; i, inconclusive data; +, positive; w+, weakly positive; n, negative), according to Frei and Würgler (1988). Light green columns (in the graph) and cells (in the table) correspond to unchallenged groups and dark green columns and cells correspond to streptonigrin-challenged groups (S). *U. rigida* (U1 or U2) was incorporated in the Instant Drosophila Medium in a percentage shown by the number preceding the letter indicative of the alga supplementation in groups' abbreviation (for comparison purposes, groups without algae incorporation were considered - C or S). n.d., not determined.

3.3.2. Effects of dietary supplementation with *F. vesiculosus*

Considering the potential to reduce spontaneous genotoxicity, the lowest concentration of *F. vesiculosus* 2 (1.25F2) showed the most marked effect, as evidenced by the significantly lower number of small and total spots, compared to control (C) (Figure 3.3). At this level of supplementation and in the absence of streptonigrin challenge, F2 batch demonstrated higher antigenotoxic potential (considering small and total spots) compared to the F1 batch. In addition, 1.25F2 group exhibited lower spontaneous genotoxicity compared to 5F2 (Figure 3.3). The IP values were 15.1, 17, 66 and 17.5% for 1.25F1, 5F1, 1.25F2 and 5F2 groups, respectively.

The genotoxic potential of streptonigrin was confirmed, since exposed groups demonstrated a significant increase in eye spots (in all parameters) relative to untreated groups (Figure 3.3). Hence, the antigenotoxic potential of *F. vesiculosus* was evident in the number of diminished small and total spots in algae-supplemented relative to non-supplemented group (S), regardless of the concentration and/or origin of the algae (Figure 3.3). The IP_S values were 23.7, 28.4, 24.3 and 27.9% for S + 1.25F1, S + 5F1, S + 1.25F2 and S + 5F2 groups, respectively.

3.3.3. Effects of dietary supplementation with *Gracilaria* species

Regarding the reduction of the spontaneous genotoxicity in *D. melanogaster* fed with *Gracilaria*, it could be noted that the lowest doses of *G. vermiculophylla* (1.25G1) and *G. gracilis* (1.25G2) showed the best results, particularly in the large and total spots parameters (Figure 3.4). In contrast, the highest concentration of *G. gracilis* (10G2) induced a significant rise of the small and total spots, compared to control (C) and to the lowest supplementation level (1.25G2). The values for the IP were 51, 56.2 and -14.1% for 1.25G1, 1.25G2 and 10G2 groups, respectively.

The streptonigrin-exposed groups presented higher values of eye spots in all parameters in comparison to non-treated flies (Figure 3.4). With respect to the antigenotoxic potential against streptonigrin insult, the lowest supplementation level, for both origins enhanced reduction of spots in particular to *G. vermiculophylla* (1.25G1) that was effective in the three parameters evaluated. The highest concentration of *G. gracilis* (10G2) also displayed beneficial effects as evidenced by inhibition of streptonigrin-induced genotoxicity expressed as small, large and total spots. The IP_S values were 48.6, 23 and 38.3%, respectively for S + 1.25G1, S + 1.25G2 and S + 10G2 groups. In the groups supplemented with the highest concentration of *G. vermiculophylla* (10G1), in both

unchallenged and streptonigrin-challenged groups, there were insufficient hatchings (i.h.) to perform the SMART assay.

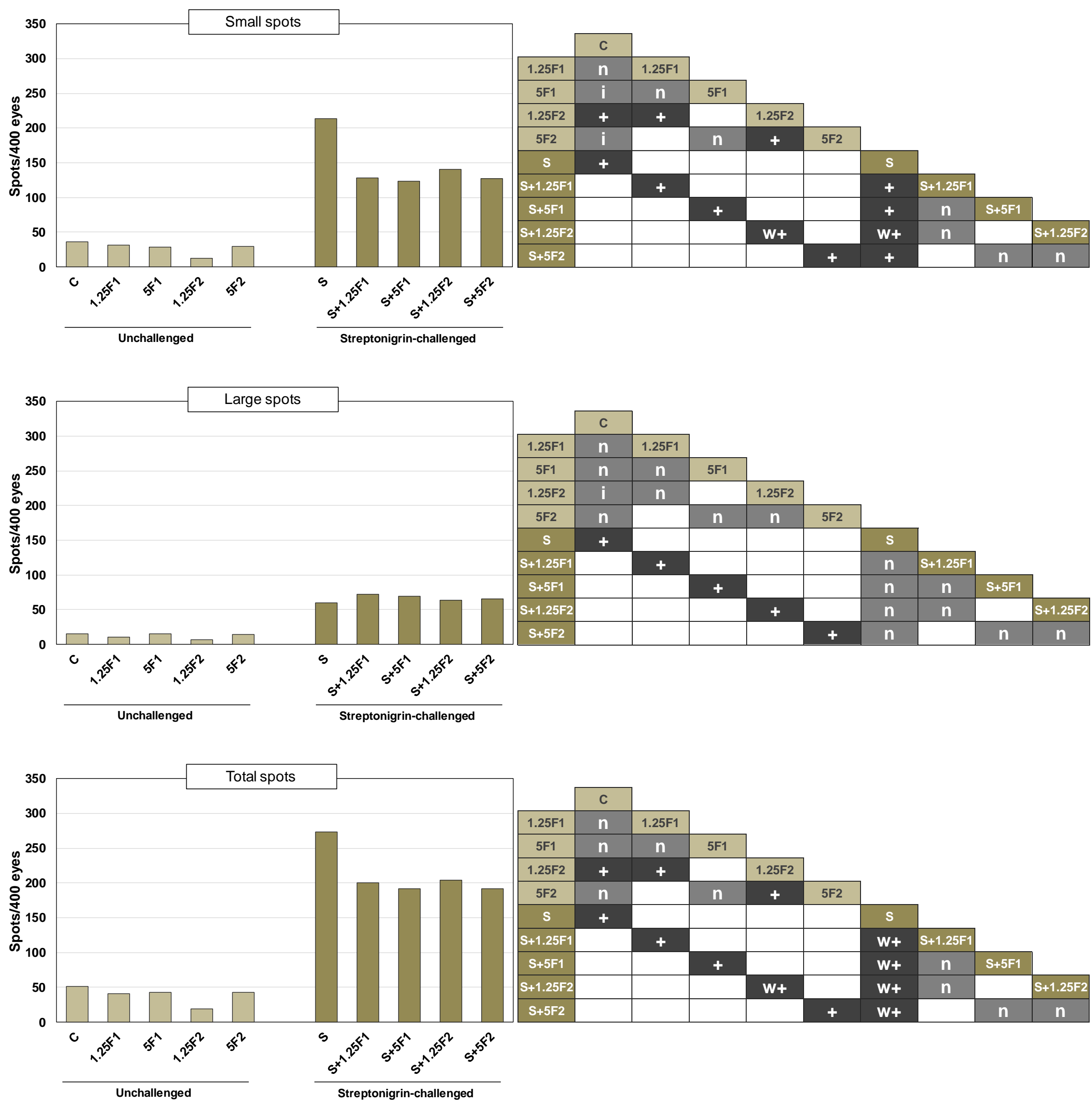

Figure 3.3. *Fucus* supplementation - number of small, large and total spots per 400 eyes observed in the *D. melanogaster* eye spot test and the respective table with statistical diagnosis (only comparable conditions were depicted; i, inconclusive data; +, positive; w+, weakly positive; n, negative), according to Frei and Würgler (1988). Light brown columns (in the graph) and cells (in the table) correspond to unchallenged groups and dark brown columns and cells correspond to streptonigrin-challenged groups (S). *F. vesiculosus* (F1 or F2) was incorporated in the Instant Drosophila Medium in a percentage shown by the number preceding the letter indicative of the alga supplementation in groups' abbreviation (for comparison purposes, groups without algae incorporation were considered - C or S).

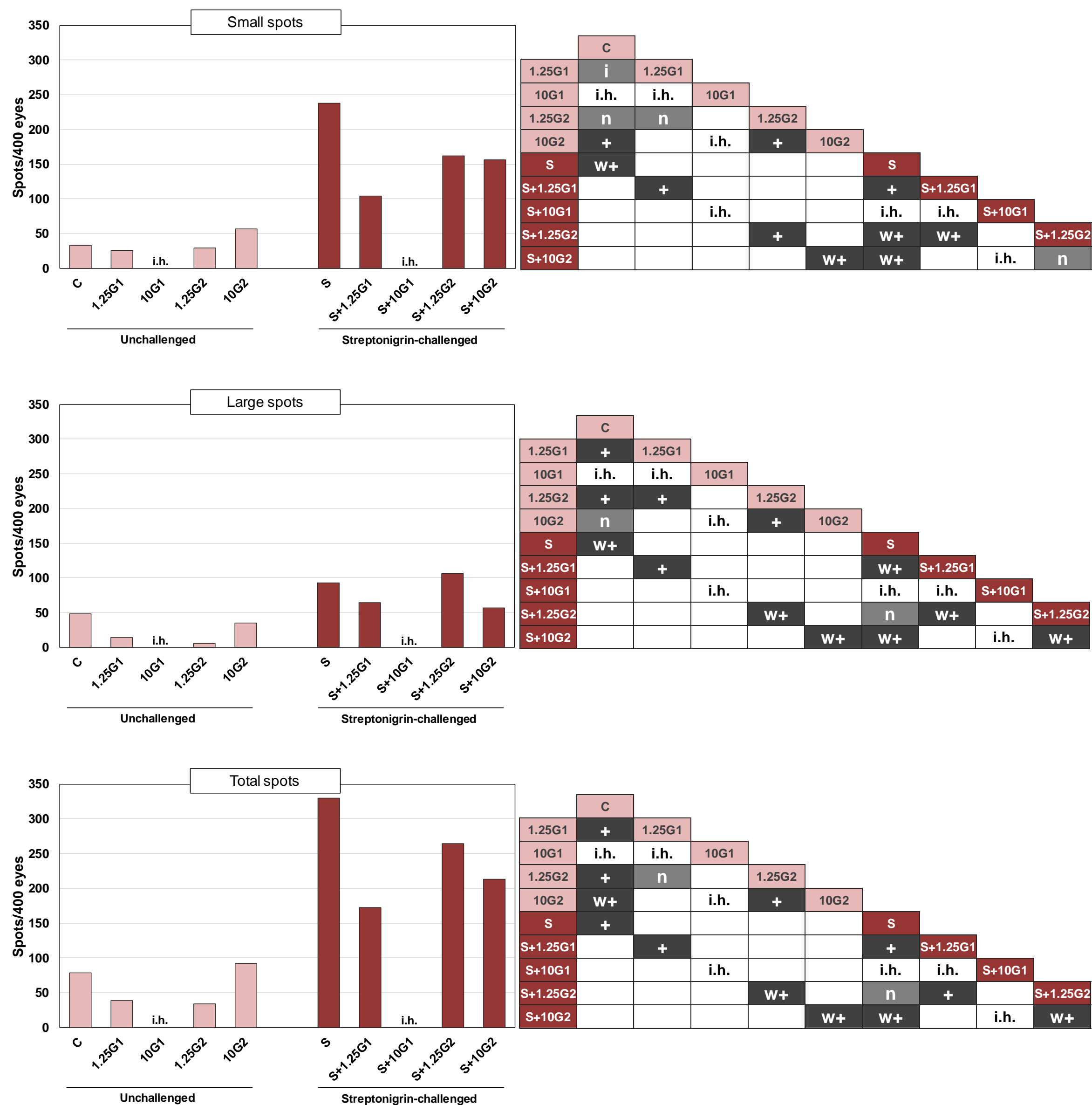

Figure 3.4. *Gracilaria* supplementation - number of small, large and total spots per 400 eyes observed in the *D. melanogaster* eye spot test and the respective table with statistical diagnosis (only comparable conditions were depicted; i, inconclusive data; +, positive; w+, weakly positive; n, negative), according to Frei and Würgler (1988). Light red columns (in the graph) and cells (in the table) correspond to unchallenged groups and dark red columns and cells correspond to streptonigrin-challenged groups (S). *G. vermiculophylla* (G1) or *G. gracilis* (G2) was incorporated in Instant Drosophila Medium in a percentage shown by the number preceding the letter indicative of the alga supplementation in groups' abbreviation (for comparison purposes, groups without algae incorporation were considered - C or S). i.h., insufficient hatchings.

Comparing both *Gracilaria* species/origins at the lowest supplementation level (*i.e.*, 1.25G1 *vs.* 1.25G2), a more pronounced protective effect of *G. vermiculophylla* (G1) against streptonigrin-mediated genotoxicity was evident.

3.4. Discussion

The link between DNA damage, mutagenesis and malignant transformation has long been established (O'Driscoll 2012). Bearing in mind the potential of macroalgae in nutraceutical applications, the current study aimed to identify the beneficial effects of recognizably edible *Ulva*, *Fucus* and *Gracilaria* species providing insights into genoprotection actions associated with dietary consumption.

It is well-established that the growing conditions of macroalgae might determine their nutritional composition (Lahaye et al. 1995; Gómez-Pinchetti et al. 1998; Abreu et al. 2009). It thus seems relevant to compare the beneficial properties of macroalgae reared in aquaculture and wild-harvested which face higher fluctuations of the growing conditions, since environmental factors such as temperature, light and salinity (varying with season and location), are known to directly influence the variety and quantity of macroalgae constituents (Marinho-Soriano et al. 2006). However, a comparative approach assessing beneficial effects in association with macroalgae origin has apparently not been reported.

Different species may present distinct phytochemical profiles and, consequently, divergent biological effects. In relation to phylogenetically close species, as the ones belonging to the same genus, different patterns were previously described (Kumari et al. 2013; Mehdinezhad et al. 2016), but this phenomenon still requires further investigation.

During the initial stages of the fly development, larvae possess precursor structures termed imaginal discs that are substantially transformed through metamorphosis into well-developed adult appendages (Beira and Paro 2016). For instance, eye-antennal discs give rise to the compound eye and the antenna of the adult flies through successive mitosis (Beira and Paro 2016). Thus, genotoxic events occurring in imaginal discs may be generated and examined at later developmental stages (Beira and Paro 2016). In fact, adoption of drosophila somatic cells for genotoxicity testing has been performed for decades since Mollet and Würgler (1974). In heterozygous individuals, certain genetic events, namely gene point mutation, deletion, non-disjunction of the homologous chromosomes and homologous recombination might lead to loss of dominant wild-type allele, with subsequent expression of the recessive marker in the form of clones or spots on the eyes of the adult flies (Graf et al. 1984; Graf and Würgler 1996).

3.4.1. Antigenotoxic potential of *U. rigida*

The current supplementation trial addressing the properties of the green alga *U. rigida* clearly revealed an antigenotoxic action especially toward the genotoxic insult induced by streptonigrin, which was particularly evident when aquacultured *U. rigida* (U2) was evaluated (small and total spots analysis). Regarding the potential against the spontaneous genotoxicity, only the lowest dose of wild-harvested *U. rigida* (2.5U1) was able to minimize the small and total spots generation. Among the algae phytochemicals with genome protective ability, Celikler et al. (2009a; 2014) and Yildiz et al. (2012) noted that chlorophyll a and b, carotenoids, vitamins A, C and E, and phenolic compounds most likely contributed to the antioxidant and antigenotoxic properties of *U. rigida*. Mezghani et al. (2016) also found that *U. rigida* extracts possessed large phenolic content with potent antioxidant activity. In addition, ulvan, a water-soluble sulphated polysaccharide extracted from green macroalgae belonging to the order Ulvales (*e.g. Ulva* genus), and its derivatives was suggested to possess antioxidant (Qi et al. 2005; Lahaye and Robic 2007; Yildiz et al. 2014; Rahimi et al. 2016) and anticarcinogenic (Abd-Ellatef et al. 2017) activities. Although antigenotoxic effects were observed in this investigation, the results did not reflect a linear relationship between efficacy and dose of *U. rigida* supplementation. This was particularly evident in the unchallenged groups, since the increase of *U. rigida* incorporation impaired the antigenotoxic protection. Further, the antigenotoxicity noted against streptonigrin was similar regardless alga dose for both algae origins. In contrast, the higher antigenotoxic potential against streptonigrin detected in aquacultured *vs.* wild-harvested *U. rigida* might be due to variations in the phytochemicals' variety and/or quantity. This seems plausible assuming that the growing conditions influence the nutritional composition of the macroalgae (Lahaye et al. 1995; Gómez-Pinchetti et al. 1998; Abreu et al. 2009).

3.4.2. Antigenotoxic potential of *F. vesiculosus*

Data also demonstrated a significant antigenotoxic potential of this brown alga, mainly in flies exposed to streptonigrin. Moreover, the lower dose of *F. vesiculosus* 2 (1.25F2) was the only condition able to diminish the spontaneous genotoxic effects as evidenced by small and total spots. Similarly in a previous study Valente et al. (2014) noted that a mixture of macroalgae containing *F. vesiculosus* exhibited antigenotoxic potential in *D. melanogaster* exposed to streptonigrin. Leite-Silva and colleagues (2007) also found that a *F. vesiculosus* extract showed antigenotoxic potential in cultured human lymphocytes against doxorubicin-induced chromosomal and DNA damage. Although a phytochemical

characterization was not addressed in the present study, *F. vesiculosus* is particularly rich in polyphenols (Díaz-Rubio et al. 2009), namely phlorotannins believed to act as antioxidants due to their redox properties (Athukorala et al. 2006; Wang et al. 2012; Farvin and Jacobsen 2013), as well as polysaccharides not digestible by the gastrointestinal enzymes, and thus considered as dietary fibre (Díaz-Rubio et al. 2009). Subsequently, these polysaccharides, including fucans (*e.g.* fucoidan), alginates, laminaranes and cellulose (Rioux et al. 2007), were demonstrated to exhibit antioxidant (Hu et al. 2001; Rupérez et al. 2002), antigenotoxic (Okai and Higashi-Okai 1994; Gamal-Eldeen et al. 2013) and anti-tumoral (Maruyama et al. 2003; Alekseyenko et al. 2007; Raafat et al. 2014) properties. In addition, several investigators identified natural pigments present in brown algae, namely fucoxanthin, as possessing antioxidant (Yan et al. 1999), and pheophytin as antigenotoxic (Okai and Higashi-Okai 1997) and anticarcinogenic (Higashi-Okai et al. 1999) effects. Hence thus seeming plausible to include them among the phytochemicals providing to *F. vesiculosus* the genome protective properties currently documented. Hence, it may be presumed that the antigenotoxic capacity of *F. vesiculosus* might be attributed to a synergistic effect of polyphenols, sulphated polysaccharides and chlorophyll-related compounds.

The increase of *F. vesiculosus* incorporation in the *D. melanogaster* diet was not accompanied by an augmented antigenotoxic protection, in relation to both spontaneous and streptonigrin-induced genotoxicity. In fact, when incorporation of *F. vesiculosus* 2 was elevated from 1.25 to 5%, the protective effect against the spontaneous genotoxicity (as small and total spots) was not detected. It seems that increasing the supplementation % of *F. vesiculosus* may limit the protective action against spontaneous genotoxicity.

The comparison of *F. vesiculosus* batches/origins yielded no marked differences. This is somewhat surprising considering that the harvesting sites are 100 km apart and differing hydrological dynamics associated to the system characteristics (ocean intertidal zone for F1 *vs*. inner lagoon area for F2). The aquacultured batch used (F2) was collected in Ria de Aveiro and kept only for a week under controlled cultivation conditions (at ALGAplus facilities), which might account for lack of differences between wild-harvested *vs.* aquacultured *F. vesiculosus*. Overall, *F. vesiculosus* seems to be less prone to alter its bioactivity (as antigenotoxic action) under varying growing conditions.

3.4.3. Antigenotoxic potential of *Gracilaria* species

Although an antigenotoxic potential was detected, the action of these red algae followed a profile distinct from the other tested algae, and not always exempted of toxicity

signs. Surprisingly, the group 10G2 produced a rise in spontaneous genotoxic events as shown by the rise in small and total eye spots. Contrarily, the 1.25G2 and 1.25G1 groups lowered spontaneous genotoxicity as evidenced by large and total spots. In addition, the antigenotoxic potential of *Gracilaria* species became more evident when streptonigrin-induced genotoxicity was examined indicating a consistent protective effect perceptible as small, large and total eye spots reduction in the groups subjected to the diet supplementation with the lower level of *G. vermiculophylla* (S + 1.25G1) and the higher level of *G. gracilis* (S + 10G2). It is noteworthy that among the tested algae *Gracilaria* species were the only ones that decreased genotoxic effects as noted by large spots. In agreement, Yang et al. (2012) demonstrated that aqueous extracts of *G. tenuistipitata* prevented oxidative DNA damage induced by H_2O_2. Accordingly, several investigators observed that extracts of various *Gracilaria* species (*viz. G. birdiae*, *G. cornea*, *G. salicornia*, *G. tenuistipitata* and *G. edulis*) possess antioxidant (Ganesan et al. 2008; Yangthong et al. 2009; Vijayavel and Martinez 2010; Souza et al. 2011) and antiproliferative (Yeh et al. 2012) potential, generally attributed to the high phenolic content. Hence, the antioxidant potential reported for this genus may be the responsible for the beneficial effects (antigenotoxicity) found in the present study, especially at the lower supplementation level.

Regarding the potential toxicity associated with *Gracilaria*, it should be noted that the fly groups grown in the presence of the highest concentration (10%) of *G. vermiculophylla*, with or without streptonigrin challenge, inhibited egg laying and/or the larvae development. Previously, Queiroz and colleagues (2014) noted that *Sparus aurata* fed with 7.5% of *Gracilaria* sp. supplementation displayed an elevation in lipid peroxidation. Al-Asgah et al. (2016) reported that *Clarias gariepinus* fed with a diet including 20 and 30% of *G. arcuata* demosntrated poorer growth, which was attributed to non-starch polysaccharides, affecting the algal digestibility. However, Al-Asgah et al. (2016) recommended that *C. gariepinus* can tolerate this ingredient up to 10% in their diets. Silva et al. (2015) found that inclusion of 10% of *G. vermiculophylla* exerted an adverse effect in diet palatability, decreasing fish feed intake and growth performance in *Oreochromis niloticus* indicating that the polysaccharides with low digestibility act in antinutritional fashion. In fact, species from the genus *Gracilaria* were also associated with a gastrointestinal disorder in humans, known as “ogonori poisoning” in Japan, which was attributed to prostaglandins (PG) (Fusetani and Hashimoto 1984). However, the properties of *Gracilaria* PG content are not consistent (Dang et al. 2010). These lipids, that distinguish the genus *Gracilaria* from other red algae, were shown to vary seasonally and with growth conditions, and wild *G.*

verrucosa, in particular, accumulated higher levels of PG in the colder season (Imbs et al. 2001). Considering that *G. vermiculophylla* in the present study was collected in the beginning of winter season, it is possible that those PG compounds, in addition to the non-starch polysaccharides, might be the reason for the observed toxicity.

To elucidate if the adverse effects of *G. vermiculophylla* (G1) on prolificacy might result from xenobiotics/contaminants bioaccumulated by alga tissues, an analysis determining levels of several elements, including metals, was carried out. The hypothesis was discarded as these analyses (Appendix II – Table 3) did not reveal any element levels to account for our findings. In addition, the enhancement of spontaneous genotoxicity in the group 10G2 (small and total spots), whose algal mass did not contain exogenous contaminants, supports the postulation that toxicity was related to intrinsic phytochemicals.

The comparison of the properties of both *Gracilaria* batches tested (only possible for the lower supplementation level) needs to consider that two variables are simultaneously present, *viz*. species and provenance (= growing conditions). In this context, data depicted some contradictory findinds. Hence, supplementation with 1.25% of *G. gracilis* (1.25G2) showed higher protective potential against spontaneous genotoxicity than that with *G. vermiculophylla* (1.25G1; as large spots), while an opposite pattern was observed when flies were challenged by the genotoxic agent streptonigrin. Under this scenario, supplementation with 1.25% of *G. vermiculophylla* (S + 1.25G1) exhibited higher antimutagenic effectiveness than the corresponding group supplemented with *G. gracilis* (S + 1.25G2), as evidenced by higher reduction of small spots provided by the former species/origin and the inability to diminish large and total spots in the latter. In addition, it may be inferred that *G. gracilis* cultivated under standardized conditions (G2) possess an algal composition (as phytochemicals' variety and/or relative quantity) with no signs of toxicity expressed as prolificacy impairments in *D. melanogaster*, in contrast to *G. vermiculophylla* – G1, which increases basal eye-spots frequency.

3.4.4. Mode of action of macroalgae as dietary supplement

In the context of SMART data interpretation, the causal association of each type of spot with specific genetic events and the respective chronology is a debatable issue (Ferreiro et al. 1995). A correlation between time of induction and spot size is plausible (Graf et al. 1984; Graf 1995; Morais et al. 2016). Hence, different frequencies are expected for each type of spot after acute or chronic treatments of *D. melanogaster* (at larval stage) with xenobiotics (Graf 1995). A higher frequency of clone induction is

predictable with aging of the genotoxic-treated larvae, while the size of the generated spots is expected to diminish with increasing age of larvae (Graf 1995), *i.e.* large spots are presumably derived from older genotoxic events, while recent insults result in development of the majority of small spots. In addition, in accordance with Graf et al. (1984), in a chronic treatment, a prevalence of small spots is expected over large ones, as observed in the present study. Further, the higher frequency of small spots in relation to large spots results in a similarity of small and total spots profiles, as particularly evident in *Ulva* and *F. vesiculosus* data. Morais et al. (2016) determined the genotoxicity of fipronil to *D. melanogaster* and noted a prevalence of small spots associated with a delayed DNA damage induction, since genotoxic events did not propagate in a large number of daughter cells through mitosis. Bearing in mind this mechanism of damage, the present study presumed that streptonigrin acted predominantly in a delayed manner, as a more evident elevation of small spots, although accentuated rises in both types of spots were detected. Nevertheless, the bioactive compounds present in the macroalgae (especially in *U. rigida* and *F. vesiculosus*) were not sufficiently strong to minimize development of large spots due to streptonigrin. Interestingly, *Gracilaria* species (both *G. vermiculophylla* and *G. gracilis*) were found to be the only macroalgae to possess an antigenotoxic potential against large spots. This might suggest that *Gracilaria* acted rapidly against DNA damage generation, which can be explained alternatively or in combination by a higher assimilation/uptake rate of algal components by flies or by a faster and/or more powerful action of their bioactive compounds. In contrast, when small spots (possibly resulting from more recent genotoxic events induced by streptonigrin) were examined, all macroalgae seem to exhibit a marked antigenotoxic capacity. Further, spontaneous and streptonigrin-induced genotoxicity implies differences in the nature and severity of the stimulus, which may also account for differences in spot sizes and frequencies.

The macroalgae currently assessed revealed specific antigenotoxicity profiles as a function of genotoxic insult, origin, species and supplementation level. If algae from different phyla (Chlorophyta, Ochrophyta or Rhodophyta), or even different species, exhibit distinct phytochemical profiles, namely polysaccharides and pigments, as well as that these profiles are also influenced by growth conditions (*e.g.* location, temperature, light incidence), it is conceivable that they might offer distinct beneficial properties. Matsukawa et al. (1997) reported that the antioxidant activity of brown algae was superior to that of red or green groups, while Yildiz et al. (2014) found that green algae have higher antioxidant activity relatively to red algae. Farvin and Jacobsen (2013) observed a red and

several brown algae as the ones presenting the highest antioxidant activity within a sample of 16 macroalgae representing the three taxonomic groups.

3.5. Conclusions

The present study demonstrated the beneficial effects of *Ulva*, *Fucus* and *Gracilaria* species included in the diet of *D. melanogaster* in providing a protection barrier against different genotoxic insults. Although each macroalga displayed specific protection patterns, a higher protective action was found against exogenous challenge (streptonigrin), To a lesser extent, the action against subtler genotoxic pressures occurring due to endogenous agents (spontaneous genotoxicity) was also observed by the three macroalgae genera. The level of supplementation found to be critical as the genome protection was only evident for the lower dose of each tested alga.

Gracilaria species provided ambivalent indications where a potent/rapid antigenotoxic action made it the only alga able to reduce the frequency of large spots) while concomitantly there was evidence of toxicity. Surprisingly, the higher doses (10%) of *G. vermiculophylla* (10G1) and *G. gracilis* (10G2) inhibited the egg laying and/or the larvae development and promoted a rise in spontaneous genotoxicity, respectively.

The functional characterization of the macroalgae according to their provenance was possible for *U. rigida* and *F. vesiculosus*. *U. rigida* demonstrated higher genoprotection against streptonigrin-induced damage when grown under aquaculture-controlled conditions, while *U. rigida* grown under wild conditions was most effective against spontaneous genotoxicity. In contrast, *F. vesiculosus* did not reveal significant differences depending upon growing conditions.

When comparing two close species from the same genus (at 1.25% supplementation level), *viz*. *G. vermiculophylla* (wild-harvested) and *G. gracilis* (aquacultured), the former displayed a greater antigenotoxic potential against the exogenous insult.

Finally, the present findings carry new perspectives likely to contribute to development of algaculture industry, as well as to the redefinition of the human eating habits, reinforcing the concept of functional food and its benefits.

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Chapter 4

Comparative genoprotection ability of naturally growing (wild) and aquacultured *Ulva rigida* coupled with phytochemical profiling

European Journal of Phycology (submitted)

4. Comparative genoprotection ability of naturally growing (wild) and aquacultured *Ulva rigida* coupled with phytochemical profiling

Abstract

Antigenotoxic properties of plant- and plant-like-derived foods may embody nutritional strategies against DNA damage. Marine macroalgae have shown DNA-protective effects, albeit underexplored regarding its nutraceutical potential and the influence of growing conditions on its properties. Hence, we aimed to assess the genoprotection potential of wild-harvested *vs.* aquacultured *Ulva rigida* on *Drosophila melanogaster*, following a dietary exposure, in the presence and absence of a genotoxic agent (streptonigrin), supported by a phytochemical profiling. DNA damage was evaluated by the comet assay improved with endonucleases. An origin-based phytochemical profile was depicted, with aquacultured alga showing higher relative amounts of fatty alcohols, sterols, sesquiterpenoids and glycerol esters. Although *U. rigida* from both origins showed a DNA-protective action, especially against streptonigrin-induced genotoxicity, aquacultured alga demonstrated higher potential, which may be linked to the distinctive phytochemical profile. Overall, this study provided scientific evidence for the genoprotection ability of *U. rigida* and its substantiation as functional food.

Keywords

Antigenotoxicity; Aquacultured; Macroalga; Phytochemical profile; Wild-harvested.

4.1. Introduction

The genome integrity is regularly threatened by several factors, resulting from endogenous processes (*e.g.* reactive oxygen species production), as well as from exogenous pressures (*e.g.* environmental genotoxicants) (Geacintov and Broyde 2010). These genetic insults could result in different lesions on the DNA molecule, namely adducts, single- and double-strand breaks and mutations, which misrepair may lead to protein dysfunction, clastogenesis, and/or oncogene activation, ultimately resulting in the development of metabolic and immunological impairments, degenerative diseases and cancer (Kurelec 1993; Shugart and Theodorakis 1996).

The human diet has been pointed either as a risk factor, contributing, for example, to the development of 30-35% of cancer cases (Ruiz and Hernández 2014), but also as a source of compounds with DNA protection ability, both against endogenous or environmental challenges (Johnson and Fenwick 2000). In the last decades, hundreds of DNA-protective and anticarcinogenic properties have been detected in several plant- and plant-like-derived foods, which has contributed to the hope of developing nutritional strategies to protect humans against DNA damage (DFG 2000; Knasmüller et al. 2002). In this framework, the consumption of marine macroalgae has been associated with several valuable properties to the human health (Smit 2004; Mohamed et al. 2012; Wells et al. 2017). In fact, macroalgae have been defended as functional food by diverse authors (Hong et al. 2007; Holdt and Kraan 2011; Mohamed et al. 2012; Tanna and Mishra 2018), which gained special relevance considering their antioxidant, antigenotoxic, antimutagenic and anticarcinogenic properties described in the literature (Athukorala et al. 2006; Yuan and Walsh 2006; Celikler et al. 2008; Celikler et al. 2009b; Zubia et al. 2009; Valentão et al. 2010; Abd-Ellatef et al. 2017; Marques et al. 2018). Moreover, the genome integrity strengthening underlying these capacities might clarify why populations whose diet include macroalgae as a significant element, namely Asian, present minor prevalence of diet-related diseases (*e.g.* cardiovascular diseases and cancer) as demonstrated in diverse epidemiological studies (Yamori et al. 2001; Pisani et al. 2002; Yuan and Walsh 2006).

The green algae (Chlorophyta) constitutes one of the most diverse group of algae, with more than 6600 species, growing in a great diversity of habitats (Guiry and Guiry 2019). These algae obtain the green colour from chlorophylls a and b, similar to the higher plants (Lee 2018). *Ulva* is a genus that includes a vast list of over 130 species (Guiry and Guiry 2019) and has a wide distribution in marine, freshwater and brackish environments throughout the world (Canter-Lund and Lund 1995; Hoek et al. 1995; Martins et al. 1999;

McAvoy and Klug 2005; Shimada et al. 2007). Among those species, one of the most studied is *U. rigida*, generally known as sea lettuce, that is an edible macroalga (Guiry and Guiry 2019) with a rich nutritional composition, consisting of polyunsaturated fatty acids, proteins, dietary fibres, sulphated polysaccharides, vitamins, minerals, phenolic compounds and pigments (Celikler et al. 2009a; Taboada et al. 2010; Tabarsa et al. 2012; Yildiz et al. 2012; Celikler et al. 2014; Mezghani et al. 2016).

Although still poorly explored, some evidence suggested the genoprotection promoted by species belonging to the *Ulva* genus. Ethanolic and aqueous extracts of *U. lactuca* revealed the potential to diminish the effects of γ-ionizing irradiation on rat liver, namely through the reduction of H_2O_2 formation, DNA fragmentation and micronuclei frequency (Alam et al. 2016). Moreover, crude extracts of *U. rigida* demonstrated antigenotoxic and anticlastogenic capacity in human lymphocytes cultured *in vitro*, through the significant decrease of chromosomal aberrations and the frequency of both sister chromatid exchanges and micronuclei induced by mitomycin-C (Celikler et al. 2008). The same research group also found that an ethanolic extract of *U. rigida* diminished the micronuclei frequency in diabetic (Celikler et al. 2009a) and hypothyroid rats (Celikler et al. 2014). In parallel, an aqueous-ethanolic extract of *U. fasciata* prevented the formation of micronucleated polychromatic erythrocytes induced in rats by benzo[a]pyrene (Rodeiro et al. 2015). Sathivel et al. (2014) found that a sulphated polysaccharide isolated from *U. lactuca* alleviates DNA fragmentation and necrosis in rats with D-galactosamine-induced liver damage. Notwithstanding, most of these studies concerns solely evaluations of extracts or compounds isolated from the algae or *in vitro* trials, which discloses the lack of knowledge relying on *in vivo* effects of the whole macroalgae ingestion on the genome integrity preservation. As defended by the European Advisory Services (2008) and echoed by Holdt and Kraan (2011), functional food should not be consumed as pills or capsules, but must remain food and be consumed as that, in realistic amounts. Furthermore, only few studies addressed *U. rigida* regarding its DNA-protective properties, which, combined with factors such as edibility, wide geographical distribution and ease of being cultivated in aquaculture, turns it on one of the most relevant *Ulva* species to study.

Environmental factors, such as light, temperature and salinity, have demonstrated to directly influence the variety and quantity of algae constituents (Marinho-Soriano et al. 2006). This legitimates the hypothesis that the genome protective properties described for *Ulva* species may be indirectly influenced by the growing conditions (Lahaye et al. 1995; Gómez-Pinchetti et al. 1998; Abreu et al. 2009), which can be particularly pronounced

when considering natural environments *vs.* conditions occurring under manipulated/controlled aquaculture rearing scenarios. Nevertheless, to the authors' knowledge, this is an underexplored perspective within the context of macroalgae genoprotective potential, despite being suggested by Marques et al. (2018) regarding *U. rigida*.

Considering the identified knowledge gaps and keeping in view the phytochemical and functional characterization of healthy foods, the present study was designed with the major goal of assessing the properties of *U. rigida* to strengthen the genome protection. Hence, the antigenotoxic potential of this green alga was evaluated in *Drosophila melanogaster* [a model organism recurrently adopted in genotoxicity/antigenotoxicity studies (Siddique et al. 2005; Carmona et al. 2011b; Carmona et al. 2011a)], following a dietary exposure, both in the presence and absence of streptonigrin, a well-known genotoxic and mutagenic agent (Gaivão et al. 1999; Bolzán and Bianchi 2001). For that purpose, the DNA damage in the flies' neuroblast cells was evaluated by the single cell gel electrophoresis (comet) assay, improved with nucleoid digestion by endonucleases FPG (formamidopyrimidine DNA glycosylase) and EndoIII (endonuclease III) to detect oxidized purines and pyrimidines, respectively, and thus allowing the discrimination of defence properties in relation to specific DNA damaging events.

Conveying an additional factor of novelty, a comparative perspective was adopted through the evaluation of naturally growing (wild) *vs.* aquacultured *U. rigida*, supported by the respective phytochemical profiling of hexane and ethanolic algae extracts.

4.2. Material and methods

4.2.1. Chemicals

The instant treatment Carolina Drosophila Medium Formula 4-24® (hereinafter referred as Instant Drosophila Medium - IDM) was purchased from Carolina Biological Supply Company (USA). Streptonigrin (CAS 3930-19-6) was obtained from Santa Cruz Biotechnology Inc (USA). DNA lesion-specific repair enzymes, namely FPG and EndoIII, were purchased from Professor Andrew Collins (University of Oslo, Norway). Solvents (of analytical grade or bi-distilled commercial products) were purchased from Panreac AppliChem (Germany) and Acros Organics, Fisher Scientific (USA). All other chemicals were obtained from Sigma-Aldrich Chemical Company (Spain).

4.2.2. Macroalgae harvesting and preparation

U. rigida from wild origin (hereinafter referred as U1) was collected, in September 2015, at the Mindelo beach, Vila do Conde, Portugal (41°18'36.8"N, 8°44'25.9"W), a location with no significant pollution sources identified (Reis et al. 2014). The *U. rigida* specimens from aquaculture (hereinafter referred as U2) were obtained from an integrated multi-trophic aquaculture (IMTA) system at ALGAplus, Lda. (certified organic production; Ílhavo, Portugal) and harvested in September 2015.

The wild-harvested and aquacultured batches of macroalgae were washed in seawater (pre-treated with UV and filtered to 5 µm), followed by 12 h in a controlled temperature chamber (25 ºC), achieving 10-12% of humidity, after which they were preserved in multiple layer packaging (paper and plastic) until further experimental procedures. Prior to the analyses and experiments, *U. rigida* from both origins was grinded with a coffee mill, obtaining particles with < 1 mm.

Species identification by DNA-barcoding was achieved on both wild-harvested and aquacultured *U. rigida* specimens, as described in Marques et al. (Marques et al. 2018).

4.2.3. Macroalgae phytochemical characterization

4.2.3.1. Extracts preparation

Algal material (U1 – 15.012 g; U2 – 15.004 g) was extracted in the dark, at room temperature, under agitation, for 72 h with hexane, renewing the solvent twice. After evaporation of the solvent from the 3 combined extractions using a rotary vacuum evaporator, 0.142 and 0.141 g were obtained, respectively, for U1 and U2 samples. The extraction yields were 0.95 and 0.94%, respectively, for U1 and U2 hexane extracts, to be analysed by gas chromatography-mass spectrometry (GC-MS). Afterwards, the remaining algal material was extracted again, in the dark, at room temperature, under agitation, for 72 h with ethanol, renewing the solvent twice. After the evaporation of the solvent from the combined extractions, using a rotary vacuum evaporator, 0.242 and 0.333 g were obtained, respectively, for U1 and U2 ethanolic extracts (extraction yields were 1.61 and 2.22%, respectively), to be analysed by ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS).

4.2.3.2. Gas chromatography-mass spectrometry (GC-MS)

Three replicates of each dried hexane extract of U1 and U2 (nearly 20 mg each) were dissolved in 0.5 mL of dichloromethane, and the internal standard (tetracosane) was added (30 mg mL^{-1} in dichloromethane). This mixture was silylated by adding 250 µL of

pyridine, 250 µL of BSTFA, and 50 µL of TMSCl, according to previously describe procedures (Isca et al. 2014; Faustino et al. 2017). Then, the mixture was heated at 70 °C for 1 h, followed by the immediate injection into the GC-MS. The reagents BSTFA and TMSCl were added in enough amounts to ensure the silylation of all hydroxyl groups present in the compounds, including those present in the carboxylic group.

The GC-MS analyses were performed on a GC-MS QP2010 Ultra Shimadzu. The separation of compounds was carried out in a DB-5 J&W capillary column (30 m × 0.25 mm inner diameter, 0.25 µm film thickness) using helium as the carrier gas (35 cm s^{-1}). The chromatographic conditions were as described by Rahmouni et al. (2018): start time at 6.5 min; initial temperature 90 ºC for 4 min; temperature rate 16 ºC min^{-1} up to 180 ºC; followed by temperature rate 6 ºC min^{-1} up to 250 ºC; followed by temperature rate 3 ºC min^{-1} up to 300 ºC, which was maintained for 5 min; injector temperature 320 ºC; transfer-line temperature 300 ºC; split ratio, 1:50. The mass spectrometer was operated in the electron impact (EI) mode with energy of 70 eV and data were collected at a rate of 1 scan s^{-1} over a range of *m/z* 33–750. The ion source was kept at 250 ºC.

The peaks from total ion chromatogram were identified by comparing their mass spectra with the equipment mass spectral library (NIST 14 Mass Spectral and Wiley Registry of Mass Spectral Data), with MS spectra and MS fragmentation pattern described in the literature (GMD; Petersson 1969; Füzfai et al. 2008; Razboršek et al. 2008; Hrabovski et al. 2012; Isca et al. 2014; Suttiarporn et al. 2015; AOCS 2019) and by comparing the retention times and mass spectra data of the standard compounds injected in the same chromatographic conditions. Moreover, when authentic standard is not available, the retention index relative to *n*-alkanes (C5–C36) was compared with retention indexes reported by NIST 14 database (when available).

4.2.3.3. Ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS)

For the UHPLC-MS analysis, U1 and U2 extracts (nearly 30 mg each) were dissolved in methanol (1 mL) and the resulting solution was filtered through a 0.2 µm Nylon membrane (Whatman). The analysis was performed using a Thermo Scientific Ultimate 3000RSLC (Dionex) equipped with a Dionex UltiMate 3000 RS diode array detector and coupled to a mass spectrometer. The column used was a Thermo Scientific Hypersil GOLD column (1000 mm x 20 mm) with a particle size of 1.9 µm and its temperature was maintained at 32 °C. The mobile phase was composed of (A) 0.1% formic acid (v/v) and (B) acetonitrile/methanol (70:30). The solvent gradient started with 85% of solvent B over

3.9 min, followed by a 6.1 min upgrade until 100% of solvent B maintained during 25.3 min, then in 31 min lowered to 85% of solvent B, which was maintained during 38 min. The injection volume was 2 µL. UV-Vis spectral data were gathered in a range of 250 to 680 nm and the chromatographic profiles were documented at 430, 450 and 650 nm. The mass spectrometer used was an LTQ XL linear ion trap 2D equipped with an orthogonal electrospray ion source (ESI). The equipment was operated in negative-ion mode with electrospray ionization source of 5.00 kV and ESI capillary temperature of 275 °C. The full scan covered a mass range of 50 to 2000 *m/z*. Collision-induced dissociation MS/MS and MS^n experiments were simultaneously acquired for precursor ions.

4.2.4. Test organisms and experimental design

D. melanogaster strain Oregon-K (*Ok*) was selected, since a study from Gaivão and Comendador (1996) noted that between 6 strains with different metabolic activities, it is one of the most suitable strains to be used for genotoxicological assays. The *Ok* strain presented the highest susceptibility for reactive oxygen species (ROS) formation when exposed to toxic chemicals and the lowest activity of antioxidant enzymes, being this last one necessary to restrict the antioxidant activity to non-enzymatic antioxidants, which are mainly obtained through diet. Stock cultures were maintained in a chamber at 25 ºC, with relative humidity of approximately 60%, in culture vials containing 20 mL of standard lab-made growth medium (10% sucrose; 10% yeast; 1.2% agar-agar; 0.2% NaCl; 0.5% propionic acid in water). Since this growth medium needs to be heated to 90 ºC, to prevent eventual denaturation of macroalgae bioactive components, the IDM (6 g of medium hydrated with 20 mL phosphate buffered saline - PBS) was used when performing the trials, as it needs neither cooking nor sterilizing.

Considering the choice of assessing the algae antigenotoxic potential on flies' neuroblasts (the precursor cells of *D. melanogaster* brain), the assay would need to be carried out on third-instar larvae, as described in Sierra et al. (2014). Hence, to obtain new individuals, 20 couples were mass-crossed in glass culture vials with approximately 20 mL of standard growth medium for 2 days to enhance the prolificacy. Then, couples were transferred to other culture vials (in triplicates for each condition) with 20 mL of IDM and divided into 2 major groups: one unchallenged and another challenged with streptonigrin (S) (see Figure 4.1). Each of the previous groups was split into 2 subgroups, corresponding to wild-harvested (U1) and aquacultured (U2) *U. rigida* trials. For each subgroup, two levels of supplementation (2.5 and 5%) and a control group (with no alga supplementation) were tested. Regarding the final medium volume, streptonigrin was

added to the IDM, dissolved in PBS, to achieve the final concentration of 20 μM. This concentration was selected according to the literature (Gaivão et al. 1999). As algae are taken here as functional foods, their levels of supplementation were expressed as % alga relatively to IDM (weight/weight), to enable a better perception of the tested amount in relation to the total food intake. This option was previously elected in diverse studies with comparable goals (Sousa et al. 2009; Rezende et al. 2013). The levels of supplementation evaluated (2.5 and 5%) were selected according with a preliminary study (Appendix II – Table 2), where a broader range of alga concentrations (1.25 - 20.0%) was tested and the prolificacy (nº of hatched individuals) per condition was recorded. The selection of the supplementation levels to test in the present study relied on the identification of the two lowest algae percentages depicting the higher prolificacy.

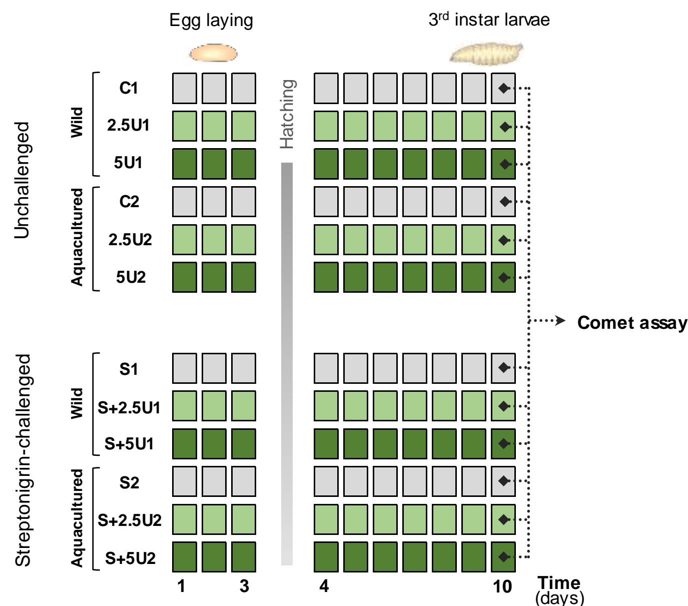

Figure 4.1. Schematic representation of the experimental design, elucidating the division of *D. melanogaster* individuals into two major groups: one unchallenged and another streptonigrin-challenged (S). Each of the previous groups was split into 2 subgroups, corresponding to wild-harvested and aquacultured *U. rigida* (U) trials (depicted in the groups codes by the last number, *i.e.*, 1 and 2, respectively). For each subgroup, two levels of supplementation (2.5 and 5%; represented in the groups codes by the number preceding the letter U) and a control group (with no alga supplementation; C or S) were tested. The comet assay was performed on larvae in the 3rd instar stage, approximately 10 days after the egg laying.

The mated flies laid eggs for 3 days, after which adults (F_0) were discarded. Vials were maintained at 25 ºC and, according to the *D. melanogaster* life cycle, it was expected that the feeding larvae (F_1) were exposed to the algae/streptonigrin *via* ingestion for approximately 5-7 days (see Figure 4.1). After this period, neuroblasts were excised (see point 4.2.5) in the third-instar stage individuals, before larvae reach the pupa stage, and the comet assay was performed.

4.2.5. Comet assay

The alkaline version of the comet assay was performed according with Collins (2004) methodology with slight modifications, as adapted by Guilherme et al. (2012), as well as the proper adjustments to the extra step, concerning the nucleoid digestion with endonucleases.

First, from each triplicate (glass vial), 4 pools with 2 larvae each were assembled. For each pool, the brain ganglia were removed from the larvae immersed in Ringer solution under a stereo microscope with diascopic illumination (Leica Wild M3Z) with the help of two dissection needles. Thus, the brain ganglia were placed in microtubes with 20 µL of Ringer solution and maintained at 4 ºC. The suspension was then centrifugated at 1.7 g for 5 min (Thermo Scientific Sorvall Legend 14 microcentrifuge), after which, the pellet composed by the brain ganglia was shredded with a dissection needle and resuspended with 140 µL of 1% low melting point agarose in PBS. Two drops of 6 µL per pool were placed vertically onto the glass microscope slides, precoated with 1% normal melting point agarose, without coverslips. Each slide confined twelve mini-gels, as six columns of 2. Then, the slides were left ± 5 min at 4 °C to solidify the agarose, immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) and kept overnight, at 4 °C. After lysis of agarose-embedded cells, slides were washed 3 times with enzyme buffer (0.1 M KCl, 0.5 mM EDTA, 40 mM HEPES, 0.2 mg mL^{-1} bovine serum albumin, pH 8) at 4 °C. Three sets of slides were prepared. Two sets were incubated with endonucleases (1) FPG and (2) EndoIII that convert oxidized purines and pyrimidines into DNA single strand breaks, respectively (Azqueta et al. 2009). The third set (3) was incubated only with the enzyme buffer. Hence, 30 µL of each enzyme diluted in enzyme buffer (and only buffer in the third set) were applied to each mini-gel, with coverslip, and the slides were incubated at 37 °C during 30 min in a humidified chamber. Then, slides were immediately placed in the electrophoresis tank, immersed in electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH > 13) for 20 min (alkaline treatment). Electrophoresis was performed during 20 min at a fixed voltage of 17 V and a current of 300 mA, which results

in approximately 0.7 V cm^{-1} (achieved by adjusting the buffer volume in the electrophoresis tank). After the electrophoresis, slides were neutralized with PBS (10 min, 4 ºC) and with distilled water (10 min, 4 ºC). Finally, slides were fixed in absolute ethanol for 15 min, air-dried and stored at room temperature.

The slides were stained with ethidium bromide (20 µg mL^{-1}) and fifty nucleoids were observed per mini-gel, using an Olympus BX 41 fluorescence microscope (400x magnification). The nucleoids were classified by visual scoring into 5 comet classes, according to the tail length and intensity from 0 (no tail) to 4 (almost all DNA in tail) (Collins 2004). The final score (expressed as "arbitrary units" in a range of 0 - 400) was obtained by multiplying the mean percentage of nucleoids in each class by the corresponding factor, according to this formula:

Genetic Damage Indicator (GDI) = [(% nucleoid class 0) x 0)] +
[(% nucleoid class 1) x 1)] + [(% nucleoid class 2) x 2)] +
[(% nucleoid class 3) x 3)] + [(% nucleoid class 4) x 4)]

The GDI and the parameters GDI_{FPG} and $GDI_{EndoIII}$ were determined. Additionally, to assess the DNA breaks corresponding specifically to the net enzyme-sensitive sites (NSS), the scores obtained with GDI slides (lacking enzyme treatment) were subtracted to GDI_{FPG} and $GDI_{EndoIII}$ values resulting in the parameters expressed as NSS_{FPG} and $NSS_{EndoIII}$, respectively.

4.2.6. Statistical analysis

Statistica 8.0 (StatSoft, Inc., USA) and Microsoft Excel (Microsoft Corporation, USA) software were used to perform the statistical analysis. First, data was tested for normality (Shapiro-Wilk test and graphical analysis) and homogeneity of variances (Levene test) and data transformation was performed when those statistical assumptions were not met. Then, a 3-way ANOVA (factors: origin of alga, streptonigrin challenge and level of alga supplementation) was performed to analyse each parameter (*e.g.* GDI). When the 3-way ANOVA revealed a significant interaction between factors, simple main effects were assessed for each factor. Finally, each ANOVA was followed by a post hoc Tukey test. Statistical differences were considered when $p < 0.05$ (Zar 1996).

4.3. Results

4.3.1. *U. rigida* phytochemical profiling

The phytochemical characterization performed allowed a semi-quantitative determination of several compounds on hexane and ethanolic extracts of wild-harvested and aquacultured *U. rigida*, U1 and U2 specimens, respectively.

In the U1 and U2 hexane extracts, 5 classes of compounds were identified: acid derivatives, fatty alcohols, sterols, sesquiterpenoids and glycerol esters (Table 4.1), being the former the most represented class and, specifically, palmitic acid was the most abundant compound, regardless the alga origin. In general, aquacultured *U. rigida* (U2) displayed major amounts of the compounds identified, especially considering the fatty alcohols, sterols, sesquiterpenoids and glycerol esters classes.

Regarding the ethanolic fractions of *U. rigida*, the metabolites identified belong to 2 compound classes: porphyrinolactones and chlorophylls, being the latter the richer group (Table 4.2). Despite some differences on the metabolites' relative determination between U1 and U2 ethanolic composition, origin-based profiles were not markedly visible.

4.3.2. Evaluation of DNA damage

Analysing GDI (Figure 4.2), slight variations were found on the unchallenged groups, since the lower supplementation level of wild-harvested *U. rigida* (2.5U1) reduced the DNA breaks, either comparing to the respective unsupplemented control group or the other level of alga supplementation (5U1). Contrarily, the same supplementation level from aquacultured *U. rigida* (2.5U2) induced an increase of GDI, comparatively to the respective unsupplemented group, as well as to the same alga supplementation level of wild-harvesting origin (2.5U1). All groups exposed to streptonigrin showed an increase of genetic damage in comparison to the unchallenged counterparts. Streptonigrin-challenged groups fed with wild-harvested *U. rigida* (regardless the supplementation level) showed a reduction on GDI relatively to the respective unsupplemented group. Flies fed with both supplementation levels of aquacultured *U. rigida* also showed lower GDI than unsupplemented group, with S + 5U2 group displaying even less DNA damage than S + 2.5U2. Differences regarding alga origin were noticed between groups S + 5U1 and S + 5U2, since the later showed a decrease on GDI relatively to the former.

Regarding the GDI_{FPG} data (Figure 4.3A), it was noticeable that the unchallenged group submitted to the higher supplementation level of wild-harvested *U. rigida* (5U1) depicted higher levels, relatively to the respective unsupplemented control and 2.5U1 groups. *D. melanogaster* fed with both supplementation levels of aquacultured *U. rigida*

Table 4.1. Compounds identified on the hexane extract of wild-harvested and aquacultured *U. rigida*, U1 and U2, respectively. Bold values on the columns U1 and U2 depict the batch/origin where higher amount was found (semi-quantitative analysis).

Ret. time (min)	**Compound# (Peak number)**	**CalRInd**	**LitRInd**	**Area% (mean ± SD)**	
				U1	**U2**
Fatty acid derivatives					
13.7	Myristic acid (4)	1779	1788	**7.3 ± 0.7**	4.7 ± 0.9
14.1	10-Undecenoic acid (5)	1477	1480	0.6 ± 0.2	**1.2 ± 0.3**
14.9	Pentadecanoic acid (6)	1886	1888	0.5 ± 0.1	0.5 ± 0.1
15.9	Palmitelaidic acid (8)	1997	1995	**1.7 ± 0.3**	1.0 ± 0.1
16.2	Palmitic acid (9)	1987	1987	**26.5 ± 6.6**	24.6 ± 4.4
18.3	(*E*)-9-Octadecenoic acid (12)	2074		0.6 ± 0.1	**0.8 ± 0.2**
18.5	(*E*)-13-Octadecenoic acid (13)	2072		**1.3 ± 0.1**	0.7 ± 0.1
18.6	Oleic acid (14)	2194	2194	**2.9 ± 1.5**	1.9 ± 0.4
18.8	Stearic acid (15)	2183	2186	1.2 ± 0.1	**1.5 ± 0.2**
19.3	(*E*)-11-Eicosenoic acid (16)	2392	2393	tr	**0.7 ± 0.3**
19.6	(*E*)-10-Hydroxy-2-decenoic acid (17)	2385		1.4 ± 0.3	**2.3 ± 0.4**
20.9	(E)-2-Octenoic acid (19)	1201	1200	**1.2 ± 0.2**	0.7 ± 0.3
24.2	Behenic acid (21)	2585	2584	0.5 ± 0.1	**0.6 ± 0.2**
Fatty alcohols					
15.1	1-Hexadecanol (7)	1896	1896	4.1 ± 0.3	**5.7 ± 1.1**
17.3	(*Z*)-9-Octadecen-1-ol (10)	2102	2103	3.7 ± 0.3	**4.2 ± 0.8**
17.7	1-Octadecanol (11)	2095	2095	2.6 ± 0.2	**3.8 ± 0.7**
32.6	1-Octacosanol (23)	3087	3089	tr	**1.1 ± 0.4**
36.1	1-Triacontanol (26)	3288	3287	0.9 ± 0.3	**1.3 ± 0.2**
Sterols					
35.9	Desmosterol (24)	2700	2703	tr	**2.4 ± 0.7**
36.0	β-Sitosterol (25)	2789	2789	3.0 ± 1.6	**3.3 ± 1.3**
36.7	Campesterol (27)	2685	2689	tr	**0.7 ± 0.2**
Sesquiterpenoids					
12.3	1-(2,6,6-Trimethylcyclohex-2-en-1-yl)propan-2-ol (1)	1587		tr	**0.9 ± 0.2**
13.1	4-(2,6,6-Trimethylcyclohex-2-en-1-yl)butan-1-ol (2)	1639		0.7 ± 0.1	**1.6 ± 0.4**
13.6	Neophytadiene (3)			**0.7 ± 0.3**	tr
Glycerol esters					
20.7	(Z)-1,3-Dimethoxypropan-2-yl octadec-11-enoate (18)	2382		1.4 ± 0.7	**2.2 ± 0.9**
23.4	1-Monopalmitin (20)	2584	2581	0.8 ± 0.1	**1.2 ± 0.2**
26.3	1-Monostearin (22)	2781	2780	tr	**0.7 ± 0.1**

#Compounds were identified by: i) comparison with pure standards; ii) comparison with GC-MS spectral libraries (NIST14.lib and WILEY229LIB); iii) comparison with spectra found in the literature; iv) interpretation of the MS fragmentation pattern. CalRInd - Retention index against C5-C35 *n* alkanes; LitRInd - NIST14.lib retention index; tr – traces.

Table 4.2. Compounds identified on ethanolic extracts of wild-harvested and aquacultured *U. rigida*, U1 and U2, respectively, and their molecular ions species and fragments (*m/z*) data. Bold values on the columns U1 and U2 depict the batch/origin where higher amount was found (semi-quantitative analysis).

Compound	Ret. time (min)	UV-Vis[a]	[M+H]+	[M+Na]+	MS2	U1 (%)[b]	U2 (%)[b]
Chlorophyll derivatives							
Chlorophyll *a* derivative	4.45	398sh, 409, 665	609	-	591, 531	**0.62**	0.13
Chlorophyll *a* derivative	5.08	398sh, 407, 490sh, 502, 532, 611, 665	645	-	627, 474, 456	**2.5**	0.79
Chlorophyll *b* derivative	12.69	366sh, 397, 493, 659	937	-	919, 850, 658, 640, 599	3.97	**4.53**
Chlorophyll *a* derivative	12.84	366sh, 404, 498, 530, 601, 658	921	-	879, 861, 834	4.07	**4.3**
Chlorophyll *a* derivative	13.04	365sh, 406, 530, 658	921	-	879, 861, 834	**3.52**	2.75
Chlorophyll *a* derivative	13.99	365sh, 399, 658	965[c]	-	947, 937, 877, 687	**2.03**	2
Chlorophyll *a* derivative	14.27	365sh, 401, 451, 539, 636, 657sh	965[c]	-	947, 933, 687	4.99	**5.95**
Chlorophyll *b* derivative	14.52	366sh, 406sh, 425, 507, 657, 678sh	917	939	899, 637, 516	**4.21**	3.57
Chlorophyll *b* derivative	15.88	365sh, 414, 657	937	-	921, 895, 877, 660,	3	**3.49**
Chlorophyll *a* derivative	16.55	366sh, 409, 505, 660, 689	909	931	915, 667, 637, 607	4.59	**6.54**
Chlorophyll *b* derivative	16.88	366sh, 431, 520, 652, 690	901	-	872, 854, 869, 823	7.21	**7.7**
Chlorophyll *b* derivative	17.1	285, 376sh, 411, 432, 654	901	-	872, 854, 857, 841	**1.76**	1.55
Chlorophyll *b* derivative	18.38	365sh, 411, 502, 658	975[c]	-	960, 957, 915, 697	1.1	**2.97**
Chlorophyll *b*	18.88	376sh, 411, 433, 523, 654	-	929	911, 869, 651	2.15	**3.26**
Chlorophyll *a* derivative	19.49	371sh, 406, 502, 531, 608, 665	887	909	607, 593, 536	30.85[d]	**32.08[d]**
Chlorophyll *b* derivative	19.62	399, 498, 529, 609, 667	903	925	859, 607, 548, 504		
Chlorophyll *a* derivative	19.98	371sh, 408, 502, 532, 609, 665	887	909	607, 593, 536	**9.5**	5.58
Porphyrinolactone derivatives							
Porphyrinolactone derivative	3.11	403sh, 414, 423sh, 658	639	-	472, 454	**0.68**	0.57
Porphyrinolactone derivative	4.85	399, 490sh, 500, 528, 668	625	-	474, 456	**0.92**	0.42
Porphyrinolactone	22.03	400, 498, 530, 613, 668	931	953	912, 871, 651, 606	6.33	**6.69**
Porphyrinolactone	22.36	366sh, 399, 497, 530, 667	931	-	912, 871, 651, 606	1.83	**1.9**
Porphyrinolactone derivative	22.69	366sh, 407, 502, 532, 665	915	937	885, 855	**4.17**	3.22

[a] UV-Vis spectra data in nm; [b] (peak area/total area) x 100; [c] although this is a [M+H]+, corresponds to an adduct with formic acid, used in the eluent; [d] sum of the peaks % of area at 19.49 and 19.62 min.

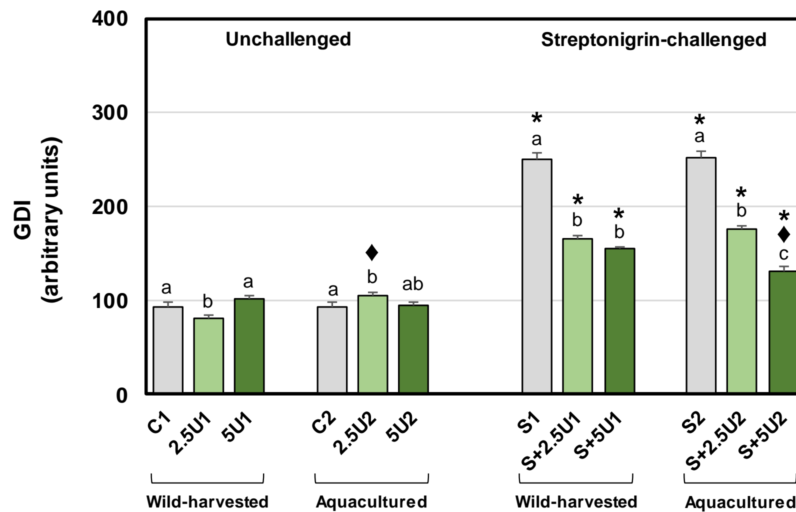

Figure 4.2. Mean values of DNA damage (GDI; expressed as arbitrary units) measured by the comet assay in neuroblast cells of *D. melanogaster* after a dietary trial with the macroalga *U. rigida* (two levels of supplementation - 2.5 and 5%, transposed to the group identifier abbreviation as the number preceding the letter U) from wild-harvesting (U1) and aquaculture (U2) origins. Two major groups were evaluated: one unchallenged and another streptonigrin-challenged. C and S represent the unsupplemented groups, respectively, unchallenged and streptonigrin-challenged. Bars represent the standard error. Statistically significant differences ($p < 0.05$) between groups submitted to the supplementation with *U. rigida* of the same origin, under the same genotoxic challenge are marked by different letters; differences between U1 and U2 groups under the same genotoxic challenge and level of supplementation are marked by black diamond (♦); differences between groups unchallenged and streptonigrin-challenged, under the same level of supplementation, from the same origin are marked by asterisk (*).

showed higher GDI_{FPG} than the respective unsupplemented control group. Moreover, 2.5U2 group showed higher GDI_{FPG} than the reciprocal group from wild origin, while the opposite pattern was visible comparing the higher supplementation level groups. Streptonigrin-challenged groups (except S + 5U1) depicted higher GDI_{FPG} values comparatively to the unchallenged ones. Flies fed with both supplementation levels of both *U. rigida* origins showed a reduction on GDI_{FPG} parameter in comparison with the respective unsupplemented groups. In the case of aquacultured *U. rigida*, a difference regarding the supplementation level was also depicted, with the higher supplementation showing lower damage. Moreover, an origin-based difference was evident concerning the lower *U. rigida* supplementation level, since S + 2.5U2 group showed higher GDI_{FPG} values comparatively to S + 2.5U1.

Through the observation of the NSS_{FPG} parameter (Figure 4.3B), unchallenged 5U1 group showed higher values of DNA breaks than the unsupplemented or 2.5U1 groups.

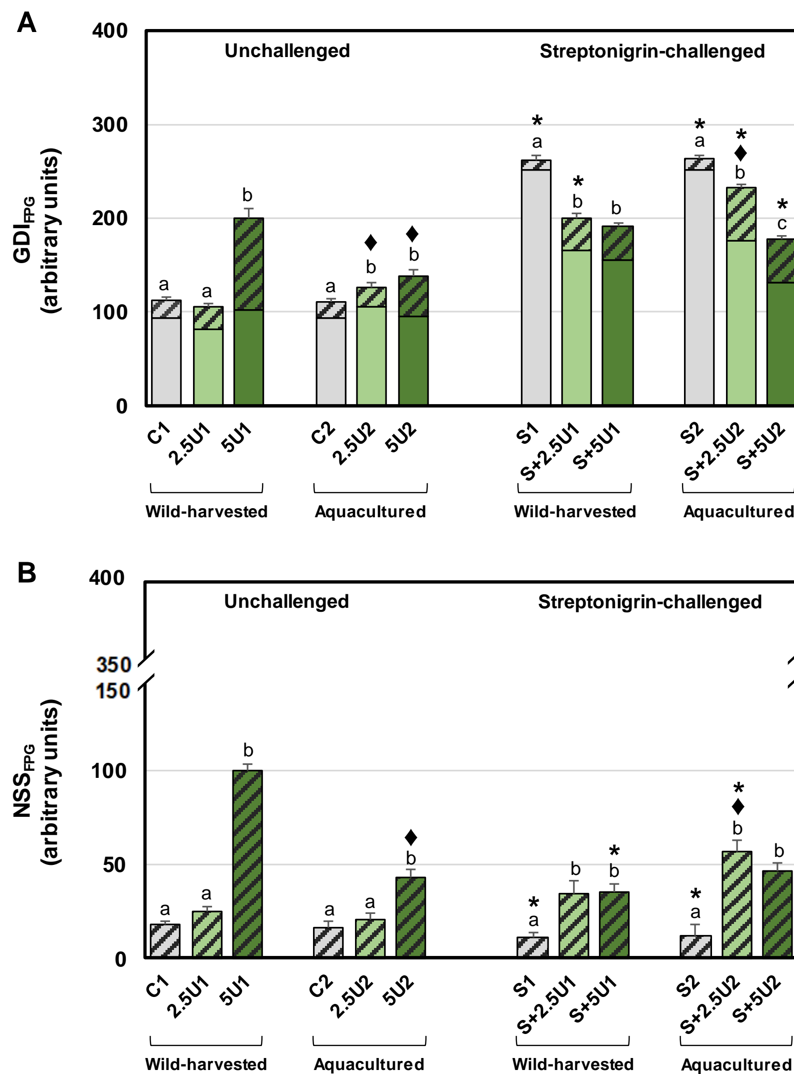

Figure 4.3. Mean values of oxidative purine DNA damage, measured by the improved comet assay in neuroblast cells of *D. melanogaster* after a dietary trial with the macroalga *U. rigida* (two levels of supplementation - 2.5 and 5%, transposed to the group identifier abbreviation as the number preceding the letter U) from wild-harvesting (U1) and aquaculture (U2) origins. Two major groups were evaluated: one unchallenged and another streptonigrin-challenged. C and S represent the unsupplemented groups, respectively, unchallenged and streptonigrin-challenged. (A) overall damage (GDI_{FPG}) and partial scores, *i.e.* GDI plus additional DNA breaks corresponding to net FPG-sensitive sites (NSS_{FPG}; dark grey diagonal stripes); (B) NSS_{FPG} alone. Bars represent the standard error. Statistically significant differences ($p < 0.05$) between groups submitted to the supplementation with *U. rigida* of the same origin, under the same genotoxic challenge are marked by different letters; differences between U1 and U2 groups under the same genotoxic challenge and level of supplementation are marked by black diamond (♦); differences between groups unchallenged and streptonigrin-challenged, under the same level of supplementation, from the same origin are marked by asterisk (*).

The same pattern was depicted concerning aquacultured *U. rigida* specimen, although to a lesser extent. Additionally, flies fed with the higher supplementation level of aquacultured *U. rigida* showed less NSS_{FPG} than the reciprocal group fed with wild-harvested alga Regarding the streptonigrin challenge, unsupplemented groups exposed to the genotoxic showed less NSS_{FPG} in comparison to non-exposed ones, as also did the group S + 5U1 comparing to 5U1 group. Differently, the group exposed to streptonigrin and which diet was supplemented with the lower level of aquacultured alga (S + 2.5U2) showed higher NSS_{FPG} values than the reciprocal unchallenged group. Concerning only streptonigrin-challenged groups, both alga supplementation levels (2.5 and 5%) showed higher NSS_{FPG} relatively to the respective unsupplemented groups, regardless its origin. Moreover, a difference concerning *U. rigida* origin was depicted between groups S + 2.5U1 and S + 2.5U2, since the latter showed higher NSS_{FPG} values than the former.

Analysing the $GDI_{EndoIII}$ results (Figure 4.4A), unchallenged group fed with the higher supplementation of wild-harvested *U. rigida* (5U1) showed higher $GDI_{EndoIII}$ values relatively to the unsupplemented group. A similar pattern was noticeable for aquacultured alga supplementation, since 5U2 revealed higher $GDI_{EndoIII}$ than unsupplemented and 2.5U2 groups, while the latter also showed lower $GDI_{EndoIII}$ than 2.5U1, highlighting a difference based on the algae origin. All streptonigrin-challenged flies depicted an increase of $GDI_{EndoIII}$ levels in comparison to unchallenged ones. Both supplementation levels of *U. rigida*, regardless its origin, showed lower $GDI_{EndoIII}$ than the respective unsupplemented groups. In addition, S + 5U2 group revealed lower values of that parameter, either comparing to S + 2.5U2 group or the reciprocal group from wild-harvesting origin (S + 5U1).

Regarding the $NSS_{EndoIII}$ parameter (Figure 4.4B), group 2.5U1 showed higher values, comparing to the respective unsupplemented group. Contrarily, the group 2.5U2 showed lower $NSS_{EndoIII}$ values relatively to the unsupplemented and 5U2 groups, as well as in comparison with 2.5U1. Considering the genotoxic challenge inflicted by streptonigrin, within wild alga supplementation, S + 2.5U1 showed lower $NSS_{EndoIII}$ levels than the corresponding unchallenged group, while the opposite was evident considering the same alga supplementation level of aquacultured alga.

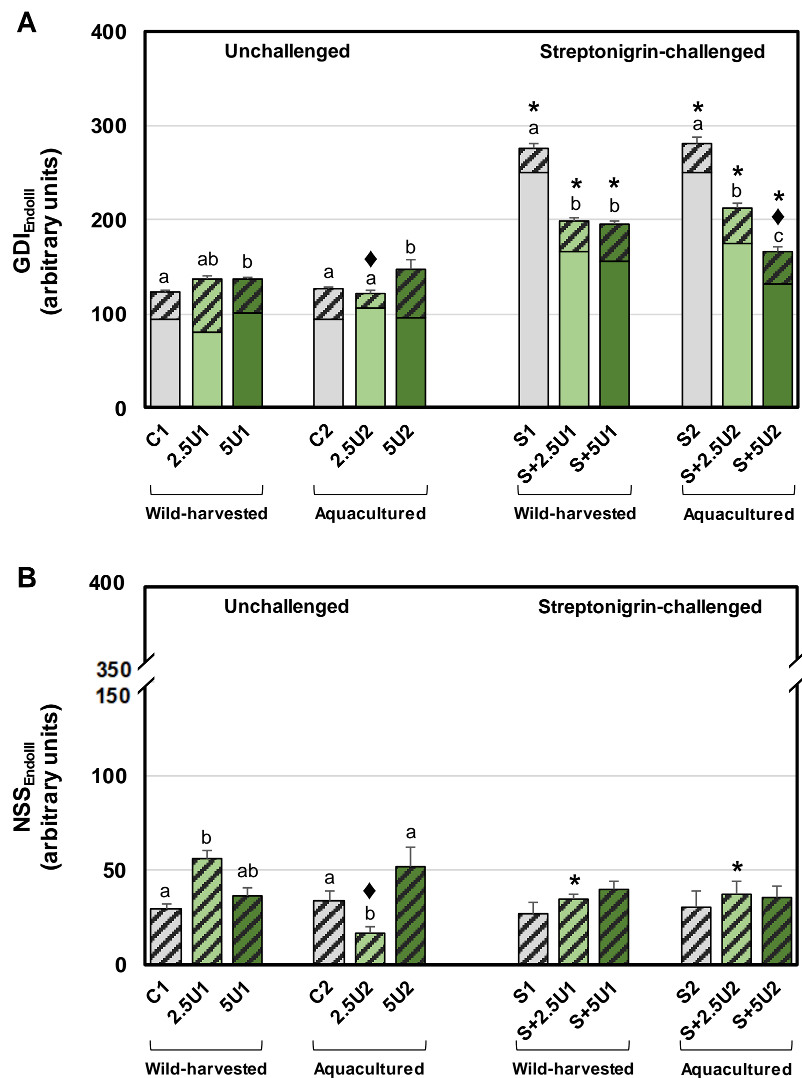

Figure 4.4. Mean values of oxidative pyrimidine DNA damage, measured by the improved comet assay in neuroblast cells of *D. melanogaster* after a dietary trial with the macroalga *U. rigida* (two levels of supplementation - 2.5 and 5%, transposed to the group identifier abbreviation as the number preceding the letter U) from wild-harvesting (U1) and aquaculture (U2) origins. Two major groups were evaluated: one unchallenged and another streptonigrin-challenged. C and S represent the unsupplemented groups, respectively, unchallenged and streptonigrin-challenged. (A) overall damage (GDI$_{EndoIII}$) and partial scores, *i.e.* GDI plus additional DNA breaks corresponding to net EndoIII-sensitive sites (NSS$_{EndoIII}$; dark grey diagonal stripes); (B) NSS$_{EndoIII}$ alone. Bars represent the standard error. Statistically significant differences ($p < 0.05$) between groups submitted to the supplementation with *U. rigida* of the same origin, under the same genotoxic challenge are marked by different letters; differences between U1 and U2 groups under the same genotoxic challenge and level of supplementation are marked by black diamond (♦); differences between groups unchallenged and streptonigrin-challenged, under the same level of supplementation, from the same origin are marked by asterisk (*).

4.4. Discussion

Marine macroalgae have been defended recently as functional food, due to their beneficial properties to the human health (*e.g.* genome protection) linked to their phytochemical profile (Holdt and Kraan 2011; Mohamed et al. 2012; Wells et al. 2017). Considering that the algae phytochemical composition varies with the macroalga growing conditions and/or origin (Lahaye et al. 1995; Gómez-Pinchetti et al. 1998; Abreu et al. 2009), it is plausible that the genome protection properties can also fluctuate according with that factor, although this hypothesis has been scarcely studied. Bearing this in mind, the current work intended to assess variations on the phytochemical profile of *U. rigida* related to its origin and, subsequently, to investigate to what extent could the antigenotoxic potential of this alga (against endogenous or exogenous insults) be differently expressed.

4.4.1. Origin-related phytochemical profiling of *U. rigida*

The phytochemical profiling (semi-quantitative determination) of wild-harvested and aquacultured *U. rigida* revealed an origin-based differential pattern, namely concerning fatty alcohols, sterols, sesquiterpenoids and glycerol esters classes, found in higher amounts in the aquacultured alga. In turn, fatty acid derivatives, as well as porphyrinolactone- and chlorophyll-related compounds, displayed an occurrence not sufficiently consistent to reveal a distinct origin-based pattern. Nevertheless, the origin-based differential pattern observed may be explained by the higher fluctuations of the growing conditions to which the wild-harvested specimens are submitted, namely environmental factors such as water temperature, light and salinity, as well as nutrients and minerals availability (which vary according to season and location). These variables are known to directly influence the variety and quantity of macroalgae constituents (Marinho-Soriano et al. 2006). Moreover, *U. rigida* specimens from aquaculture were farmed on a certified organic IMTA system, *i.e.*, the macroalga could assimilate fish-excreted ammonia, phosphate and CO_2, converting them into potentially valuable biomass (Abreu et al. 2011). In fact, the cultivation of seaweeds in IMTA systems promotes higher productivity levels, allowing less variability than the natural seaweed beds due to higher and more constant nutrients availability (Abreu et al. 2009).

4.4.2. Streptonigrin-induced DNA damage

Albeit not being the main purpose of the current study, it seems important to address streptonigrin-induced genotoxicity, as formerly stated by some authors (Bolzán and

Bianchi 2001; Gaivão et al. 1999), namely to confirm the generation of an external genotoxic pressure that, hypothetically, can be counteracted by the macroalga supplementation. This metabolite produced by *Streptomyces flocculus* has antibiotic and antitumor activities and exerts genotoxic effects at the DNA level, mainly through three different modes of action: (i) the production of single and double strand breaks; (ii) the inhibition of topoisomerase II (by stabilizing cleavable complexes), which enhances the primary action; (iii) the induction of covalent DNA adducts (Bolzán and Bianchi 2001). Supporting the first mode of action mentioned, streptonigrin presents multiple metal complexation sites, through which binds irreversibly to the DNA, *via* the formation of streptonigrin-metal-DNA complexes, in the presence of certain metal ions, namely zinc, copper, iron, manganese, cadmium and gold (Bolzán and Bianchi 2001). Besides that, streptonigrin is an aminoquinone that, through auto-oxidation of the quinone moiety to a semiquinone (in the presence of NADH), results in the production of free radical species to produce its DNA-damaging effects (Cone et al. 1976; Lown and Sim 1976; Shiloh et al. 1983; Sugiura et al. 1984; Bolzán and Bianchi 2001). Again, the presence of metal ions catalyses that reaction, producing hydroxyl radicals through a Fenton-type reaction, which are assumed to be the ultimate source of streptonigrin-induced DNA damage (Cone et al. 1976; Lown et al. 1978; DeGraff et al. 1994; Testoni et al. 1997; Bolzán et al. 2001; Bolzán and Bianchi 2001).

In the current study, and regarding the non-specific DNA damage evaluated through the standard alkaline comet assay, *D. melanogaster* individuals exposed to streptonigrin confirmed the previous literature, since higher levels of DNA damage in the form of single and/or double strand breaks were depicted. In fact, unsupplemented fly groups exposed to that agent (S1 and S2) have more than doubled the levels of DNA damage. Surprisingly, the data interpretation regarding the oxidative DNA damage revealed somewhat unexpected details. Hence, S1 and S2 groups, though showing a damage increase as overall damage (GDI_{FPG}) in line with GDI, revealed lower levels of oxidized purine bases (NSS_{FPG}; sensitive sites converted into DNA breaks by FPG enzyme) than non-exposed groups. This results pattern was not perceptible regarding pyrimidine bases (which oxidation would be detected by EndoIII and converted into DNA breaks), demonstrating that purines and pyrimidines are differently affected. Considering that streptonigrin has been described as a pro-oxidant agent (as referred before), one can hypothesize that (i) it may induce different types of oxidative DNA lesions not detected by FPG or EndoIII, but also that (ii) the level of DNA damage induced was so significantly strong that most of it was already depicted in the form of single and/or double strand

breaks (and expressed as GDI), since oxidative stress may directly induce DNA strand breaks, beyond the oxidation of the DNA bases (Bertoncini and Meneghini 1995; Lloyd and Phillips 1999; Ciccia and Elledge 2010). This seems plausible especially considering that the *D. melanogaster* strain adopted in this study (*Ok* strain) is highly susceptible to the ROS attack and presents low activity of antioxidant enzymes (Gaivão and Comendador 1996). In the same direction, the lower or equal level of oxidative DNA damage, measured respectively as NSS_{FPG} and $NSS_{EndoIII}$ parameters in flies treated with streptonigrin comparatively to the unchallenged ones, should not be attributed to an activation of the antioxidant defences.

Globally, the streptonigrin genotoxicity was demonstrated by the current data, thereby allowing the exploitation of the antigenotoxic protection of *U. rigida* against an exogenous challenge.

4.4.3. *U. rigida* genoprotection potential and association with phytochemical profiling

Regarding the antigenotoxic potential of *U. rigida* against basal non-specific DNA damage, slight and ambivalent variations were depicted concerning the lower level of supplementation of both alga origins, translated into a damage reduction for 2.5U1 and an increase for 2.5U2. This contradictory pattern must be interpreted cautiously taking into consideration that those subtle variations resulted from a sensible balance between genotoxic and antigenotoxic pressures. The susceptibility of this balance, especially when the genotoxic *vs.* antigenotoxic potential of certain food, beverages, extracts or isolated compounds is considered, was formerly proved by several authors, when substances apparently described as beneficial showed a slight genotoxic action (Yen et al. 2002; Miyaji et al. 2004; Lambert and Elias 2010; Leandro et al. 2013; Ho et al. 2013; Alves et al. 2014; Oyeyemi et al. 2015; Azqueta and Collins 2016).

Interestingly, previous research revealed that those food, beverages or compounds previously mentioned as able to slightly reduce the DNA integrity baselines in the absence of a genotoxic challenge, frequently displayed antigenotoxic ability against a multiplicity of genotoxic agents (Yen et al. 2002; Miyaji et al. 2004; Leandro et al. 2013; Alves et al. 2014; Oyeyemi et al. 2015). Likewise, in the current study, *U. rigida* demonstrated its antigenotoxic capacity against the streptonigrin-induced DNA damage, also displaying a dose-response effect in the case of aquacultured specimens. Furthermore, similar outcomes were observed by our research group (Marques et al. 2018) when assessing the antigenotoxic potential of *U. rigida* on *D. melanogaster*, under the same experimental

conditions but through the presence of white spots on red eyes as a signal of genotoxicity. In this previous study (Marques et al. 2018), both alga origins showed strong genoprotection against the streptonigrin insult and, particularly, the aquacultured alga promoted even lower levels of small and total spots on flies' eyes.

Furthermore, when the oxidative DNA damage was under analysis, the parameters concerning the overall damage, as GDI_{FPG} and $GDI_{EndoIII}$, showed protection profiles identical to that disclosed by GDI, specifically concerning the genoprotection against streptonigrin. On the other hand, somewhat surprising details came to light regarding merely the DNA breaks resulting from the endonucleases activity (NSS_{FPG} and $NSS_{EndoIII}$ parameters). In general, *U. rigida* from both origins enhanced the DNA breaks resulting from FPG endonuclease activity, either in the absence or presence of streptonigrin. This was only punctually evident regarding EndoIII endonuclease activity. As mentioned before, genoprotection involves a complex net of processes and should be regarded as a multiphasic action (a resistance to simplification should be practiced). In line, certain food, beverages or isolated compounds that are usually described as antioxidants, also display pro-oxidant actions (Podmore et al. 1998; Yen et al. 2002; Lambert and Elias 2010; Ho et al. 2013). In fact, that pro-oxidant potential has been pointed to be possibly beneficial, since a mild oxidative stress may trigger cell antioxidant defences and xenobiotic-metabolising enzymes (Halliwell 2008), activating essential cell signalling pathways (Procházková et al. 2011), and, ultimately, preventing the development of certain diseases, such as cancer (Lambert and Elias 2010; Forester and Lambert 2011; Carocho and Ferreira 2013). To the authors' knowledge, no study investigated the pro-oxidant potential of *U. rigida*. Nonetheless, diverse studies pointed a pro-oxidant activity of certain phytocompounds, namely carotenoids (Astorg 1997; El-Agamey et al. 2004), vitamin C (Podmore et al. 1998), polyphenols (Halliwell 2008) [previously determined on *U. rigida* (Yildiz et al. 2012)] and β-sitosterol (Rosenblat et al. 2013) (determined on *U. rigida* in the current study), as well as unsaturated fatty acids extracted from *U. lactuca* (Wang et al. 2013). Hence, it is reasonable to assume that *U. rigida* may possess a mild pro-oxidant potential, which probably contributes to its antioxidant action and, indirectly, to the antigenotoxic capacity observed in the current study and in the previous ones (Celikler et al. 2008; Celikler et al. 2009a; Celikler et al. 2014; Marques et al. 2018). On the other hand, the pro-oxidant potential of those compounds depends on the dose (de Roos and Duthie 2015), as well as on their chemical structure and metal-chelating activity, which will influence their redox properties (Yen et al. 2002; El-Agamey et al. 2004). Thus, considering that the amount of FPG-induced DNA breaks on unchallenged individuals in

the higher supplementation level of wild-harvested *U. rigida* more than doubled regarding the same supplementation level of aquacultured alga or comparing with the groups exposed to streptonigrin, it cannot be ensured that this still corresponds to a beneficial mild pro-oxidant effect as described before.

Bearing in mind the specificities related to the algae origin, 5% of the aquacultured *U. rigida* revealed to be the condition which offered higher protection against the genotoxic insult inflicted by streptonigrin, pushing the DNA breaks almost to the unchallenged level, which can be consistently observed on GDI, GDI_{FPG} and $GDI_{EndoIII}$ parameters. This must be related with the higher production of certain phytocompounds in aquaculture context observed in the current study, resulting from the controlled cultivation conditions of this alga in an organic IMTA system and the genome protective properties ascribed to them. According to the literature, sterols, namely β-sitosterol and campesterol, showed anticarcinogenic (Shahzad et al. 2017; Sharmila and Sindhu 2017), antioxidant (Yoshida and Niki 2003) and antigenotoxic (Zeiger and Tice 1997; Paniagua-Pérez et al. 2008; Sharmila and Sindhu 2017) properties. In turn, sesquiterpenoids have shown antioxidant, anticarcinogenic and anti-inflammatory potential (Zhang et al. 2005; Chakraborty and Paulraj 2010; Ghantous et al. 2010; Merfort 2011). Natural pigments, such as chlorophyll, and pigment-related compounds previously showed antioxidant ability (Pangestuti and Kim 2011). Moreover, compounds belonging to the fatty alcohols class, especially octacosanol, displayed antioxidant (Ohta et al. 2008) and anti-inflammatory (Guo et al. 2017) capacities.

In general, the phytocompounds of *U. rigida* determined in the current study may explain the observed effects as well as the putative mechanisms regarding its genoprotection potential. Nevertheless, it should be considered the existence of other compounds not determined in the current study (*e.g.* sulphated polysaccharides, carotenoids, polyphenols), mainly because *U. rigida* was tested *in vivo* as a whole food. Thus, the observed effects were probably due, as well, to synergisms between the algal phytocompounds. In that context, and aiming the scrutiny of macroalgae as functional food, further studies should be pursued, either targeting a more comprehensive phytochemical analysis, as well as the unveiling of the genoprotection mechanisms triggered by *U. rigida* and other edible macroalgae.

4.5. Conclusions

U. rigida showed a clear DNA-protective action, especially against the genotoxic insult inflicted by streptonigrin. Paradoxically, this antigenotoxic potential seems to rely,

partially, on a subtle pro-oxidant potential (depicted on NSS_{FPG} and $NSS_{EndoIII}$ parameters) that could be triggering *D. melanogaster* neuroblast cells' antioxidant and antigenotoxic defences. Moreover, an origin-based genoprotection capacity was notorious on streptonigrin-challenged flies, where the higher supplementation level of aquacultured alga pushed the DNA breaks close to the unchallenged level.

This is in line with the demonstration that the growing conditions affect the phytochemical composition of *U. rigida*, since naturally growing (wild) and aquacultured specimens revealed some differences on that concern, especially on the lipophilic profile (hexane extract). Hence, aquacultured *U. rigida* showed higher relative amounts of fatty alcohols, sterols, sesquiterpenoids and glycerol esters.

Overall, in the context of reviewing macroalgae as functional food, particularly regarding DNA-protective properties, the current study depicted promising results though further studies should be pursued, since a more comprehensive phytochemical analysis can be advantageous, complementing a full disclosure of the genoprotection mechanisms.

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Chapter 5

Marine macroalgae as a dietary factor promoting DNA integrity – Assessing potential benefits of direct consumption and indirect intake of algae-borne phytocomponents using fish as a vehicle

5. Marine macroalgae as a dietary factor promoting DNA integrity – Assessing potential benefits of direct consumption and indirect intake of algae-borne phytocomponents using fish as a vehicle

Abstract

Marine macroalgae are an important element of the human diet in East Asia, while, in Europe, this practice is marginal. Contrarily, fishery and aquaculture products are often consumed in Europe. Moreover, the improvement of the nutritional value of farmed fish through their diets' manipulation has been suggested. Particularly, aquafeeds supplementation with macroalgae to promote the transference of their phytocompounds and related bioactivities, such as genoprotection, is currently hypothesized. Thus, our aim was to evaluate the genoprotection potential of *Ulva rigida*, *Fucus vesiculosus* and *Gracilaria gracilis* (as a mix), through a direct consumption and an indirect intake using fish (*Sparus aurata*) as a vehicle of the phytocompounds. Mice (*Mus musculus*) were subjected to a diet supplementation, for one month, with 5% of the macroalgae mix or 10% of the fillet of fish previously fed with an algae-supplemented aquafeed (for comparison purposes, mice were also fed with a 10% fish-supplemented diet), followed by a treatment with a genotoxic challenge, methyl methanesulfonate (MMS). The genetic damage was evaluated through the comet and the micronucleus assays. Complementarily, haematological, energy metabolism and oxidative stress related parameters were assessed to infer about the animals' general physiological status. Macroalgae promoted genoprotection against MMS-induced genetic damage, which was not verified regarding basal levels of damage. Genoprotection properties were not transferred *via* fish fed with the algae-enriched aquafeed, though the transmission of a healthier antioxidant status was depicted. Further, the supplemented diets tendentially smoothed down the antioxidant system, which is an evidence of a lower pro-oxidant challenging condition, alongside a compensatory process of saving cellular resources. In line, the algae-enriched diet was the only decreasing the levels of lipid peroxidation. No toxicity signals were associated to the supplemented diets. The decreased activity of hepatic lactate and isocitrate dehydrogenases promoted by the supplemented diets potentially reflects an improved energy balance. Overall, this study highlighted the genoprotection afforded by macroalgae, likely promoted by desmutagenic factors.

Keywords

Antigenotoxicity; Fish consumption; Functional food; Marine macroalgae consumption.

5.1. Introduction

Marine macroalgae have been used for human consumption for thousands of years (Fleurence and Levine 2016). In East Asian countries, such as Japan, South Korea and China, marine macroalgae have been an important element of the diet for long, which can be a factor underlying the lower prevalence of diet-related diseases and higher life expectancies (Yamori et al. 2001; Yuan and Walsh 2006). In turn, in Europe, this dietary habit is less recurrent and only recently macroalgae have gained social attention as functional food (Plaza et al. 2008; Holdt and Kraan 2011; Mohamed et al. 2012; Fleurence and Levine 2016; Wells et al. 2017).

On the other hand, fishery and aquaculture products comprise an important nutritional source of protein, amino acids, polyunsaturated fatty acids (PUFAs), vitamins and minerals (Khalili Tilami and Sampels 2018). Thus, fish or seafood is a crucial component of a healthy diet in Europe, reaching 25.1 kg per person per year (almost 4 kg more than the rest of the world), and particularly in Portugal, where the average value reaches 55.9 kg (FAO et al. 2015). Furthermore, a hypothesis concerning an eventual increase of the nutritional value of fish under farming conditions through the manipulation of their diet has been suggested (Bourre 2005). Particularly, macroalgae may be included in fish feeds to promote the transference of phytocomponents to the final consumer (Valente et al. 2015), especially in societies where fish is consumed more often than algae. This hypothesis was already confirmed with rainbow trout (*Oncorhynchus mykiss*), whose muscle accumulated higher levels of PUFAs after the diet supplementation with 3% to 6% of *Macrocystis pyrifera* (Dantagnan et al. 2009), as well as a higher iodine content after the incorporation of 5% of *Gracilaria* sp. (Valente et al. 2015). Nevertheless, this topic still requires further corroboration, particularly targeting the eventual transference to fish fillet of the macroalgae beneficial properties that have been previously described (*e.g.* Noda 1993; Patarra et al. 2011; Liu et al. 2012; Yende et al. 2014; Cornish et al. 2015; Fleurence and Levine 2016) and justified their definition as functional food (Holdt and Kraan 2011; Mohamed et al. 2012).

One of the beneficial properties attributed to macroalgae is related with their capacity to promote genome protection. In fact, some studies have highlighted the antioxidant, antigenotoxic and antimutagenic potential of macroalgae (*e.g.* Leite-Silva et al. 2007; Celikler et al. 2009b; Zubia et al. 2009; Valentão et al. 2010; Marques et al. 2018). Nevertheless, their mode of action in this context remains unclear, namely the elucidation on the involvement of desmutagen or bio-antimutagen factors. In fact, it has been proposed that the antigenotoxic factors acting before the mutagenic attack to the DNA,

through the partial or full inactivation of the compound, are designated desmutagens (Bhattacharya 2011; Izquierdo-Vega et al. 2017). In turn, bio-antimutagens (or true antimutagens) suppress the mutation process after the damage is inflicted to the genome, improving the repair and replication processes of the mutagen-damaged DNA (Bhattacharya 2011; Izquierdo-Vega et al. 2017).

Considering that the genome integrity and stability are crucial for the survival and proper functioning of living organisms, as well as the causal linkage between DNA damage and various diseases, it emerges the importance of studying the liaison between genome protection and macroalgae consumption, either directly or indirectly through the ingestion of fish as a vehicle of the algae phytocompounds. Nevertheless, to the authors' knowledge, the hypothesis concerning the prospective transference of the macroalgae genome protection ability to consumer *via* fish submitted to a macroalgae-supplemented diet was never approached.

Bearing in mind the identified gaps of knowledge, the main goal of this study was to evaluate the genoprotection potential of a mix of three marine macroalgae, *viz. Ulva rigida*, *Fucus vesiculosus* and *Gracilaria gracilis*, as well as to test the hypothesis that fish (*Sparus aurata*) fed with an aquafeed supplemented with the same algae mix can be an indirect vector of the algae-borne phytocomponents. To do that, a dietary trial was designed with mice (*Mus musculus*), in which animals were subjected, for 1 month, to 5% supplementation of the macroalgae mix or 10% of fillet from fish fed with an algae-supplemented aquafeed. Then, mice were exposed to a genotoxic challenge, methyl methanesulfonate (MMS), to allow the discrimination of the potential protection against basal and exogenously induced genetic damage. The single cell gel electrophoresis (comet) and the micronucleus (MN) assays were selected to assess the genotoxicity/antigenotoxicity capacities. Complementarily, to detect any potential toxicity of the supplementation and infer about the interference on the general physiological condition of the animals, a battery of haematological, energy metabolism and oxidative stress related parameters, as well as growth performance, was evaluated. Moreover, the evaluation of the antioxidant system status may allow the elucidation of antigenotoxicity mechanisms promoted by the different experimental diets *via* desmutagen factors.

5.2. Material and methods

5.2.1. Chemicals

Methyl methanesulfonate (MMS; CAS 66-27-3) was obtained from Acros Organics™, Fisher Scientific (USA). Anaesthetics ketamine (Imalgen® 1000) and xylazine (Rompun®

2%) were obtained from Merial SAS (France) and Bayer Healthcare SA (Germany), respectively. All other reagents were obtained from Sigma-Aldrich Chemical Company (Spain) and VWR International, LLC (USA).

5.2.2. Test organisms

Mice (*Mus musculus*) of FVB/n strain were generously donated by Dr. Jeffrey Arbeit and Dr. Douglas Hanahan, from the University of California, through the USA National Cancer Institute Mouse Repository. Animals were bred in-house and the study was authorized by the University of Trás-os-Montes e Alto Douro ethics committee (approval no. 10/2013) and the Portuguese Veterinary Directorate (approval no. 0421/000/000/2014).

5.2.3. Diets preparation

Four experimental diets were considered and prepared from the standard laboratory mice diet 4RF21® (Mucedola, Italy) at the Department of Genetics and Biotechnology of the University of Trás-os-Montes and Alto Douro. First, the standard diet was ground to powder in a mill (Retsch Mill SM1). Then, the four experimental diets were individually prepared by mixing each diet main ingredients with 5% of water, in a horizontal helix ribbon mixer (Mano, 100 L capacity, CPM) and dry pelleted using a laboratory pellet press (CPM, C-300) with a 4.8 mm die. Thus, the experimental diets obtained were (as detailed on Table 5.1): (i) S diet, composed only by the standard diet 4RF21® re-pelleted; (ii) A diet, composed by 95% of standard diet 4RF21® plus 5% of a mix of dried and ground macroalgae *U. rigida*, *F. vesiculosus* and *G. gracilis* (approximately 1.67% of each alga); (iii) F diet, composed by 90% of standard diet 4RF21® plus 10% of dried and ground fillet of *S. aurata* fed during 1 year with standard aquafeed; (iv) FA diet, composed by 90% of standard diet 4RF21® plus 10% of dried and ground fillet of *S. aurata* fed during 1 year with algae-supplemented aquafeed (incorporation of *U. rigida*, *F. vesiculosus* and *G. gracilis* at approximately 1.67% of each alga, totalizing 5% of macroalgae). All diets were kept at 4 ºC throughout the trial.

Macroalgae used on this study were reared at ALGAplus, Lda. (Ílhavo, Portugal), a certified organic and integrated multi-trophic aquaculture (IMTA) company and, for each alga, the same batch was used to produce both fish and mice feeds. Following harvesting, macroalgae were washed in seawater (treated with UV and filtered to 5 µm) and then dried during 12 h in a chamber with controlled temperature (25 ºC), achieving 10-12% of humidity, after which they were preserved in multiple layer packaging (paper and plastic).

Before the fish and mice feeds production, macroalgae were grinded to powder and incorporated in the respective feeds. The total percentage of the macroalgae incorporated on fish aquafeed fits within the macroalgae percentages already tested to increase the nutritional value of fish to its final consumer, through the increase of algae phytocompounds (Dantagnan et al. 2009; Valente et al. 2015). Considering that an average adult ingests about 1,800 g of food per day, that Asian populations consume between 4 and 8.5 g of dried macroalgae (Hwang et al. 2010; Zava and Zava 2011; Chen et al. 2018) and that some macroalgae species may have a swelling capacity of nearly ten times their dry volume (Zava and Zava 2011), it is realistic to evaluate 5% of macroalgae incorporation on the mice diet.

Table 5.1. Proximate composition of the different experimental diets: standard (S), algae-supplemented (A), fish-supplemented (F) and algae fed fish-supplemented (FA).

	Experimental diet			
Composition	**S**	**A**	**F**	**FA**
Standard laboratory mice diet 4RF21®	100%	95%	90%	90%
Dried mix of *U. rigida*, *F. vesiculosus* and *G. gracilis*	-	5% (equally divided for each alga species)	-	-
Dried fillet of fish fed with standard aquafeed	-	-	10%	-
Dried fillet of fish fed with algae-supplemented aquafeed (at 5%)	-	-	-	10%

Fish (*S. aurata*) were purchased from a local fish farm (Nasharyba, Lda., Figueira da Foz, Portugal) as fingerlings (8-10 g) and maintained in 500-L cylindric PVC tanks, under a natural photoperiod, as open systems, from July 2017 to July 2018 (2 tanks per condition, 25 fish per tank). Fish growth was carried out in the installations of ALGAplus, Lda. (Ílhavo, Portugal). Both aquafeeds for *S. aurata* were produced by SPAROS, Lda. (Olhão, Portugal), composed by the same basic ingredients, corresponding to a standard diet formulated according to *S. aurata* nutritional requirements, and an algae-enriched diet, incorporating the three different macroalgae species, as described before. Fish were hand-fed once a day at a daily rate of approximately 3.8, 3.5 and 3.0% [as percentage of

fish total weight; in accordance with Henriques (1998)] on the first, second and remaining months, respectively, except during the winter months, mainly between December and February, when fish reduced the responsiveness to feeding, being in this period fed 1-2 times per week. Following the animals' sacrifice, fish were conserved at -20 ºC until further processing. The fillets were removed from each group of fish, weighed and dried in a ventilated chamber at 35 ºC during 24 h achieving approximately 20% of humidity. Then, the dried fillets were ground in a food processor and incorporated in the respective mice feeds. Once more, aiming that an average person eats daily 1,800 g of food and, particularly, Portuguese population eats about 55.9 kg of fishery and aquaculture products per year (FAO et al. 2015), a daily 10% of fish in the diet fits within these standards.

5.2.4. Experimental design

Animals were maintained in accordance with the Portuguese (Decreto-Lei 113/2013) and European (EU Directive 2010/63/EU) legislation and the ARRIVE guidelines, under controlled conditions of temperature (23 ± 2 °C), light-dark cycle (12 h light/12 h dark) and relative humidity (50 ± 10%). Mice were housed in hard polycarbonate cages coated with corn cob, with adequate environmental enrichment, and health checks were performed daily. Water and food were provided *ad libitum*. Body weight (b.w.) was determined at the beginning and ending of the trial and weekly recorded (Appendix III – Table 1), together with food intake (f.i.) and water intake (w.i.). The ponderal gain [$PG = (W_2 - W_1) / W_2 \times 100$, where W_1 is the initial b.w. and W_2 the final b.w.] and the ponderal homogeneity index [$PH = 2W_L / (W_L + W_H)$, where W_L being the lowest animal weight and W_H the highest animal weight] were also calculated.

Eighty females at the age between 13 and 14 weeks were divided into eight experimental groups (10 animals per cage/group), corresponding to two groups for each dietary background (2 x 4 = 8 groups; see Figure 5.1): S, A, F and FA (detailed on 5.2.3.). After the gradual introduction of the experimental diets, the dietary trial was extended for 1 month. Then, for each dietary background, one group was intraperitoneally injected with the genotoxic compound MMS (at 40 mg kg^{-1} b.w. dissolved in saline solution), thus, setting the MMS-treated major group, and the other injected with saline solution, thus, setting the untreated major group. To calculate the volume to inject, respecting the desired MMS dose as a function of b.w., animals were weighed before the injection and the volume of the MMS stock solution (at 3.286 mg mL^{-1}) was proportionally defined according with a ratio of approximately 300 µL/25 g b.w. The MMS concentration selected

was based on previous studies (Kliesch et al. 1981; Pereira et al. 2005; Carvalho et al. 2011; Leffa et al. 2012).

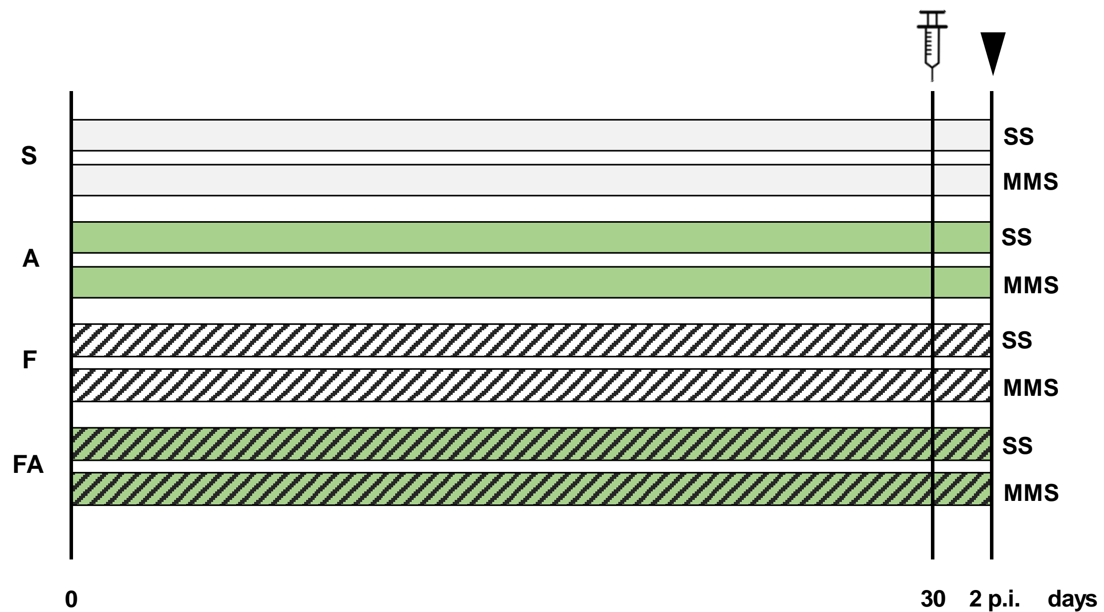

Figure 5.1. Schematic representation of the experimental design, depicting a 30-day period of dietary background with the following diets: standard (S), algae-supplemented (A), fish-supplemented (F) and algae fed fish-supplemented (FA). Then, one group for each dietary background was injected with saline solution (SS) or methyl methanesulfonate (MMS), and sampling carried out (arrowhead) two days post-injection (p.i.).

Two days after the injections, animals were weighed (see Table 5.2) and subjected to deep anaesthesia induced by ketamine/xylazine, as indicated by the Federation for Laboratory Animal Science (FELASA). Blood was first collected to heparinized capillary tubes (2 per animal) to perform microhematocrit test. Blood smears were immediately prepared for MN test. Animals' sacrifice was achieved by total exsanguination through cardiac puncture and, therefore, the remaining blood was collected to microtubes (without anticoagulant) and centrifuged (Heraeus Labofuge 400R; at 1,500 g for 15 min at 4 ºC) to isolate blood cells and serum, which were conserved at -80 °C, for comet assay and biochemical analysis, respectively. Liver was collected and conserved at -80 ºC for further biochemical analysis.

Table 5.2. Initial and final body weight (b.w.), ponderal gain (PG) and ponderal homogeneity index (PH) obtained for the different experimental groups (n = 10) fed with standard (S), algae-supplemented (A), fish-supplemented (F) or algae fed fish-supplemented (FA) diets, either untreated or treated with methyl methanesulfonate (MMS). Mean values ± standard errors are represented.

	Experimental diet	Initial b.w. (g)	Final b.w. (g)	PG	PH
Untreated	S	28.79 ± 0.60	29.64 ± 0.97	2.46 ± 1.74	0.96 ± 0.01
	A	28.07 ± 0.47	28.26 ± 0.35	0.68 ± 0.86	0.97 ± 0.00
	F	26.49 ± 0.67	27.43 ± 0.78	3.29 ± 1.24	0.96 ± 0.01
	FA	25.17 ± 0.44	26.70 ± 0.33	5.70 ± 1.45	0.96 ± 0.01
MMS-treated	S	26.40 ± 0.88	27.65 ± 0.95	4.46 ± 0.68	0.97 ± 0.00
	A	24.85 ± 0.80	26.23 ± 0.90	5.13 ± 1.21	0.96 ± 0.00
	F	27.78 ± 0.64	28.79 ± 0.52	3.48 ± 1.49	0.97 ± 0.01
	FA	26.21 ± 0.98	28.61 ± 1.04	8.20 ± 2.67	0.94 ± 0.01

5.2.5. Evaluation of genetic damage

5.2.5.1. Comet assay

The alkaline version of the comet assay was performed according with Collins (2004) methodology with slight modifications, as adapted by Guilherme et al. (2012). First, 5 µL of the pellet containing blood cells (including leucocytes, targeted by this assay) were added to 200 µL of phosphate buffered saline (PBS). From this cell suspension, 50 µL were added to 100 µL of 1% low melting point agarose in PBS, and then, two drops of 6 µL were placed vertically onto the glass microscope slides, precoated with 1% normal melting point agarose, without coverslips. In each slide twelve mini-gels were placed, as six columns of two, corresponding to six animals per slide. Then, the slides were left 5 min at 4 °C to solidify the agarose, immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for at least 1 h, at 4 °C. After lysis of agarose-embedded cells, slides were immediately placed in the electrophoresis tank, immersed in the electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH > 13) for 20 min (alkaline denaturation). Electrophoresis was performed during 20 min at a fixed voltage of 25 V and a current of 300 mA (achieved by adjusting the buffer volume in the electrophoresis tank). After the electrophoresis, slides were neutralized with PBS (10 min, 4 ºC) and with distilled

water (10 min, 4 ºC). Finally, slides were fixed in absolute ethanol for 10 min, air-dried and stored at room temperature.

Slides were stained with ethidium bromide (20 µg mL^{-1}) and fifty nucleoids were observed per mini-gel, using a Leica DMLS fluorescence microscope (400x magnification). The nucleoids were classified by visual scoring into 5 comet classes, according to the tail length and intensity from 0 (no tail) to 4 (almost all DNA in tail) (Collins 2004). The final score (expressed as "arbitrary units" in a range of 0-400) was obtained by multiplying the mean percentage of nucleoids in each class by the corresponding factor, according to this formula:

Genetic Damage Indicator (GDI) = [(% nucleoid class 0) x 0)] +
[(% nucleoid class 1) x 1)] + [(% nucleoid class 2) x 2)] +
[(% nucleoid class 3) x 3)] + [(% nucleoid class 4) x 4)].

The inhibition percentage (IP) afforded by supplemented A, F and FA diets was calculated, for untreated and MMS-treated groups, according to the following formula:

IP (%) = [(S diet - supplemented diet)/S diet)] x 100.

5.2.5.2. Micronucleus test

One blood smear for each animal was fixed with methanol for 10 min and stained with Giemsa (5%) for 30 min. Slides, previously coded, were subsequently blind scored. From each smear, 2000 erythrocytes were scored, under 1000x magnification (microscope Olympus BX50) to assess the presence of micronuclei (MN). The results obtained were expressed as the frequency of MN per 1000 cells (‰).

Again, the percentage of genotoxicity inhibition was calculated as described for the genetic damage assessed through the comet assay.

5.2.6. Haematological evaluation

Microhaematocrit test was performed following capillary tubes centrifugation (PrO-Vet Centurion, Scientific Limited; at 13,500 g for 5 min), after which, it was determined the ratio (%) of the volume of packed red blood cells (RBC) to the volume of whole blood.

Total protein content was determined in serum, through the Biuret method (Gornall et al. 1949), using bovine serum albumin as a standard, in a SpectraMax 190 microplate reader.

Serum alanine transaminase (ALT) activity, as a direct measure of formed pyruvate, was determined based on Reitman and Frankel (1957) method, adapted to microplate. Briefly, 0.1 mL of phosphate buffer (0.1 M; pH 7.4) with DL-alanine (0.2 M) and 2-oxoglutaric acid (0.002 M) at 37 ºC was added to 0.02 mL of each sample and incubated at the same temperature for 30 min. Then, 0.1 mL of chromogenic solution with 2,4-dinitrophenylhydrazine (0.001 M) and hydrochloric acid (HCl; 1 M) were added to the mixture, followed by a 20 min period at 25 ºC. After this, 1 mL of 1:11 diluted solution of sodium hydroxide (NaOH; 4,4 M) was added and, after 5 min, the change of absorbance was measured in a SpectraMax 190 microplate reader at 530 nm. ALT activity was calculated with a standard curve obtained with pyruvate standards.

5.2.7. Hepatic biochemical evaluation

Liver samples were homogenized using a Potter-Elvehjem homogenizer, in chilled potassium phosphate buffer (0.1 M, pH 7.4) in a 1:10 ratio [tissue mass (mg):buffer volume (mL)]. The resulted homogenate was then divided into two aliquots for lipid peroxidation (LPO) measurement and post-mitochondrial supernatant (PMS) preparation. The PMS fraction was obtained by centrifugation in a refrigerated centrifuge (Eppendorf 5415R) at 13,400 g for 25 min at 4 ºC. Total protein content was determined in PMS, as described before for serum protein. Aliquots of PMS were then divided into microtubes and stored at -80 ºC until further analyses.

5.2.7.1. Energy metabolism parameters

Lactate dehydrogenase (LDH) activity was determined in hepatic PMS according to Vassault (1983), adapted by Diamantino (2001), with some modifications. First, samples were all diluted to the same total protein concentration, 0.9 mg mL^{-1}. Briefly, 0.01 mL of sample, 0.25 mL of Tris (0.0813 M), sodium chloride (NaCl; 0.2033 M) and nicotinamide adenine dinucleotide hydrogen (NADH; 0.00025 M) solution, and 0.04 mL of Tris (0.0813 M), NaCl (0.2033 M) and pyruvate (0.012 M) solution were introduced in a microplate well, in triplicate. LDH activity was determined at 340 nm in a SpectraMax 190 microplate reader by following the decrease of absorbance, for a period of 2.5 min, due to the simultaneous oxidation of NADH and consumption of the substrate pyruvate. LDH activity was expressed in µmol NADH oxidized min^{-1} mg $prot^{-1}$, using a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

Isocitrate dehydrogenase (IDH) activity was determined in hepatic PMS according to Ellis and Goldberg (1971) and Lima et al. (2007), with some modifications. Briefly, 0.05

mL of sample (diluted at 0.9 mg mL^{-1} of total protein), 0.2 mL of reaction solution [Tris (0.05 M; pH 7.8), manganese chloride ($MnCl_2$; 0.002 M) and DL-isocitric acid (0.007 M)] and 0.05 mL of $NADP^+$ (0.0005 M) were introduced in a microplate well, in triplicate. IDH activity was determined at 340 nm during 2.5 min in a SpectraMax 190 microplate reader by following the increase of NADPH, when IDH decarboxylates isocitrate (DL-isocitric acid). IDH activity was expressed in nmol NADPH formed min^{-1} mg $prot^{-1}$, using a molar extinction coefficient of 6.22 x 10^3 M^{-1} cm^{-1}.

5.2.7.2. Antioxidant system and lipid peroxidation

Catalase (CAT) activity was assayed in PMS by Claiborne (1985) method, with slight modifications. Briefly, the assay mixture consisted of 0.19 mL potassium phosphate buffer (0.05 M, pH 7.0) with hydrogen peroxide (H_2O_2; 0.01 M) and 0.01 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 in a SpectraMax 190 microplate reader at 240 nm and CAT activity was calculated in terms of μmol H_2O_2 consumed min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 43.5 M^{-1} cm^{-1}.

Superoxide dismutase (SOD) activity 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), forming a red formazan dye determined at 505 nm in a SpectraMax 190 microplate reader. Then, SOD activity was measured by the degree of inhibition of this reaction, considering that one SOD unit causes a 50% inhibition of the INT reduction rate, 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 described by Mohandas et al. (1984) and modified by Athar and Iqbal (1998). The assay mixture consisted of 0.09 mL potassium phosphate buffer (0.05 M, pH 7.0), 0.03 mL of PMS, 0.03 mL glutathione reductase (GR; 2.4 U mL^{-1}), 0.03 mL reduced glutathione (GSH; 0.01 M), 0.03 mL sodium azide (NaN_3; 0.01 M), 0.03 mL ethylenediaminetetraacetic acid (EDTA; 0.01 M), 0.03 mL NADPH (0.0015 M) and the reaction was initiated with 0.03 mL H_2O_2 (0.0025 M) in a total volume of 0.3 mL. Oxidation of NADPH to $NADP^+$ was recorded at 340 nm in a SpectraMax 190 microplate reader and GPx activity was calculated in terms of nmol NADPH oxidized min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 6.22 x 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. Briefly, the assay mixture contained 0.05 mL of PMS fraction and 0.25 mL of reaction medium consisted of NADPH (0.0002 M), glutathione disulphide (GSSG; 0.001 M) and diethylenetriaminepentaacetic acid (DTPA; 0.0005 M) dissolved in potassium phosphate buffer (0.05 M, pH 7.0). The enzyme activity was determined by measuring the oxidation of NADPH at 340 nm in a SpectraMax 190 microplate reader and calculated as nmol NADPH oxidized min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 6.22 x 10^3 M^{-1} cm^{-1}.

Glutathione-S-transferase (GST) activity was determined in PMS using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate according to the method of Habig et al. (1974). The assay mixture consisted in 0.1 mL of PMS fraction, 0.17 mL of GSH (0.001765 M) in potassium phosphate buffer (0.2 M, pH 7.9). The reaction was initiated by adding 0.03 mL of CDNB (0.01 M) and the increase in absorbance was recorded at 340 nm in a SpectraMax 190 microplate reader. GST activity was calculated as nmol CDNB conjugate formed min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 9.6 x 10^3 M^{-1} cm^{-1}.

Total glutathione content (GSH_t) in PMS was precipitated with trichloroacetic acid (TCA; 12 %) for 1 h (at 4 ºC) and then centrifuged at 12,000 g for 5 min at 4 ºC (Eppendorf 5415R). The content on GSH_t was determined in the resulting supernatant (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) producing a yellow coloured 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, in turn, is directly proportional to the GSH concentration in the sample. The assay mixture consisted in 0.04 mL of deproteinated PMS and 0.2 mL of DTNB (0.001 M) and NADPH (0.00034 M) diluted in sodium phosphate (0.143 M) and EDTA (0.0063 M) buffer (pH 8). The reaction was initiated with 0.04 mL of GR (8.5 U mL^{-1}). Formation of TNB was measured in a SpectraMax 190 microplate reader at 415 nm. It should be noted that GSSG is converted to GSH by GR in this system, which consequently measures total GSH content. The results were expressed as nmol TNB formed min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 14.1 x 10^3 M^{-1} cm^{-1}.

As an estimation of LPO, the quantification of thiobarbituric acid reactive substances (TBARS) was carried out in the previously prepared homogenate according to the procedure of Ohkawa et al. (1979) and Bird and Draper (1984) and adapted by Wilhelm Filho et al. (2001a; 2001b). Briefly, 0.005 mL of butylatedhydroxytoluene (BHT; 4% in methanol) and 0.045 mL of potassium phosphate buffer (0.05 M, pH 7.4) were added to

0.075 mL of homogenate and mixed well to prevent oxidation. To 0.05 mL of this mixture, 0.25 mL of TCA (12%) were added and vortexed, and 0.225 mL of Tris–HCl (0.06 M) and DTPA (0.0001 M) (pH 7.4) and 0.25 mL of thiobarbituric acid (TBA; 0.73%) were added. This mixture was heated for 1 h in a water bath set at 100 ºC and then cooled to room temperature and centrifuged (Eppendorf 5415R) at 15,700 g for 5 min. The absorbance of each sample supernatant was measured at 535 nm in a SpectraMax 190 microplate reader. LPO was expressed in nmol of TBARS formed mg tissue^{-1} using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

5.2.8. Statistical analysis

Statistica 8.0 software (StatSoft, Inc., USA) was used for the statistical analysis. First, all data were tested for normality (Shapiro–Wilk test and graphical analysis) and homogeneity of variances (Levene’s and Brown-Forsythe tests) and, when necessary, transformed to meet these statistical assumptions. A two-way ANOVA was applied to test the effects of the factors “experimental diet” and “MMS treatment”, as well as the interactions between them, for each parameter evaluated, followed by a post-hoc Newman-Keuls test for all pairwise comparisons. Statistically significant differences between groups were considered when $p < 0.05$ (Zar 1996).

5.3. Results

5.3.1. Evaluation of genetic damage

5.3.1.1. Comet assay

Regarding the untreated major group, A and F dietary groups depicted higher GDI values than S group (Figure 5.2A). The MMS treatment increased the GDI values of the four dietary backgrounds (S, A, F and FA). Among these, individuals fed with A, F and FA diets depicted lower GDI values than those fed with S diet (Figure 5.2A), revealing IP values of 13.8, 14.5 and 14.1%, respectively. A significant effect of the factor “MMS treatment” on GDI parameter and a significant interaction between the two factors are depicted on Table 5.3.

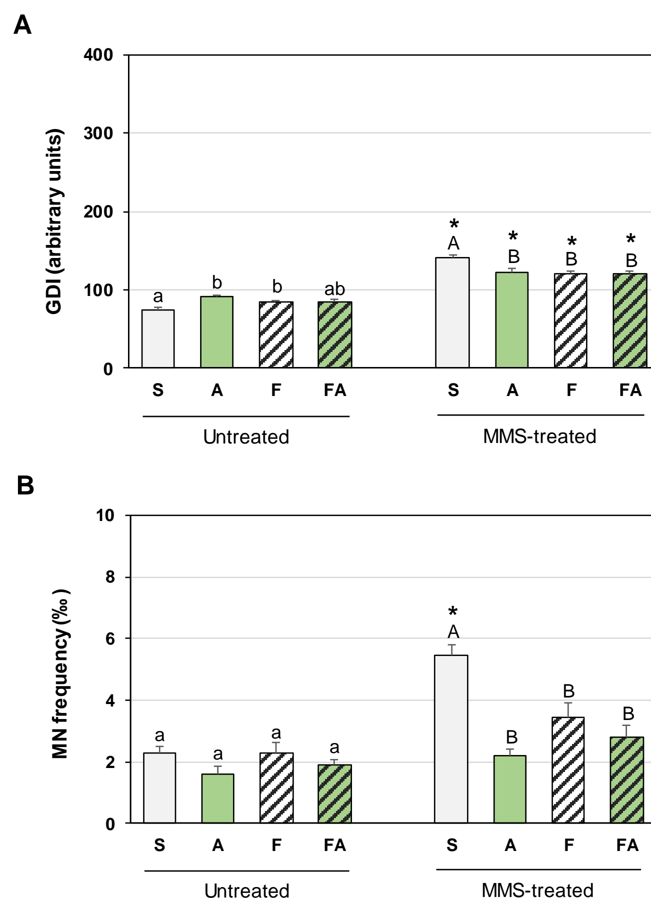

Figure 5.2. Mean values of genetic damage, measured through the (A) genetic damage indicator (GDI) and the (B) micronuclei (MN) frequency in the different experimental groups (n = 10) fed with standard (S), algae-supplemented (A), fish-supplemented (F) or algae fed fish-supplemented (FA) diets, either untreated or treated with methyl methanesulfonate (MMS). Bars represent standard errors. Different lower case or capital letters correspond to statistically significant differences ($p < 0.05$) between dietary backgrounds, within untreated or MMS-treated major groups, respectively; (*) corresponds to statistically significant differences ($p < 0.05$) between untreated and MMS-treated major groups, within the same experimental diet.

5.3.1.2. Micronucleus test

The group MMS-treated and fed with S diet revealed higher frequency of MN relatively to the reciprocal untreated group. Following the treatment with MMS, animals fed with A, F and FA diets showed lower MN frequency than the group fed with S diet (Figure 5.2B), translated in IP values of 59.6, 36.7 and 48.6%, respectively. Significant effects of the factors "experimental diet" and "MMS treatment" on the MN frequency are depicted on Table 5.3, as well as the significant interaction between the two factors.

Table 5.3. Results of the two-way ANOVA testing the effects of factors "experimental diet" and "methyl methanesulfonate (MMS) treatment", as well as the interactions between them on the different parameters evaluated.

Parameter	**Factors**				**Interaction**	
	Experimental diet		**MMS treatment**		**Experimental diet x MMS treatment**	
	F	***p***	**F**	***p***	**F**	***p***
GDI	1.037	ns	306.767	<0.05	11.592	<0.05
MN	12.226	<0.05	36.291	<0.05	3.956	<0.05
PG	2.981	<0.05	4.462	<0.05	0.66	ns
PH	1.3	ns	0.1	ns	1	ns
Haematocrit	2.4	ns	0.3	ns	2.4	ns
Total serum protein	3.22	<0.05	0	ns	0.66	ns
ALT	8.766	<0.05	5.604	<0.05	3.324	<0.05
LDH	22.817	<0.05	0.743	ns	4.81	<0.05
IDH	26.36	<0.05	0.03	ns	0.96	ns
CAT	7.736	<0.05	0.348	ns	0.612	ns
SOD	7.573	<0.05	4.264	<0.05	2.31	ns
GPx	14.225	<0.05	0.428	ns	1.044	ns
GR	27.241	<0.05	0.001	ns	2.589	ns
GST	11.913	<0.05	0.246	ns	4.444	<0.05
GSH_t	5.468	<0.05	1.434	ns	1.681	ns
LPO	10.804	<0.05	0.001	ns	0.807	ns

5.3.2. Assessment of the overall physiological status

No mortality occurred during the experimental trial. Furthermore, no visual alterations were visible regarding animals' behaviour or macroscopic features. Initial and final b.w. are depicted in Table 5.2, showing no statistical differences regarding PG and PH indexes, although significant effects of the factors "experimental diet" and "MMS treatment" were observed on PG index (Table 5.3).

For each dietary group, total food intake (f.i.) and water intake (w.i.) per animal per day (mean ± standard error) were the following: S diet, f.i. = 4.18 ± 0.03 g and w.i. = 4.99 ± 0.22 g; A diet, f.i. = 4.21 ± 0.12 g and w.i. = 5.72 ± 0.00 g; F diet, f.i. = 3.71 ± 0.15 g and w.i. = 4.18 ± 0.35 g; FA diet, f.i. = 4.23 ± 0.39 g and w.i. = 5.22 ± 0.52 g.

5.3.2.1. Haematological evaluation

No differences were depicted regarding haematocrit and total serum protein (Figures 5.3A and 5.3B), although a significant effect of the factor "experimental diet" was observed on the total serum protein parameter (Table 5.3). ALT activity showed no differences between the dietary groups within the untreated major group. Instead, MMS-treated animals fed with A diet showed higher ALT activity than the reciprocal untreated group. Moreover, MMS-treated individuals fed with A and FA diets depicted higher ALT activity relatively to S diet (Figure 5.3C). Significant effects of the factors "experimental diet" and "MMS treatment", as well as a significant interaction between them were observed for this parameter (Table 5.3).

5.3.3. Hepatic biochemical evaluation

5.3.3.1. Energy metabolism parameters

Regarding LDH activity on the untreated major group, the experimental diets A, F and FA showed lower levels in comparison with S diet group (Figure 5.4A). Moreover, while the MMS treatment increased this parameter on F group (relatively to the untreated reciprocal one), the opposite pattern was depicted on the A group. Additionally, mice treated with MMS and fed with A and FA diets showed lower LDH activity than animals fed with S and F diets (Figure 5.4A). A significant effect of the factor "experimental diet" and a significant interaction between the two factors were observed for this parameter (Table 5.3).

Considering IDH parameter, A, F and FA groups revealed lower values than S group, regarding both the untreated and MMS-treated major groups (Figure 5.4B). A significant effect of the factor "experimental diet" was observed for IDH (Table 5.3).

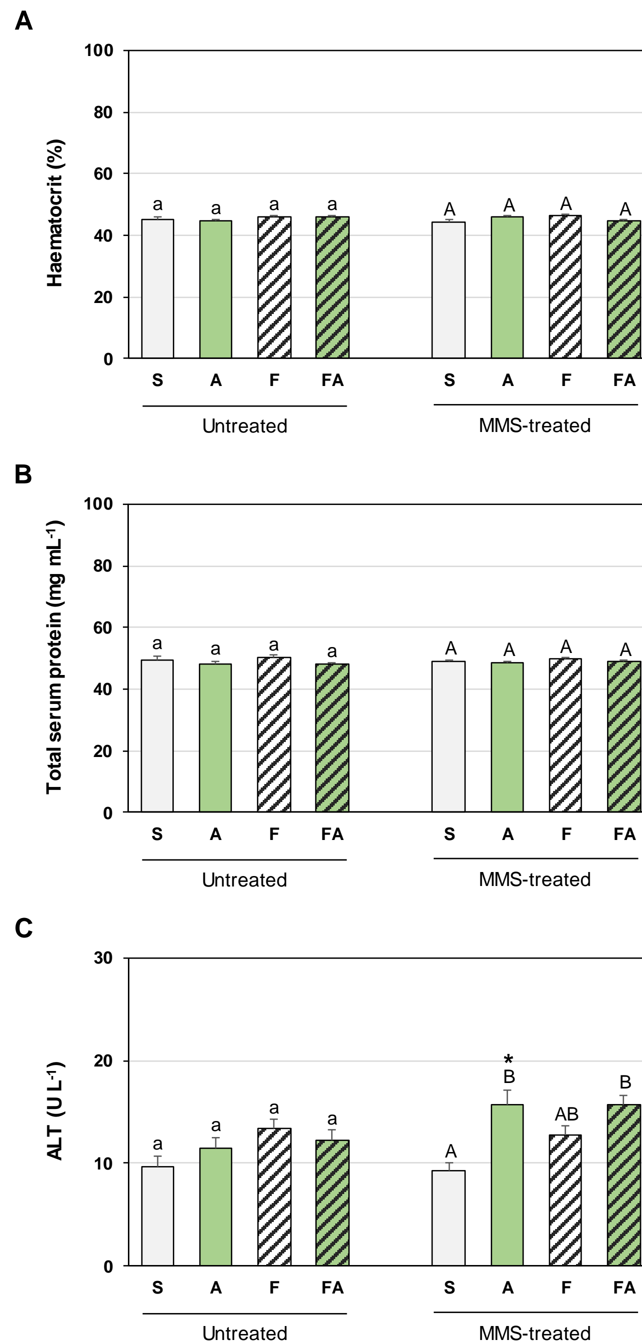

Figure 5.3. Mean values of haematological parameters, namely (A) haematocrit, (B) total serum protein levels and (C) serum alanine transaminase (ALT) activity, determined in the different experimental groups (n = 10) fed with standard (S), algae-supplemented (A), fish-supplemented (F) or algae fed fish-supplemented (FA) diets, either untreated or treated with methyl methanesulfonate (MMS). Bars represent standard errors. Different lower case or capital letters correspond to statistically significant differences ($p < 0.05$) between dietary backgrounds, within untreated or MMS-treated major groups, respectively; (*) corresponds to statistically significant differences ($p < 0.05$) between untreated and MMS-treated major groups, within the same experimental diet.

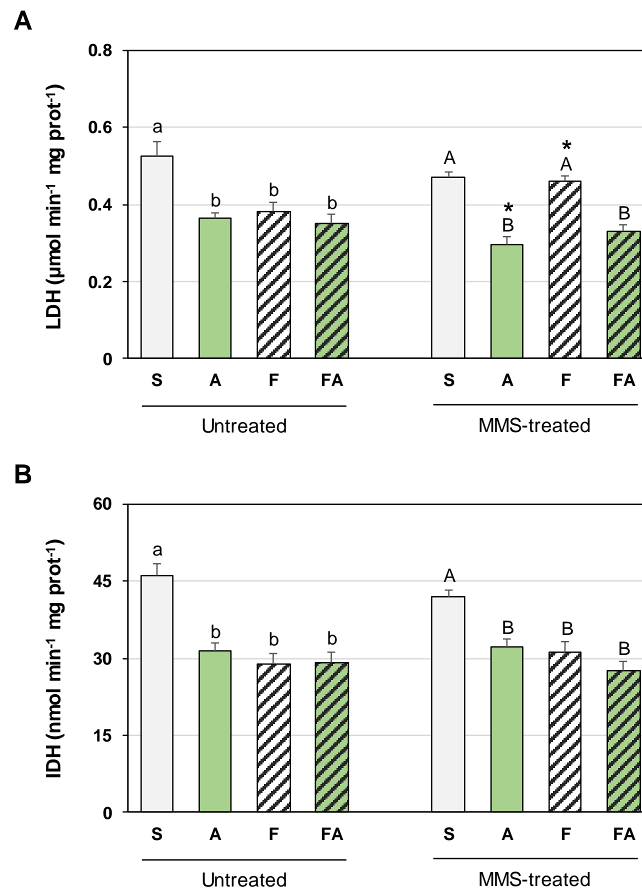

Figure 5.4. Mean values of energy metabolism parameters, namely (A) lactate dehydrogenase (LDH) and (B) isocitrate dehydrogenase (IDH) activities, determined in the different experimental groups (n = 10) fed with standard (S), algae-supplemented (A), fish-supplemented (F) or algae fed fish-supplemented (FA) diets, either untreated or treated with methyl methanesulfonate (MMS). Bars represent standard errors. Different lower case or capital letters correspond to statistically significant differences (p < 0.05) between dietary backgrounds, within untreated or MMS-treated major groups, respectively; (*) corresponds to statistically significant differences (p < 0.05) between untreated and MMS-treated major groups, within the same experimental diet.

5.3.3.2. Antioxidant system and lipid peroxidation

The untreated major group showed differences on CAT activity between the dietary backgrounds, namely animals fed with F and FA diets revealed lower values than animals fed with S diet. No alterations were depicted regarding the MMS treatment (Figure 5.5A).

The same pattern was observed on SOD activity within the untreated major group, while MMS-treated animals fed with A diet depicted a lower activity relatively to S group (Figure 5.5B).

Regarding GPx activity, untreated mice fed with the experimental diets A, F and FA showed decreased levels in comparison with S diet, while MMS-treated individuals fed with A and FA diets demonstrated lower GPx activity relatively to S diet, and A group lower activity in comparison with F (Figure 5.5C).

A similar pattern was observed regarding GR activity of the untreated major group, since mice fed with A, F and FA diets revealed lower activity than S group. Animals fed with F diet and treated with MMS showed higher GR activity than the untreated reciprocal group. On the MMS-treated major group, individuals fed with A and FA diets depicted lower activity levels of this enzyme relatively to S and F dietary groups (Figure 5.5D).

Regarding GST activity, no differences were observed between diets on untreated individuals, but following the MMS treatment, mice fed with A and FA diets showed lower enzyme activity relatively to S and F groups (Figure 5.5E).

Moreover, no differences between the experimental diets were visible on GSH_t content of the untreated mice, while after the MMS treatment, the A group depicted a lower value of this parameter in comparison with S and F groups (Figure 5.5F).

Regarding the evaluation of LPO, untreated animals fed with A diet showed lower levels of this indicator, relatively to S and F dietary groups. The MMS treatment did not expressively change this profile, since the same dietary group (A) revealed lower TBARS levels relatively to all the others (Figure 5.5G).

Significant effects of the factors “experimental diet” and “MMS treatment” on the different oxidative stress related parameters are depicted on Table 5.3, as well as the significant interactions between them. Briefly, the factor “experimental diet” showed significant effects on all the oxidative stress related parameters evaluated and the “MMS treatment” only revealed significant effect on the SOD activity, while GST was the only parameter demonstrating a significant interaction between the two factors.

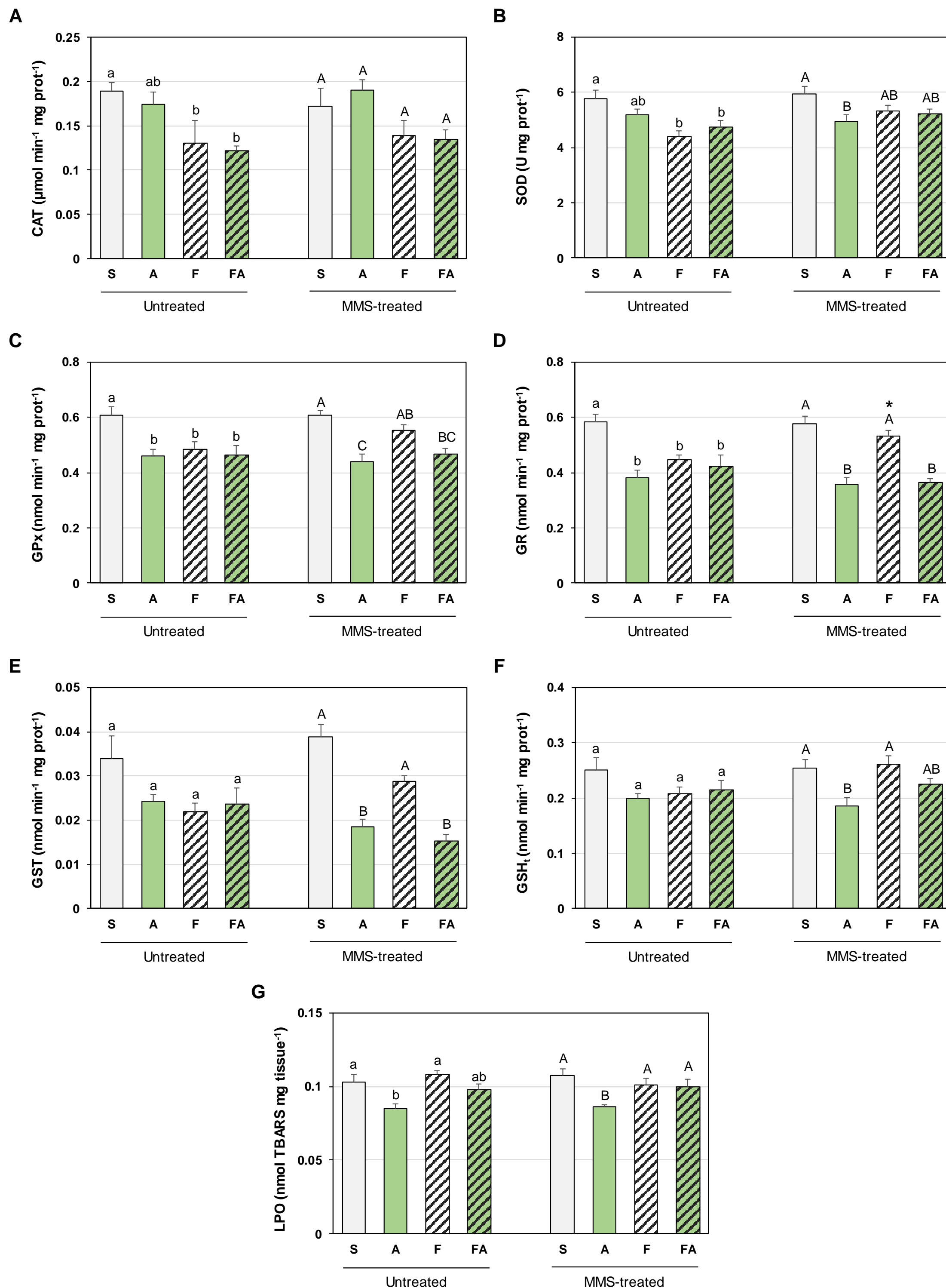

Figure 5.5. Mean values of hepatic oxidative stress parameters, namely (A) catalase (CAT), (B) superoxide dismutase (SOD), (C) glutathione peroxidase (GPx), (D) glutathione reductase (GR) and (E) glutathione-S-transferase (GST) activities, (F) total glutathione content (GSH_t), and (G) lipid peroxidation (LPO), determined in the different experimental groups (n = 10) fed with standard (S), algae-supplemented (A), fish-supplemented (F) or algae fed fish-supplemented (FA) diets, either untreated or treated with methyl methanesulfonate (MMS). Bars represent standard errors.

Different lower case or capital letters correspond to statistically significant differences ($p < 0.05$) between dietary backgrounds, within untreated or MMS-treated groups, respectively; (*) corresponds to statistically significant differences ($p < 0.05$) between untreated and MMS-treated major groups, within the same experimental diet.

5.4. Discussion

5.4.1. Antigenotoxic protection promoted by marine macroalgae

The dietary supplementation with marine macroalgae, either directly incorporated on the diet or indirectly consumed (using fish as a vehicle), was not able to diminish the basal levels of genetic damage in mice blood cells. In fact, the diet A showed a small decrease of DNA integrity measured through the comet assay, which was not matched by a MN frequency increase. MN translate a more severe and less transient type of genetic damage, while the DNA breaks measured by the comet assay are more prone to be repaired. Moreover, the GDI levels determined in those animals should be interpreted cautiously, taking into consideration that it resulted from a delicate balance between genotoxic and antigenotoxic pressures. Previous studies demonstrated that substances described as DNA integrity promotors depicted a slight genotoxic action, which may be a path to trigger the antigenotoxic defences (*e.g.* Yen et al. 2002; Leandro et al. 2013; Alves et al. 2014; Azqueta and Collins 2016).

In turn, the suitability of the compound MMS as a genotoxic challenge was confirmed, demonstrating a high specificity to preferentially attack the DNA molecule. Indeed, all the parameters determined on the S group (except the ones assessing genotoxicity) showed no effects caused by this agent, which contrasts with the genetic damage, since MMS induced both DNA strand breaks (measured by the comet assay) and chromosomal damage (measured by MN test). Accordingly, MMS have previously demonstrated its genotoxic effects on mice, after the injection at the same concentration currently used (Madrigal-Bujaidar et al. 1998; Carvalho et al. 2011; Leffa et al. 2012).

From that starting point, it was possible to address the antigenotoxic potential of the macroalgae against an exogenous source of genetic damage. Thus, it was clear that the direct intake of macroalgae by mice allowed the decrease of the DNA damage. Still, this genoprotective effect was not enough to push the genetic damage (measured as GDI) to the basal levels. On the contrary, this outcome was observed when MN frequency was interpreted, since the A diet was able to completely block the MMS genotoxic effects. Accordingly, several marine macroalgae have recently demonstrated DNA protection properties (Celikler et al. 2009b; Vatan et al. 2011; Gamal-Eldeen et al. 2013; Rodeiro et al. 2015; Ferreira et al. 2019). Particularly, the macroalgae species adopted in the current

study have already shown to enhance the genome protection, either in a mix incorporated in *S. aurata* aquafeed (Pereira et al. 2019) or individually tested: *F. vesiculosus* extract in cultured human lymphocytes (Leite-Silva et al. 2007), *U. rigida* extract on diabetic (Celikler et al. 2009a) and hypothyroidic mice (Celikler et al. 2014), and *U. rigida*, *F. vesiculosus* and *G. gracilis* dietary supplementation in the fruit fly (Marques et al. 2018).

Additionally, even though there were no statistically significant differences between the experimental diets tested, a decreasing tendency was observed on the MN frequency (S > F > FA > A), with group A (of both untreated and MMS-treated major groups) depicting the lowest values. Moreover, within the untreated major group, the A group showed the highest IP (30.4%), depicting almost the double value than FA (17.4%) and strongly contrasting with the IP revealed by the F group (0%). In line, on the MMS-treated major group, the A group showed, once more, the higher IP, allowing the inhibition of 59.6% of the genetic damage induced by MMS, (against only 48.6 and 36.7% promoted by FA and F diets, respectively). Hence, even though these signs were not translated into significant differences between the dietary supplementations, they may underline a higher genoprotection predisposition achieved by the direct intake of marine macroalgae. Eventually, this was not fully disclosed in the current study due to the relatively short period of the nutritional trial and/or to the level of macroalgae supplementation tested.

On the other hand, considering that both genotoxicity endpoints revealed that FA diet showed statistically similar antigenotoxic potential to the A one, but which were not superior to the benefits depicted by the F diet, it seems that the antigenotoxic ability provided by the fish fillet in FA group was not enhanced by the previous macroalgae supplementation of *S. aurata* aquafeed. Therefore, the hypothesis concerning the eventual transference of the macroalgae genome protection ability through the consumption of fish as a vehicle of the algae-borne phytocompounds was not confirmed. Nevertheless, it must be assumed that both fish groups originating F and FA diets were raised with top quality aquafeeds and optimal water conditions and this could be masking the full biotechnological potential of macroalgae-fed fish on that regard. Hence, considering that feeding farmed fish with high quality aquafeeds may not always be the rule in the aquaculture industry, and that this is known to influence *S. aurata* growth and feed efficiency rates (Aksnes et al. 1997; Vergara et al. 1999), it would be relevant to test the same experimental design with poorer quality aquafeeds. Furthermore, the dietary trial period of 1 month may have not been enough to disclose the eventual genome protective properties promoted by fillet of the macroalgae-fed fish, especially considering that nutritional habits with potential health benefits are usually advised to be lifelong routines

and not temporary practices. In addition, aiming the parallel to humans, it is important to bear in mind that the actual balance between nutritional aspects and genotoxic challenges that one is exposed to, may significantly vary in comparison with mice.

5.4.2. Impact of the experimental diets on the overall physiological status

First, none of the experimental diets influenced the animals' behaviour and morphological characteristics nor induced mortality or any impact on the weight related indexes evaluated (PG and PH), thereby providing a rough evidence of the basic safety of the diets.

Furthermore, according to Serfilippi et al. (2003), who performed a comprehensive evaluation of serum clinical chemistry and haematology parameters to establish reference values for different mice strains, female FVB mice of about 16 weeks old would depict normal values of haematocrit between 40.2 and 45.4% and total serum protein between 48 and 53 mg mL^{-1}. Moreover, Schneck et al. (2000) also evaluated several haematological and serum chemistry parameters in non-transgenic FVB female mice (7-9 weeks old) and revealed the haematocrit to be established between 36.9 and 50.0%. Thus, the haematocrit and total serum protein levels determined on the current study properly fitted in those ranges, highlighting that the experimental diets tested had no impact on those parameters. Moreover, this profile was not altered by the MMS injection, suggesting that the macroalgae-supplemented, as well as both fish- and macroalgae fed fish-supplemented diets, allowed a homeostatic maintenance of these basic haematological parameters and/or that the compound MMS (at 40 mg kg^{-1} b.w.) did not induce alterations on those biomarkers. Likewise, Oshida et al. (2008) found similar results, considering that, 4 and 24 h after the treatment with MMS (50-150 mg kg^{-1} b.w.), mice showed no alterations on haematocrit and total plasma protein levels.

In turn, current results related to the serum ALT showed no impact caused by the A, F and FA diets on the untreated major group, pointing out the absence of hepatotoxicity. Moreover, the same was revealed for the MMS injection alone (S group). Again, Oshida et al. (2008) showed no effects of MMS on mice plasma ALT activity either 4 or 24 h after the injection (50-150 mg kg^{-1} b.w.). In line, Nicolella et al. (2017) also demonstrated no effects on mice serum ALT activity 24 h after one MMS injection (40 mg kg^{-1} b.w.). Nonetheless, in the current work, within MMS-treated major group, this parameter was enhanced on the A and FA groups, which also occurred with one of the doses of fruit *Persea americana* pulp oil tested on mice by Nicolella et al. (2017). This must be regarded as a mild effect, since current values are, actually, lower than the reference values

depicted by Serfilippi et al. (2003) and Schneck et al. (2000) for FVB female mice (24-48 U L^{-1} and 51-270 U L^{-1}, respectively). In fact, ALT is considered a biomarker of hepatic damage caused by diseases or numerous substances, which may be translated on leakage of that enzyme (among others) from injured hepatocytes into the blood (Lehninger et al. 2005). Nicolella et al. (2017) attributed the augment of ALT levels induced by the higher dose of *P. americana* fruit pulp oil to its fatty acids (FAs) profile, in particular, the elevated composition on palmitic acid. In fact, the excessive amount of long-chain saturated FAs, such as the palmitic, myristic or stearic acids may induce cell toxicity (Nicolella et al. 2017), probably due to alterations in the phospholipid composition of the endoplasmic reticulum membrane, compromising its structure and integrity (Borradaile et al. 2006), which, in turn, may contribute to apoptotic cell death, lipotoxicity and liver injury (Cao et al. 2012; Ogawa et al. 2018). Accordingly, the macroalgae used in this study have a rich composition on FAs (with both saturated and unsaturated chains) (Gómez-Pinchetti et al. 1998; Francavilla et al. 2013; Schmid et al. 2014), being the palmitic acid one of the most representative. Additionally, the fillet of *S. aurata* that was fed with algae-supplemented aquafeed may present higher FAs accumulation [as observed by Fountoulaki et al. (2009) who replaced fish oil by vegetable oils in *S. aurata* diet] and consequent higher bioavailability, which could explain why both A and FA groups were the only ones displaying the increment on ALT levels. Nevertheless, it must be reinforced that this alteration only occurred on the MMS-treated A and FA groups and not on the untreated comparable ones and that a significant interaction between factors "experimental diet" and "MMS treatment" was noticed. Thus, it should be taken into consideration that a combination of those factors was behind the ALT increment and that the respective diets should be considered harmless regarding this parameter.

Usually, LDH activity is determined as an index of cell damage, namely hepatic or heart lesions, when it is leaked from those cells, raising its level in the serum (Deters et al. 1998). However, in the current study, LDH activity was determined on the liver homogenate, therefore contributing to infer about the energy metabolism pathways that the hepatic cells could be employing. Indeed, pyruvate, after production through glycolysis, can be turned into lactate through the LDH activity (in a reversible reaction), especially in anaerobic conditions, to produce energy (Lehninger et al. 2005). Thus, mice from untreated groups subjected to the diets A, F and FA, as well as those MMS-treated and subjected to the diets A and FA, depicting lower LDH activity than S group, will, eventually, benefit from a higher energy balance (favourably produced through the Krebs cycle), though hypothetically more oxygen-dependent and less tolerant to hypoxia. This

aspect deserves some further investigation, especially considering that recent studies have associated low or inhibited LDH activity with tumour progression inhibition (Granchi et al. 2010; Le et al. 2010). In fact, most invasive tumour phenotypes depict the so-called *Warburg effect*, that is a switch from oxidative phosphorylation to an increased anaerobic energy metabolism through the upregulation of LDH genes, together with oncogene activation and vascularization increase, creating hypoxic regions, where, ultimately, only those tumour cells can survive (Granchi et al. 2010). Hence, some LDH inhibitors have been studied to prevent this effect and disclose their full potential as anticancer agents (Granchi et al. 2010; Le et al. 2010). Furthermore, it is known that LDH levels in the rat tissues vary with age, decreasing in senescent animals (Singh and Kanungo 1968). On the opposite, the mice used in the current study are young and the LDH activity reduction is only visible on certain groups. A similar result was noticed by Özen and Korkmaz (2003), who described a significant decrease on the hepatic LDH activity on mice treated with *Urtica dioica* hydroalcoholic extract. These authors suggested that this could be due to a cytoprotective action, including against pro-oxidation-induced membrane damage, promoted by this plant. Additionally, some studies suggested that an increase on vitamin C consumption may lead to a decrease of LDH activity, but, since those studies concerned rats (Swamy et al. 2011) and humans (Tauler et al. 2003) sera, the effect was related to a hepatic damage prevention. The macroalgae currently tested possess vitamin C and/or other compounds with similar biological properties (antioxidant) (Díaz-Rubio et al. 2009; Taboada et al. 2010; Francavilla et al. 2013) that also are present in fish like *S. aurata* (Harlioğlu et al. 2016; Öztürk et al. 2019).

Two IDH different forms are identified, catalysing identical reactions, but one requiring NAD^+ as electron acceptor, occurring in the mitochondrial matrix and helping in the Krebs cycle, and the other requiring $NADP^+$, found in both the mitochondrial matrix and the cytosol, which main function is the regeneration of NADPH (Lehninger et al. 2005). Thus, the $NADP^+$-dependent isozyme, which activity was determined in the current study, plays essential roles, providing NADPH for reductive anaerobic reactions (Lehninger et al. 2005) (as the one catalysed by LDH), or in the maintenance of the cellular redox status, supplying NADPH that works as a cofactor of GR and needs to be regenerated (Jo et al. 2001; Lee et al. 2002; Lima et al. 2007). Hence, since the LDH activity measured in some experimental groups of this study depicted lower levels (due to the eventual enzyme activity inhibition or reduced synthesis), a negative feedback mechanism could be causing the reduced levels of IDH observed in the respective groups, except on the MMS-treated F group. In fact, MMS-treated animals fed with F diet depicted levels of GR and LDH

activities like S diet group and values of IDH activity lower than the same group, which may suggest that, in this case, LDH was providing NAPDH to GR. Moreover, GR activity on the other groups (untreated A, F and FA and MMS-treated A and FA groups) showing levels under the control, supports the idea that a feedback mechanism may have influenced the LDH/IDH expression/activity.

Furthermore, the experimental diets A, F and FA induced a similar profile on the antioxidants of the untreated major group, since animals fed with those diets revealed tendentially lower levels. This could suggest either an inhibition or a minor expression/synthesis of those low molecular weight scavengers and antioxidant enzymes. Nevertheless, considering that oxidative damage arises when the critical equilibrium between free radical generation and antioxidant defences is unfavourable (Rock et al. 1996; Lobo et al. 2010), the lack of augmented LPO damage (as TBARS) lead to the interpretation that the current antioxidants' profile reflects a lower pro-oxidant challenge rather than a defence impairment. On the other hand, only A diet was able to decrease LPO levels on the liver, both on the untreated and MMS-treated mice, even though MMS itself did not induce LPO. Hence, in this aspect, the macroalgae-supplemented diet revealed to be advantageous, which must be linked to their unique phytochemicals' profile, provided directly to the mice, and which antioxidant and radical scavenging activity was already demonstrated (*e.g.* Celikler et al. 2009b; Francavilla et al. 2013; Kang et al. 2013; Wang et al. 2013; Andrade et al. 2013; Yildiz et al. 2014). Moreover, the levels of GR and GST activities showed by the MMS-treated FA group (similar to A and lower than F groups) suggest the indirect transference of a minor pro-oxidant status *via* consumption of fish previously fed with a macroalgae-supplemented diet, due to the eventual accumulation of the algae bioactive compounds in the fish fillet, as it was found to occur with iodine (Valente et al. 2015) or PUFAs (Dantagnan et al. 2009).

5.4.3. Potential mechanisms contributing to the antigenotoxic protection promoted by the marine macroalgae

Aiming to the concept of desmutagens and bio-antimutagens (Bhattacharya 2011; Izquierdo-Vega et al. 2017), it may be possible to classify the antigenotoxic mechanisms afforded by the macroalgae, although the concept of (anti)genotoxicity is broader than the (anti)mutagenicity one. Thus, bearing in mind the results concerning the antioxidant system, especially the lower antioxidant activities concomitant with the absence of lipid peroxidation, one could assume that the genome protection observed was achieved before the attack occurred, thus, being the direct macroalgae intake a source of

desmutagen substances. In fact, the cause behind the antioxidants' profile may be acting upstream the antioxidants' synthesis, namely if the macroalgae-supplemented diet (A) have reduced the generation of reactive oxygen species (ROS). For example, Alam et al. (2016) revealed that ethanolic and aqueous extracts of *U. lactuca* showed the potential to diminish the H_2O_2 generation on rat liver after treatment with γ-ionizing irradiation. Moreover, additional studies reported that macroalgae (or extracts) may protect against oxidative stress (*e.g.* Senevirathne et al. 2006; Yuan and Walsh 2006; Godard et al. 2009; Farvin and Jacobsen 2013; Eo et al. 2015), and, at least, one study revealed a decrease in GPx activity and the reduction in SOD and GPx genes expression in rat liver after the ingestion of *U. linza* for 8 weeks (Ramirez-Higuera et al. 2014). In addition, Pereira et al. (2019) reported a decrease on GSHt content in *S. aurata* blood after a dietary supplementation with *U. rigida*, *F. vesiculosus* and *G. gracilis*. Some other studies showed comparable outcomes but with different dietary elements: apples [decrease of CAT activity and total antioxidant status in blood of volunteers undergoing haemodialysis after a daily consumption of 2 *Fuji* apples] and flavonoids [decrease of GR, CAT and GPx activities in blood of rat after the oral administration of chrysin, quercetin and genistein (Breinholt et al. 1999)]. An eventual explanation for the decrease observed on the antioxidant enzymes is a negative feedback mechanism, as it was also defended by Breinholt et al. (1999) and Pereira et al. (2019). Accordingly, following the introduction of antioxidants provided by the A diet, such as vitamins, flavonoids, pigments, sterols or PUFAs, a compensatory process of saving cellular resources may occur, as there is lesser need to maintain the same expression/synthesis levels of the antioxidants. Moreover, even though the genoprotection capacity of marine macroalgae may have not been transferred to mice through their indirect intake *via* fish fillet (a vehicle of the algae phytocompounds), considering GST and GR activity levels depicted by FA *vs.* F groups, a transference of phytocomponents able to promote a healthier oxidant-antioxidant status was demonstrated. Subsequently, the eventual transference of desmutagen substances (among other beneficial compounds) should not be completely disregarded.

Furthermore, and considering that MMS is a direct-acting alkylating agent (IARC 1999), the macroalgae components must afford other beneficial properties besides the antioxidant potential, namely substances that were able to inactivate the compound, through the direct interaction with it or the modulation of metabolic pathways to inactivate it (Słoczyńska et al. 2014). On the other hand, the eventual presence of substances with bio-antimutagenicity capacity should not be completely overlooked, especially considering that some foods and supplements may also modulate the cellular concentration of

micronutrients required as cofactors in DNA synthesis and repair (Fenech and Bonassi 2011).

Ultimately, the possible occurrence of substances with potential to counteract invasive tumours, through the inhibition of LDH activity and, therefore, the neoplasia progression should be taken into consideration, as marine macroalgae could be a promising source of these anticancer agents as some literature have been suggesting (Murphy et al. 2014; Abd-Ellatef et al. 2017; Santos et al. 2019).

Hence, supplementary studies should be pursued, targeting the full disclosure of the genome protection mechanisms, as well as the processes contributing to the antioxidant and anticancer properties potentially promoted by diets supplemented with marine macroalgae.

5.5. Conclusions

The current study confirmed the genoprotection ability of the marine macroalgae *U. rigida*, *F. vesiculosus* and *G. gracilis* when directly incorporated, as a mixture, in the *M. musculus* diet, particularly against the MMS-induced genetic damage, translated in the decrease of both DNA breaks and chromosomal lesions. However, the macroalgae mix was not able to diminish basal levels of genetic damage. Moreover, the hypothesis concerning the transposition of the genoprotection potential through the indirect intake of the algae-borne phytocompounds *via* fish (previously fed with a macroalgae-supplemented diet) was not confirmed. Anyhow, a food chain transference of phytocomponents able to promote a favourable oxidant-antioxidant status was demonstrated.

No toxicity signals were associated to the supplemented diets, since the general physiological condition of the untreated animals was not negatively affected, namely as haematocrit, serum ALT activity and total protein, and growth performance indexes. In turn, the profile of hepatic LDH and IDH disclosed a reduced activity promoted by the supplemented diets, potentially reflecting an improved energy balance. Lower levels of hepatic antioxidants were tendentially promoted by the supplemented diets, which is an evidence of a healthier and lower pro-oxidant challenging condition, accompanied by a compensatory process of saving cellular resources. The macroalgae-supplemented diet was the only able to decrease the levels of LPO, either on the untreated or MMS-treated animals.

Overall, the current findings carry new perspectives regarding the genome protection afforded by marine macroalgae, involving desmutagenic substances. Thus, it was

reinforced the definition of macroalgae as functional food, promoting their inclusion on the human nutritional habits and, ultimately, contributing to the development of the algaculture industry and disclosing promising prospection actions in the applied phycology field.

Ethical statement

This study was conducted in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes, authorized by the University of Trás-os-Montes e Alto Douro ethics committee (approval no. 10/2013) and the Portuguese Veterinary Directorate (approval no. 0421/000/000/2014) and with a certified operator (approval no. 0421/000/000).

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Chapter 6

General discussion and final remarks

6. General discussion and final remarks

The present chapter aims to integrate the main findings of chapters 2 to 5, as well as to build a bridge to the general and specific objectives previously delineated for this thesis. In general, the current work focused on assessing the properties of three marine macroalgae, namely *Ulva rigida* (sea lettuce or *alface-do-mar*), *Fucus vesiculosus* (bladderwrack or *bodelha*) and *Gracilaria* (ogonori or *cabelo-de-velha*), towards the genome integrity promotion. Moreover, it was intended to evaluate the genoprotection afforded by those macroalgae incorporated on the diets of both human driven models and farmed fish, thus validating their concept as functional food/feed and exploring the underlying mechanisms. While those macroalgae were tested isolated on *Drosophila melanogaster* diet (Chapter 3), they were tested as a mixture on *Sparus aurata* (Chapter 2) and *Mus musculus* (Chapter 5) diets. Besides, a comparative approach of the genome properties of *U. rigida* linked to the phytochemical profiling was performed, using *D. melanogaster*, to address the influence of the growing conditions on that regard (Chapter 4).

In accordance with Holdt and Kraan (2011), who echoed the recommendation of the European Advisory Services, functional foods should not be consumed as pills or capsules, but must remain foods, and demonstrate their effects in amounts that can normally be expected to be consumed in the diet (EAS 2008). This aspect is often ignored in the literature, with most of the studies evaluating only the potential benefits of food extracts or isolated components. Regardless, a few studies pointed in the opposite direction, elucidating about eventual synergisms between food constituents, suggesting that different bioavailability or bioefficacy capacities could be afforded by whole food, or even dietary patterns, and isolated food constituents, ultimately, advocating the concept of "thinking food first" (Jacobs Jr et al. 2009; Bondonno et al. 2017). In line with this concept, throughout the current thesis the macroalgae were always incorporated in the diets of the several model organisms as whole biomass, instead of algae extracts or isolated compounds.

6.1. Marine macroalgae as functional food/feed in the context of genome integrity

Marine macroalgae have been described as functional food by diverse authors, as they have been showing to ensure health benefits, besides their basic nutritional value, namely depicting antiviral, anti-inflammatory, antioxidant, antidiabetic,

hypocholesteraemic, hypolipidaemic and anticancer potential, among other properties (Holdt and Kraan 2011; Mohamed et al. 2012). As previously stated, this thesis aimed to strength the concept of marine macroalgae as functional food, specifically, in the context of aquaculture and human nutrition. While the concept of functional food is generalized and well accepted on human nutrition, it is underexplored regarding animal production and, more precisely, aquaculture. Nevertheless, there are a few studies suggesting some advantages of using functional feed ingredients or additives on aquafeeds formulations, namely to improve animals' growth, performance, immune system or resistance to stress and diseases (Soto et al. 2015; Encarnação 2016). Accordingly, marine macroalgae had demonstrated to potentiate these parameters after their incorporation on fish diets (Wassef et al. 2005; Valente et al. 2015; Peixoto et al. 2016) and, specifically, to enhance their genome integrity (Nagarani et al. 2012; Zinadah et al. 2013; Pereira et al. 2019). As described in Chapter 2, gilthead seabream (*S. aurata*) aquafeed was supplemented with the three marine macroalgae mentioned (totalizing up to 5% of supplementation) and fish were subjected to realistic treatments with aqua-medicines frequently adopted in aquaculture to prevent and/or treat diseases. While those therapeutic agents induced chromosomal damage on *S. aurata* erythrocytes, the macroalgae-supplemented aquafeed was able to counteract that effect, providing a genoprotective action, besides promoting the rejuvenation of the erythrocytes' population. Hence, aiming to these findings along with the previous studies defending additional beneficial properties, it seems clear that marine macroalgae can be validated as functional feed ingredients in the aquaculture context. Therefore, their utilization as aquafeed additives by fish farmers, especially when aqua-medicines need to be applied, should be encouraged, as promotors of the genome integrity that, ultimately, may influence the overall fish production. Despite being difficult to establish specific concentrations suitable to each species solely based on this study, it can be suggested that, in general, higher concentrations of marine macroalgae might have negative repercussions on fish. In fact, the incorporation of *Porphyra purpurea* at 16 and 33% in the diets of mullet (*Chelon labrosus*) was found to suppress growth and feed efficiency rates (Davies et al. 1997), while 7.5% of *Gracilaria* sp. supplementation on *S. aurata* diet increased lipid peroxidation (Queiroz et al. 2014). In turn, *G. vermiculophylla* also showed adverse effects at 10% on Nile tilapia (*Oreochromis niloticus*) diet (Silva et al. 2015) and *G. arcuata* at 20 and 30% on catfish (*Clarias gariepinus*) diet (tolerating up to 10% of the alga) (Al-Asgah et al. 2016). Therefore, it seems critical to evaluate the most beneficial macroalgae species, as well as the respective concentrations for each fish (or other aquatic) species of commercial interest under an aquaculture framework.

On the other hand, two human driven models were adopted to address the concept of marine macroalgae as functional food in the context of human nutrition. Thus, two dietary trials were performed with *D. melanogaster* (Chapters 3 and 4). The macroalgae antigenotoxic potential was tested either against basal or streptonigrin-induced genetic damage. In the first trial (Chapter 3), the macroalgae *U. rigida*, *F. vesiculosus* and two *Gracilaria* species were tested individually, performing also a comparison between wild-harvested and aquacultured batches. While *U. rigida* showed differences on the antigenotoxic potential associated with the growing conditions (higher genoprotective action afforded by the aquacultured alga), *F. vesiculosus* revealed similar responses between origins. In turn, *Gracilaria* species depicted contradictory indications, since the lowest supplementation level enhanced the genome integrity, while the highest showed toxicity signals. Accordingly, the second trial with *D. melanogaster* (Chapter 4) confirmed different antigenotoxic potential promoted by wild-harvested and aquacultured *U. rigida* specimens. Moreover, the phytochemical profiling suggested that the growing conditions influenced the relative quantity of certain *U. rigida* phytocompounds, namely fatty alcohols, sterols, sesquiterpenoids and glycerol esters, which, ultimately, may have affected the genome protection ability of the two algae specimens. In both trials, the macroalgae antigenotoxic potential became more evident against streptonigrin-induced damage, which may be due to their strong genoprotection capacity, but also to the higher genetic damage expression, that would more easily allow the observation of the antigenotoxic potential. In the *D. melanogaster* trials, macroalgae supplementation levels were selected based on a preliminary trial, in which a broad range of concentrations (1.25-20%) was evaluated, and the two lowest algae supplementation levels showing the higher prolificacy (nº of hatched individuals) were selected. Nevertheless, the supplementation levels selected (*U. rigida* – 2.5 and 5%, *F. vesiculosus* – 1.25 and 5%, and *G. gracilis* – 1.25 and 10%) may be regarded as realistic in the human nutrition framework, considering the macroalgae ingestion habits of Asian populations (Hwang et al. 2010; Zava and Zava 2011; Chen et al. 2018).

Additionally, an experimental trial was performed through the supplementation of *M. musculus* diet with *U. rigida*, *F. vesiculosus* and *G. gracilis* (of aquaculture origin) (Chapter 5). In this trial, macroalgae genoprotection properties were evaluated either directly incorporated in the mice diet or indirectly, using fish (*S. aurata*) fillet as a vehicle of the algae-borne phytocompounds, and both against basal and methyl methanesulfonate-induced genotoxicity. Thus, the direct incorporation of the three macroalgae on the mice diet (totalizing up to 5% of supplementation) lead to a decrease on the genetic damage

induced by the genotoxic compound. In turn, the genoprotection promoted by the fillet of fish previously fed with an algae-supplemented aquafeed was like the one induced by the direct incorporation of the macroalgae and not superior to the potential afforded by standardly fed fish. Therefore, it can be stated that, even if the algae phytocompounds accumulate in the macroalgae-fed fish, which seemed to have been translated in an improvement of the antioxidant status, the genoprotection properties did not seem to increase in this fortified product. This nutritional trial lasted 30 days and can be regarded as a preliminary approach. Nevertheless, it must be contemplated that it is a relatively short period, especially considering that nutritional habits with potential health effects are usually advised to be lifelong routines. Consequently, this aspect may be considered when interpreting the genoprotective potential afforded by macroalgae, either directly or indirectly incorporated on human nutrition. Moreover, the genoprotection observed was only achieved against the genetic damage induced by methyl methanesulfonate. Even though this is not a typical source of genotoxicity, humans frequently face multiple exogenous genotoxic challenges (*e.g.* tobacco smoke, pesticides and pernicious dietary substances), besides the endogenously generated ones. The levels of macroalgae (5%) and fish fillet (10%) incorporation on the mice diet, as performed in this chapter, must be considered realistic, aiming the dietary habits of Asian individuals regarding macroalgae consumption (Hwang et al. 2010; Zava and Zava 2011; Chen et al. 2018), as well as European, particularly, Portuguese population on the fish consumption regard (FAO et al. 2015). Hence, the main findings obtained with the human driven models highlighted the beneficial properties of marine macroalgae regarding the genome protection, endorsing their inclusion on the human nutritional habits and validating their status as functional food.

The two organisms *D. melanogaster* and *M. musculus* are regularly adopted as human driven models, both offering suitable conditions to perform basic and applied research. *M. musculus* is preferentially elected as a vertebrate mammalian model, sharing common genetic characteristics as well as physiological and metabolic traits with humans. However, practical and ethical issues impose limits to the utilization of this model organism. In that direction, *D. melanogaster* appears as a suitable alternative, considering its classification as invertebrate (avoiding ethical obstacles), their easy and low-cost laboratory maintenance and propagation, and the homology of fundamental biological mechanisms and genetic pathways with humans. Nevertheless, in this thesis, while the fruit fly revealed to be advantageous on studies with large number of individuals (Chapters

3 and 4), the mice adoption allowed a more comprehensive analysis of the effects of the macroalgae-supplemented diets (Chapter 5).

6.2. Mechanisms of genome protection

Despite some studies defending macroalgae beneficial properties towards the genome integrity (*e.g.* Leite-Silva et al. 2007; Celikler et al. 2008; Celikler et al. 2009; Ferreira et al. 2019), little is known regarding the underlying mechanisms. Some authors pointed the antioxidant capacity of macroalgae as one important factor contributing to their genoprotection capacity, attributing this property to specific algae phytocompounds, namely vitamins, phenolic compounds and pigments (Celikler et al. 2009; Yildiz et al. 2012). Moreover, an antimutagenic potential have also been described as a beneficial effect promoted by macroalgae (Okai and Higashi-Okai 1994; Higashi-Okai et al. 1999).

This thesis represents a step forward towards the partial disclosure of the mechanisms associated to the macroalgae antigenotoxic effects. Yet, bearing in mind the current findings, along with the literature, a few modes of action can be hypothesized, namely (i) the radical scavenging activity/antioxidant potential, (ii) the ability of inducing subtle pro-oxidant/pro-genotoxic effects, triggering the organisms' antioxidant and antigenotoxic defences, (iii) anticlastogenic and antimutagenic activities and/or (iv) DNA repair enhancement. Moreover, antimutagenic substances have been classified either as desmutagens and bio-antimutagens (Izquierdo-Vega et al. 2017). The first group refers to substances able to promote the elimination of the genotoxic compound from the organism, as well as agents capable of partial or fully inactivate the mutagens by enzymatic or chemical interaction before the mutagen attacks the genes (also considered as apparent antimutagens). In turn, the bio-antimutagens (or true antimutagens) can suppress the process of mutation after genes are damaged by mutagens, acting on the repair and replication processes of the mutagen-damaged DNA, resulting in a mutation frequency decline (Ferguson et al. 2004; De Flora and Ferguson 2005). To the author knowledge, no study addressed the influence of macroalgae on the DNA repair mechanisms, despite this being a plausible genoprotective mechanism displayed by them. Therefore, the antigenotoxic potential afforded by marine macroalgae may result from the concomitant expression of those different protection mechanisms, especially bearing in mind that they promoted genoprotection against several genotoxic challenges with distinctive modes of action.

6.3. Biotechnological potential

In general, the biotechnological potential of this thesis concerned two fields: algaculture and pisciculture. Contributing for the first one, this thesis highlighted the phytochemical and functional differences between macroalgae specimens belonging to the same species (or genus), resulting from distinctive growing conditions. Consequently, the factors causing those differences may be manipulated and/or potentiated to achieve macroalgae specimens with specific beneficial profiles.

Moreover, farmed fish could beneficiate from macroalgae-supplemented aquafeeds, at least when exogenous sources of genotoxic stress are applied, as the aqua-medicines used to prevent/treat diseases and aiming that genetic damage may have a negative impact on fish fitness, with subsequent repercussion on the aquaculture productivity (Silva et al. 2011) mainly due to greater energy expenditure to DNA repair processes (Olson and Mitchell 2006) and homeostasis achievement (Pacheco and Santos 2002).

The hypothesis based on the supplementation of fish aquafeed with marine macroalgae to achieve the transference of genome protective properties to the final consumer was not confirmed, considering the tested conditions, despite the eventual transference of phytocomponents able to promote a favourable oxidant-antioxidant status. Moreover, the concept of fortified farmed fish has been gaining public awareness and acceptance, especially if fish are fortified with antioxidants and *n*-3 fatty acid (Ribeiro et al. 2019). Hence, this is a very promising field, especially in geographical areas which populations preferentially consume fishery and aquaculture products in comparison with macroalgae, namely in Europe.

6.4. Remaining knowledge gaps and future trends

The concept of functional food and the thematic revolving around foods, herbs or substances with beneficial properties for human health face the challenge of building a scientifically supported knowledge in order to attain the public trust. This thesis, set with this intent, performed a pilot yet solid approach on that regard.

Nevertheless, further studies are needed to complement the knowledge obtained herein. For instance, the three macroalgae *U. rigida*, *F. vesiculosus* and *Gracilaria* species might be tested in a mixture on *D. melanogaster* diet, to address eventual synergistic activities, and otherwise isolated in the fish and mice models. Moreover, a thorough phytochemical profiling could be pursued, namely on *F. vesiculosus* and *Gracilaria* species, comparing the influence of growing conditions on that profile, as it was performed with *U. rigida* specimens. In fact, specifically considering the wild-harvested macroalgae, it

may be of great interest to evaluate algae specimens from multiple geographic locations, as well as at different seasonality periods, always targeting the phytochemical profiling alongside a functionality characterization. Moreover, it would be pertinent to perform a phytochemical screening on the fillet of the differently fed fish, elucidating about the eventual higher bioavailability of specific phytocompounds on the fish group fed with the macroalgae-supplemented aquafeed, despite this was not translated on the genoprotective properties transference.

In addition, aiming to fully disclose the mechanisms responsible for the antigenotoxic potential of marine macroalgae, further studies should be designed. For example, the involvement of the DNA repair machinery ought to be investigated.

Overall, considering the great biodiversity of marine macroalgae, an almost infinite field of possibilities arises.

6.5. Final remarks

The general findings obtained in this thesis support the assumption of marine macroalgae as functional food, reinforcing their inclusion on the human diet, and as functional feed ingredients, encouraging the reformulation of fish aquafeeds, especially when aqua-medicines are applied under realistic rearing scenarios.

The macroalgae *U. rigida*, *F. vesiculosus* and *Gracilaria* species, individually or in a mixture, demonstrated to hold genoprotective action in farmed fish and human driven models, especially against damage induced by genotoxic challenges.

The growing conditions of *U. rigida* influenced its genoprotection properties. Particularly, aquacultured *U. rigida* showed higher genoprotective potential, mainly against streptonigrin-induced genetic damage, which may be attributed to the higher relative amount of fatty alcohols, sterols, sesquiterpenoids and glycerol esters.

A direct intake of a mix of aquacultured *U. rigida*, *F. vesiculosus* and *G. gracilis* demonstrated to enhance the genoprotection on *M. musculus*. Yet, their antigenotoxic properties were not transferred to mice *via* the intake of fish fed with the macroalgae-supplemented, despite the eventual transference of phytocomponents able to promote a favourable oxidant-antioxidant status.

Several protection mechanisms could be enhancing genome integrity on the different model organisms fed with the marine macroalgae, namely antioxidant and anticlastogenic properties, or even the capacity of triggering the organisms' antioxidant and antigenotoxic defences, among other eventual mechanisms.

Overall, the present findings convey new perspectives likely to contribute to the development of algaculture and pisciculture industries, as well as to the redefinition of human nutritional habits, reinforcing and validating the concept of marine macroalgae as functional food and subsequent health benefits.

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Appendices

Appendix I

Table 1. Mean frequency (‰) of each nuclear abnormality category (± standard error) determined in peripheral erythrocytes of *S. aurata* after the differential dietary background.

Dietary background		Kidney Shaped (K)	Segmented (S)	Lobed (L)	Vacuolated (V)	Micronuclei (MN)
	t_0	26.88 ± 1.48	8.38 ± 1.24	6.13 ± 1.09	0.00 ± 0.00	0.00 ± 0.00
	S	30.25 ± 2.46	4.25 ± 0.59	7.88 ± 1.26	0.00 ± 0.00	0.00 ± 0.00
	A	25.75 ± 3.45	4.88 ± 0.97	5.38 ± 1.19	0.00 ± 0.00	0.00 ± 0.00

Appendix I

Table 2. Mean frequency (‰) of each nuclear abnormality category (± standard error) determined in peripheral erythrocytes of *S. aurata* at the two sampling moments considering the cyclophosphamide (CP) sub-trial. (*) = statistically significant differences ($p < 0.05$; n = 8) between groups, within each time.

Cyclophosphamide sub-trial		Kidney Shaped (K)	Segmented (S)	Lobed (L)	Vacuolated (V)	Micronuclei (MN)
4 days	S	24.38 ± 0.86	5.50 ± 1.00	12.00 ± 1.72	0.25 ± 0.25	0.13 ± 0.13
	A	16.38 ± 2.49	4.75 ± 1.31	9.88 ± 1.46	0.75 ± 0.49	0.25 ± 0.25
	S_{CP}	48.88 ± 2.57	9.13 ± 1.33	15.50 ± 2.21	0.00 ± 0.00	0.38 ± 0.18
	A_{CP}	26.13 ± 1.23	4.13 ± 0.64	6.13 ± 0.93	0.38 ± 0.26	0.00 ± 0.00
18 days	S	25.50 ± 1.88	3.25 ± 1.26	12.63 ± 2.15	0.00 ± 0.00	0.00 ± 0.00
	A	25.00 ± 1.54	1.50 ± 0.53	8.63 ± 1.45	0.00 ± 0.00	0.00 ± 0.00
	S_{CP}	38.88 ± 1.98	3.38 ± 1.25	15.25 ± 2.70	0.00 ± 0.00	0.00 ± 0.00
	A_{CP}	30.13 ± 0.77	3.13 ± 0.69	7.50 ± 1.21	0.00 ± 0.00	0.00 ± 0.00

Significance brackets (*) as printed: 4 days — K: S vs A; S vs S_{CP}; S_{CP} vs A_{CP}; A vs A_{CP}; S: S_{CP} vs A_{CP}; L: S_{CP} vs A_{CP}. 18 days — K: S vs S_{CP}; S_{CP} vs A_{CP}; L: S_{CP} vs A_{CP}.

Appendix I

Table 3. Mean frequency (‰) of each nuclear abnormality category (± standard error) determined in peripheral erythrocytes of *S. aurata* at the two sampling moments considering the oxytetracycline (OTC) sub-trial. (*) = statistically significant differences ($p < 0.05$; n = 8) between groups, within each time.

Oxytetracycline sub-trial		Kidney Shaped (K)	Segmented (S)	Lobed (L)	Vacuolated (V)	Micronuclei (MN)
4 days	S	20.88 ± 1.49	2.88 ± 1.04	6.00 ± 1.25	0.13 ± 0.13	0.25 ± 0.16
	A	20.63 ± 1.18	3.88 ± 0.67	4.63 ± 1.16	0.13 ± 0.13	0.25 ± 0.16
	S_{OTC}	46.29 ± 2.72	7.86 ± 1.59	17.29 ± 1.79	0.14 ± 0.13	0.00 ± 0.00
	A_{OTC}	36.38 ± 3.12	5.00 ± 0.89	10.13 ± 2.37	0.13 ± 0.13	0.00 ± 0.00
18 days	S	25.44 ± 2.03	2.33 ± 0.43	7.44 ± 0.90	0.00 ± 0.00	0.00 ± 0.00
	A	23.38 ± 1.86	2.63 ± 0.78	5.00 ± 1.05	0.00 ± 0.00	0.00 ± 0.00
	S_{OTC}	37.25 ± 2.24	3.88 ± 1.13	6.25 ± 1.18	0.00 ± 0.00	0.38 ± 0.26
	A_{OTC}	25.25 ± 1.70	4.50 ± 1.12	6.38 ± 0.89	0.00 ± 0.00	0.00 ± 0.00

(Significance brackets, 4 days: K — S vs S_{OTC} *, A vs A_{OTC} *, S_{OTC} vs A_{OTC} *; S — S vs S_{OTC} *; L — S vs S_{OTC} *. 18 days: K — S vs S_{OTC} *, S_{OTC} vs A_{OTC} *.)

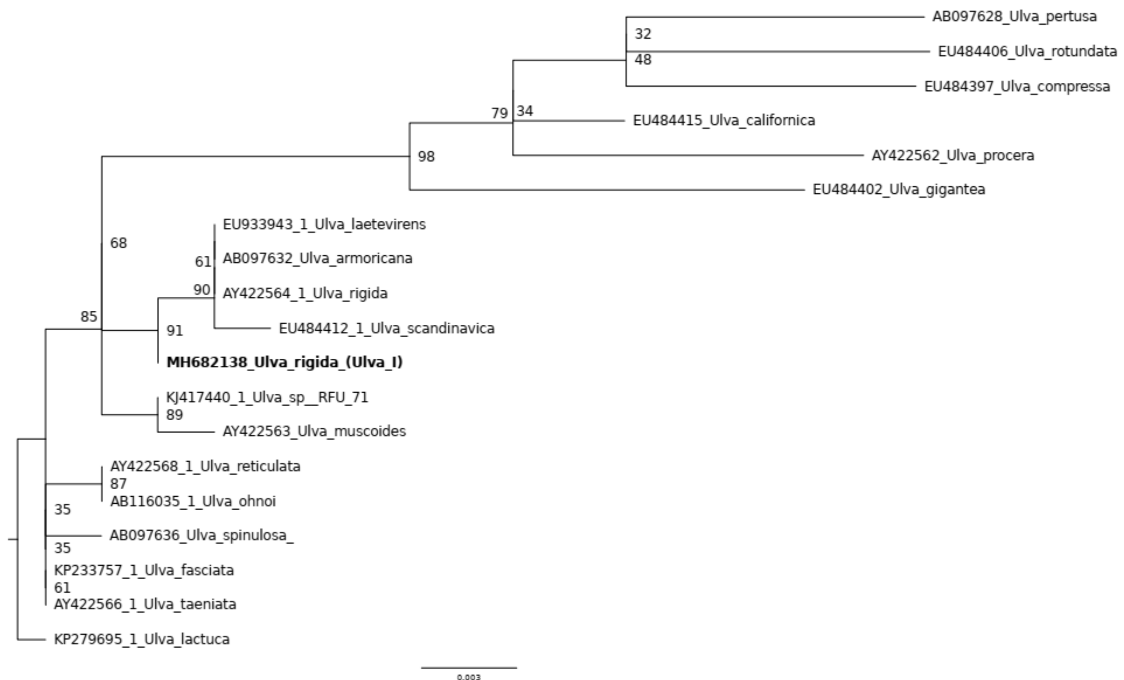
Appendix I

Table 4. Mean frequency (‰) of each nuclear abnormality category (± standard error) determined in peripheral erythrocytes of *S. aurata* at the two sampling moments considering the formalin (FOR) sub-trial. (*) = statistically significant differences ($p < 0.05$; n = 8) between groups, within each time.

Formalin sub-trial		Kidney Shaped (K)	Segmented (S)	Lobed (L)	Vacuolated (V)	Micronuclei (MN)
4 days	S	18.33 ± 1.30	3.00 ± 0.50	8.71 ± 2.58	0.43 ± 0.40	0.00 ± 0.00
	A	22.30 ± 1.56	3.78 ± 0.66	5.67 ± 1.33	0.00 ± 0.00	0.25 ± 0.25
	S_{FOR}	42.63 ± 1.28	5.63 ± 0.91	13.63 ± 1.93	0.00 ± 0.00	0.57 ± 0.35
	A_{FOR}	29.30 ± 3.45	2.70 ± 0.83	6.10 ± 1.54	0.00 ± 0.00	0.00 ± 0.00
18 days	S	26.00 ± 1.67	2.63 ± 0.78	6.75 ± 0.96	0.00 ± 0.00	0.00 ± 0.00
	A	26.33 ± 1.76	4.29 ± 0.86	5.13 ± 1.42	0.00 ± 0.00	0.00 ± 0.00
	S_{FOR}	41.38 ± 4.03	4.75 ± 0.75	10.00 ± 1.81	0.00 ± 0.00	0.00 ± 0.00
	A_{FOR}	26.17 ± 1.08	7.50 ± 1.35	5.75 ± 0.98	0.00 ± 0.00	0.00 ± 0.00

Significance brackets (*): 4 days — K: S vs S_{FOR} *, S_{FOR} vs A_{FOR} *; S: S_{FOR} vs A_{FOR} *; L: S_{FOR} vs A_{FOR} *. 18 days — K: S vs S_{FOR} *, S_{FOR} vs A_{FOR} *.

Appendix II



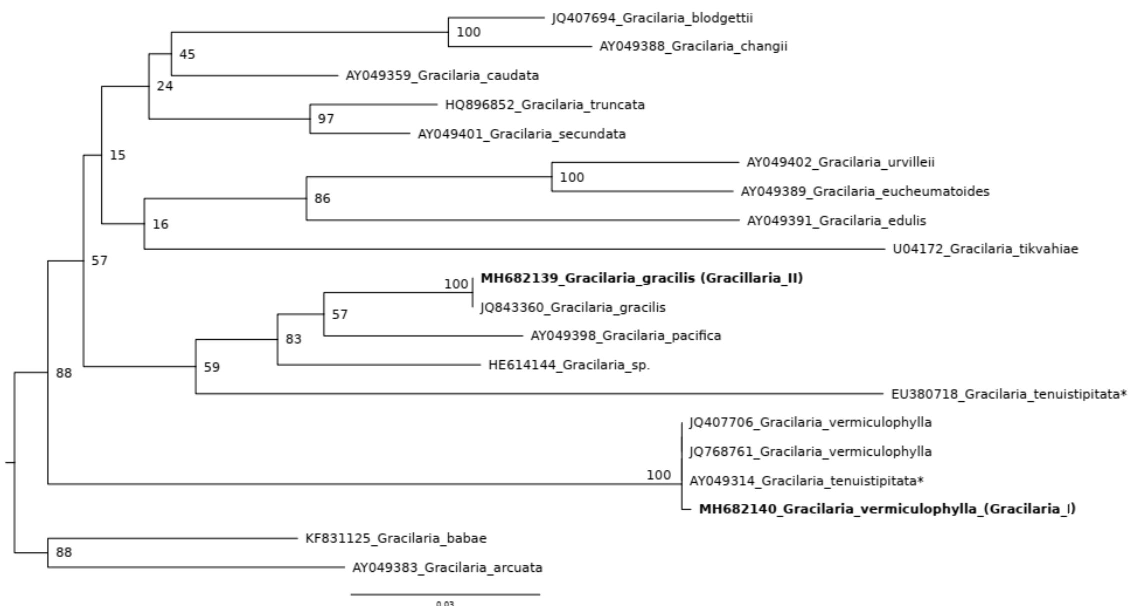


Figure 1. Maximum likelihood tree of partial *rbcL* sequences of *Ulva* (A) and *Gracilaria* (B). Species that were identified in this study by DNA-barcoding are highlighted in bold. The *G. tenuistipitata* specimen marked with an asterisk is supposed to be a miss-identification.

Appendix II

Table 1. *p*-distances of *Ulva* and *Gracilaria* species. Specimens that were identified in this study are highlighted in bold.

		a	b	c	d	e	f	g	**h**	i	j	k	l	m	n	o	p	q	r	s
EU484412_Ulva_scandinavica	a		0.00902	0.00723	0.0072	0.0018	0.00902	0.00723	0.0036	0.0018	0.00902	0.0312	0.0018	0.00902	0.02936	0.009	0.03119	0.02607	0.0312	0.022
KP279695_Ulva_lactuca	b	0.00902		0.00182	0.00542	0.00723	0.00361	0.00182	0.00542	0.00723	0.00361	0.02939	0.00723	0.00361	0.02755	0.00721	0.02939	0.02426	0.02939	0.02019
KP233757_Ulva_fasciata	c	0.00723	0.00182		0.00363	0.00544	0.00179	0	0.00363	0.00544	0.00179	0.0276	0.00544	0.00179	0.02576	0.00542	0.02759	0.02247	0.0276	0.0184
KJ417440_Ulva_sp	d	0.0072	0.00542	0.00363		0.00541	0.00542	0.00363	0.0036	0.00541	0.00542	0.0276	0.00541	0.00542	0.02576	0.00179	0.02759	0.02247	0.02759	0.01839
EU933943_Ulva_laetevirens	e	0.0018	0.00723	0.00544	0.00541		0.00723	0.00544	0.00181	0	0.00723	0.0294	0	0.00723	0.02757	0.0072	0.0294	0.02428	0.0294	0.0202
AY422568_Ulva_reticulata	f	0.00902	0.00361	0.00179	0.00542	0.00723		0.00179	0.00542	0.00723	0	0.02939	0.00723	0.00359	0.02755	0.00721	0.02938	0.02426	0.02939	0.02019
AY422566_Ulva_taeniata	g	0.00723	0.00182	0	0.00363	0.00544	0.00179		0.00363	0.00544	0.00179	0.0276	0.00544	0.00179	0.02576	0.00542	0.02759	0.02247	0.0276	0.0184
MH682138_(Ulva_I)	h	0.0036	0.00542	0.00363	0.0036	0.00181	0.00542	0.00363		0.00181	0.00542	0.0276	0.00181	0.00542	0.02576	0.00539	0.02759	0.02247	0.0276	0.0184
AY422564_Ulva_rigida	i	0.0018	0.00723	0.00544	0.00541	0	0.00723	0.00544	0.00181		0.00723	0.0294	0	0.00723	0.02757	0.0072	0.0294	0.02428	0.0294	0.0202
AB116035_Ulva_ohnoi	j	0.00902	0.00361	0.00179	0.00542	0.00723	0	0.00179	0.00542	0.00723		0.02939	0.00723	0.00359	0.02755	0.00721	0.02938	0.02426	0.02939	0.02019
AB097628_Ulva_pertusa	k	0.0312	0.02939	0.0276	0.0276	0.0294	0.02939	0.0276	0.0276	0.0294	0.02939		0.0294	0.02939	0.02382	0.02939	0.01836	0.02765	0.01838	0.01644
AB097632_Ulva_armoricana	l	0.0018	0.00723	0.00544	0.00541	0	0.00723	0.00544	0.00181	0	0.00723	0.0294		0.00723	0.02757	0.0072	0.0294	0.02428	0.0294	0.0202
AB097636_Ulva_spinulosa	m	0.00902	0.00361	0.00179	0.00542	0.00723	0.00359	0.00179	0.00542	0.00723	0.00359	0.02939	0.00723		0.02756	0.00721	0.02939	0.02427	0.02939	0.02019
AY422562_Ulva_procera	n	0.02936	0.02755	0.02576	0.02576	0.02757	0.02755	0.02576	0.02576	0.02757	0.02755	0.02382	0.02757	0.02756		0.02755	0.02382	0.02582	0.02382	0.01462
AY422563_Ulva_muscoides	o	0.009	0.00721	0.00542	0.00179	0.0072	0.00721	0.00542	0.00539	0.0072	0.00721	0.02939	0.0072	0.00721	0.02755		0.02938	0.02426	0.02939	0.02019
EU484397_Ulva_compressa	p	0.03119	0.02939	0.02759	0.02759	0.0294	0.02938	0.02759	0.02759	0.0294	0.02938	0.01836	0.0294	0.02939	0.02382	0.02938		0.02765	0.01838	0.01643
EU484402_Ulva_gigantea	q	0.02607	0.02426	0.02247	0.02247	0.02428	0.02426	0.02247	0.02247	0.02428	0.02426	0.02765	0.02428	0.02427	0.02582	0.02426	0.02765		0.02765	0.01845
EU484406_Ulva_rotundata	r	0.0312	0.02939	0.0276	0.02759	0.0294	0.02939	0.0276	0.0276	0.0294	0.02939	0.01838	0.0294	0.02939	0.02382	0.02939	0.01838	0.02765		0.01644
EU484415_Ulva_californica	s	0.022	0.02019	0.0184	0.01839	0.0202	0.02019	0.0184	0.0184	0.0202	0.02019	0.01644	0.0202	0.02019	0.01462	0.02019	0.01643	0.01845	0.01644	

		a	b	**c**	d	e	f	g	**h**	i	j	k	**l**	m	n	o	p	q	r	s	t
JQ407706_Gracilaria_vermiculophylla	a		0	0.00145	0	0.19197	0.18601	0.18542	0.2108	0.21174	0.15417	0.16338	0.15377	0.15375	0.13423	0.14375	0.1479	0.1447	0.16678	0.17183	0.13579
AY049314_Gracilaria_tenuistipitata	b	0		0.00145	0	0.19197	0.18601	0.18542	0.2108	0.21174	0.15417	0.16338	0.15377	0.15375	0.13423	0.14375	0.1479	0.1447	0.16678	0.17183	0.13579
MH682140_Gracilaria_I	c	0.00145	0.00145		0.00145	0.19341	0.18745	0.18687	0.21225	0.21319	0.15561	0.16483	0.15521	0.1552	0.13568	0.1452	0.14935	0.14615	0.16823	0.17328	0.13724
JQ768761_Gracilaria_vermiculophylla	d	0	0	0.00145		0.19197	0.18601	0.18542	0.2108	0.21174	0.15417	0.16338	0.15377	0.15375	0.13423	0.14375	0.1479	0.1447	0.16678	0.17183	0.13579
AY049391_Gracilaria_edulis	e	0.19197	0.19197	0.19341	0.19197		0.1552	0.15461	0.17999	0.15471	0.13737	0.14658	0.13697	0.13695	0.14476	0.15428	0.14458	0.14138	0.16346	0.16851	0.13247
AY049402_Gracilaria_urvilleii	f	0.18601	0.18601	0.18745	0.18601	0.1552		0.05597	0.15127	0.17497	0.13141	0.14062	0.13101	0.13099	0.1388	0.14832	0.13862	0.13542	0.1575	0.16255	0.12651
AY049389_Gracilaria_eucheumatoides	g	0.18542	0.18542	0.18687	0.18542	0.15461	0.05597		0.15069	0.17439	0.13082	0.14004	0.13042	0.13041	0.13821	0.14773	0.13803	0.13484	0.15691	0.16196	0.12592
U04172_Gracilaria_tikvahiae	**h**	0.2108	0.2108	0.21225	0.2108	0.17999	0.15127	0.15069		0.19977	0.1562	0.16541	0.1558	0.15579	0.16359	0.17311	0.16341	0.16021	0.18229	0.18734	0.1513
EU380718_Gracilaria_tenuistipitata	i	0.21174	0.21174	0.21319	0.21174	0.15471	0.17497	0.17439	0.19977		0.15714	0.16636	0.15674	0.15673	0.16453	0.17405	0.16435	0.16116	0.18323	0.18828	0.15224
HE614144_Gracilaria_sp.	j	0.15417	0.15417	0.15561	0.15417	0.13737	0.13141	0.13082	0.1562	0.15714		0.06973	0.06012	0.06011	0.10696	0.11648	0.10677	0.10358	0.12566	0.13071	0.09467
AY049398_Gracilaria_pacifica	k	0.16338	0.16338	0.16483	0.16338	0.14658	0.14062	0.14004	0.16541	0.16636	0.06973		0.05084	0.05083	0.11617	0.12569	0.11599	0.1128	0.13487	0.13992	0.10388
MH682139_Gracilaria_II	l	0.15377	0.15377	0.15521	0.15377	0.13697	0.13101	0.13042	0.1558	0.15674	0.06012	0.05084		0.00001	0.10656	0.11608	0.10638	0.10318	0.12526	0.13031	0.09427
JQ843360_Gracilaria_gracilis	m	0.15375	0.15375	0.1552	0.15375	0.13695	0.13099	0.13041	0.15579	0.15673	0.06011	0.05083	0.00001		0.10654	0.11606	0.10636	0.10317	0.12524	0.13029	0.09425
KF831125_Gracilaria_babae	n	0.13423	0.13423	0.13568	0.13423	0.14476	0.1388	0.13821	0.16359	0.16453	0.10696	0.11617	0.10656	0.10654		0.08195	0.10069	0.0975	0.11957	0.12462	0.08858
AY049383_Gracilaria_arcuata	o	0.14375	0.14375	0.1452	0.14375	0.15428	0.14832	0.14773	0.17311	0.17405	0.11648	0.12569	0.11608	0.11606	0.08195		0.11021	0.10701	0.12909	0.13414	0.0981
HQ896852_Gracilaria_truncata	p	0.1479	0.1479	0.14935	0.1479	0.14458	0.13862	0.13803	0.16341	0.16435	0.10677	0.11599	0.10638	0.10636	0.10069	0.11021		0.0352	0.10136	0.10641	0.07037
AY049401_Gracilaria_secundata	q	0.1447	0.1447	0.14615	0.1447	0.14138	0.13542	0.13484	0.16021	0.16116	0.10358	0.1128	0.10318	0.10317	0.0975	0.10701	0.0352		0.09817	0.10322	0.06717
JQ407694_Gracilaria_blodgettii	r	0.16678	0.16678	0.16823	0.16678	0.16346	0.1575	0.15691	0.18229	0.18323	0.12566	0.13487	0.12526	0.12524	0.11957	0.12909	0.10136	0.09817		0.03607	0.08026
AY049388_Gracilaria_changii	s	0.17183	0.17183	0.17328	0.17183	0.16851	0.16255	0.16196	0.18734	0.18828	0.13071	0.13992	0.13031	0.13029	0.12462	0.13414	0.10641	0.10322	0.03607		0.08531
AY049359_Gracilaria_caudata	t	0.13579	0.13579	0.13724	0.13579	0.13247	0.12651	0.12592	0.1513	0.15224	0.09467	0.10388	0.09427	0.09425	0.08858	0.0981	0.07037	0.06717	0.08026	0.08531	

Appendix II

Table 2. *D. melanogaster* prolificacy registered for the different levels of supplementation with the macroalgae.

	% supplementation	Prolificacy
Control	0	50
U1	1.25	55
	2.5	107
	5	88
	10	80
	20	59
U2	1.25	56
	2.5	101
	5	73
	10	75
	20	84
G1	1.25	98
	2.5	5
	5	9
	10	3
	20	1
G2	1.25	18
	2.5	38
	5	13
	10	59
	20	49
F1	1.25	64
	2.5	78
	5	67
	10	38
	20	61
F2	1.25	48
	2.5	40
	5	58
	10	63
	20	51

Appendix II

Table 3. Concentration levels of several elements, including metals, determined in dried samples of the macroalgae.

Analyte Symbol	Li	Na	Mg	Al	P	K	Ca	V	Cr	Mn	Fe	Co
Unit Symbol	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Detection Limit	0.04	18.3	1.3	0.2	0.95	29	2.3	0.001	0.003	0.01	0.1	0.002
Analysis Method	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS
G1 I	2.936	20956.288	3816.617	356.528	2485.688	81233.745	1780.108	8.742	1.553	552.389	2340.738	5.205
G1 II	1.013	21839.485	3986.103	363.318	2465.211	85451.192	1858.150	9.003	1.032	543.117	2182.986	5.077
G2 I	2.029	17656.433	3437.423	931.756	2867.469	74996.426	2252.001	2.811	2.901	245.946	1617.322	1.502
G2 II	4.980	17048.599	3330.438	929.611	3566.781	75956.031	2079.148	2.673	2.700	238.853	1598.209	1.524
U1 I	0.771	17467.135	33216.684	913.707	1744.442	22702.801	5568.597	1.764	3.573	47.183	1362.483	0.619
U1 II	3.080	16768.862	34374.959	859.154	1762.308	22904.956	5559.646	2.006	3.628	45.250	1329.399	0.587
U2 I	0.908	40331.140	28532.921	245.028	1515.412	28680.910	4653.356	0.598	1.031	20.171	445.021	0.349
F1 I	0.949	41964.488	8351.330	10.654	1323.958	37893.167	7789.172	0.260	0.197	64.972	84.733	0.729
F2 I	<0.04	37309.189	8887.966	40.851	1359.792	41940.919	10829.294	0.584	0.526	73.221	118.423	0.851

Analyte Symbol	Ni	Cu	Zn	As	Mo	Ag	Cd	Sn	Ba	W	Pb	Th
Unit Symbol	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Detection Limit	0.01	0.01	0.2	0.02	0.02	0.05	0.005	0.01	0.1	0.03	0.02	0.01
Analysis Method	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS
G1 I	1.922	4.142	98.913	23.918	<0.02	<0.05	<0.005	13.311	1.424	<0.03	5.142	0.133
G1 II	1.492	3.971	94.773	23.670	<0.02	<0.05	<0.005	13.510	1.297	<0.03	4.987	0.160
G2 I	3.684	23.318	26.542	9.471	<0.02	<0.05	<0.005	22.860	2.320	<0.03	2.469	0.171
G2 II	3.890	23.606	27.078	10.078	<0.02	<0.05	<0.005	22.445	1.925	<0.03	2.404	0.188
U1 I	4.039	18.974	14.251	2.000	<0.02	<0.05	<0.005	14.953	2.398	<0.03	1.307	0.302
U1 II	4.248	19.074	13.838	1.543	<0.02	<0.05	<0.005	14.628	2.517	<0.03	1.264	0.233
U2 I	2.341	16.768	15.587	0.982	<0.02	<0.05	<0.005	15.101	0.643	<0.03	0.394	<0.01
F1 I	2.021	0.620	29.723	18.878	<0.02	<0.05	0.680	16.997	6.866	<0.03	<0.02	<0.01
F2 I	3.247	3.326	38.769	32.853	<0.02	<0.05	0.475	16.678	7.690	<0.03	<0.02	<0.01

Appendix III

Table 1. Mean (± standard error) weight per experimental group weekly recorded

		Weight per group (g)					
		day 0	**week 1**	**week 2**	**week 3**	**week 4**	**week 5**
Untreated	**S**	28.8 ± 0.6	28.9 ± 0.7	29.5 ± 0.7	30.2 ± 1.0	29.4 ± 0.8	29.6 ± 1.0
	A	28.1 ± 0.5	28.1 ± 0.4	29.5 ± 0.4	28.9 ± 0.4	28.6 ± 0.4	28.3 ± 0.4
	F	26.5 ± 0.7	26.6 ± 0.7	27.1 ± 0.6	27.7 ± 0.7	27.4 ± 0.7	27.4 ± 0.8
	FA	25.2 ± 0.4	25.8 ± 0.5	26.8 ± 0.4	26.6 ± 0.4	26.5 ± 0.5	26.7 ± 0.3
MMS-treated	**S**	26.4 ± 0.9	26.9 ± 0.9	27.2 ± 0.9	27.1 ± 0.9	27.0 ± 0.9	27.6 ± 1.0
	A	24.8 ± 0.8	25.1 ± 0.8	26.0 ± 0.8	25.7 ± 0.9	26.4 ± 0.9	26.2 ± 0.9
	F	27.8 ± 0.6	28.2 ± 0.6	28.5 ± 0.6	28.8 ± 0.6	28.5 ± 0.5	28.8 ± 0.5
	FA	26.2 ± 1.0	26.8 ± 0.8	26.9 ± 0.8	27.6 ± 1.0	26.9 ± 0.9	28.6 ± 1.0