Universidade de Trás-os-Montes e Alto Douro

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Efeitos Benéficos em *Drosophila melanogaster*

Potential of Seaweeds from the Portuguese Coast as Functional Food:

Beneficial Effects in *Drosophila melanogaster*

Dissertação de Mestrado em Biotecnologia para as Ciências da Saúde

João Miguel Mendes Ferreira

Orientadora: Professora Doutora Isabel O'Neill de Mascarenhas Gaivão
Coorientador: Professor Doutor Mário Guilherme Garcês Pacheco

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Resumo

Palavras-chave: Macroalgas marinhas; *Drosophila melanogaster*; Toxicidade; Antitoxicidade; Longevidade; Antigenotoxicidade

As macroalgas marinhas são um dos melhores exemplos de organismos marinhos com grandes aplicações considerando a sua composição rica em compostos bioativos com efeitos benéficos para a saúde. Assim, elas têm sido defendidas como alimento funcional e a sua inclusão na dieta humana é uma realidade, principalmente em países do Este Asiático. Com especial atenção aos seus efeitos toxicológicos, incluindo as ações de proteção como antioxidantes e antigenotóxicos, o número de estudos está a aumentar. No entanto, constata-se que estudos anteriores mediram apenas efeitos de compostos específicos ou extratos das macroalgas marinhas, principalmente *in vitro*, expondo um hiato relativamente às avaliações *in vivo* dirigidas aos impactos da sua ingestão integral. Foram assim avaliadas cinco macroalgas marinhas colhidas da costa portuguesa, *Gracilaria* sp. (confirmação de DNA pendente entre *Gracilaria gracilis* e *Gracilaria vermiculophylla*), *Grateloupia turuturu*, *Porphyra umbilicalis*, *Ulva* sp. (confirmação de DNA pendente entre *Ulva lactuca* e *Ulva rigida*) e *Fucus vesiculosus*, quanto aos seus efeitos toxicológicos em *Drosophila melanogaster* com o objetivo de demonstrar os seus potenciais como alimento funcional. As macroalgas marinhas foram primeiramente testadas num rastreio toxicológico inicial, através da incorporação em meios a 1,25, 2,5, 5, 10 e 20%. Em seguida, as duas macroalgas marinhas com os melhores efeitos antitóxicos foram avaliadas num ensaio de longevidade. Num terceiro ensaio foi realizado o teste de mutação e recombinação somática (SMART) para manchas nos olhos, no qual se avaliou o potencial genotoxicológico das macroalgas marinhas do ensaio de longevidade (mas apenas as duas melhores concentrações por macroalga marinha) aquando da exposição da *Drosophila* a macroalgas marinhas e macroalgas marinhas com estreptonigrina (SN). Os resultados do rastreio inicial demonstraram que todas as algas promoveram efeitos antitóxicos em pelo menos uma das concentrações testadas. *Grateloupia* e *Porphyra* foram as mais promissoras em termos de antitoxicidade. Para as moscas-da-fruta expostas às concentrações de *Gracilaria* testadas superiores a 1,25% verificou-se toxicidade. *Porphyra* a 10% e *Grateloupia* a 20% foram capazes de aumentar a longevidade da *Drosophila*, com *Grateloupia* atingindo o maior potencial. No SMART foram provadas as ações antigenotóxicas para as concentrações selecionadas, *Porphyra* a 5% e 10% e *Grateloupia* a 10% e 20%, com 20% de *Grateloupia* revelando o maior potencial, demostrando potencial para a prevenção de eventos cancerígenos. Assim, *Grateloupia turuturu* e *Porphyra umbilicalis* colhidas na costa Portuguesa demonstraram o seu potencial como alimento funcional. Estudos adicionais são necessários para as restantes macroalgas marinhas testadas.

Abstract

Keywords: Seaweeds; *Drosophila melanogaster*; Toxicity; Antitoxicity; Longevity; Antigenotoxicity

Seaweeds are one of the best examples of marine organisms with large applications considering their rich composition in bioactive compounds with beneficial effects to health. Therefore, they have been defended as functional food and their inclusion in the human diet is a reality mainly in East Asian countries. With special attention to their toxicological effects, including the protective actions such as antioxidants and antigenotoxics, the number of studies is rising. Nevertheless, it should be noted that previous studies measured only effects from specific compounds or extracts from seaweeds, mainly *in vitro*, exposing a gap relatively to *in vivo* evaluations directed to impacts of its full intake. Were thus evaluated five seaweeds collected from the Portuguese coast, *Gracilaria* sp. (pendent DNA confirmation between *Gracilaria gracilis* and *Gracilaria vermiculophylla*), *Grateloupia turuturu*, *Porphyra umbilicalis*, *Ulva* sp. (pendant DNA confirmation between *Ulva lactuca* and *Ulva rigida*), and *Fucus vesiculosus*, for their toxicological effects in *Drosophila melanogaster* with the purpose of demonstrating their potentials as functional food. The seaweeds were first tested in an initial toxicological screening, through the incorporation in the media at 1.25, 2.5, 5, 10, and 20%. Then, the two seaweeds with the best antitoxic effects were evaluated in a longevity assay. In a third assay was performed the eye-spot somatic mutation and recombination test (SMART), which consisted in measuring the genotoxicological potential of the two seaweeds from the longevity assay (but only the two best concentrations per seaweed) when exposure of *Drosophila* to seaweeds and seaweeds plus streptonigrin (SN). The results of the initial screening demonstrated that all seaweeds promoted antitoxic effects in at least one of the concentrations tested. *Grateloupia* and *Porphyra* were the most promising in terms of antitoxicity. For the fruit flies exposed to the tested concentrations of *Gracilaria* higher than 1.25%, toxicity was verified. *Porphyra* at 10% and *Grateloupia* at 20% were able to enhance the longevity of *Drosophila*, with *Grateloupia* reaching the highest potential. In SMART, the antigenotoxic actions for the concentrations selected were proved, *Porphyra* at 5% and 10% and *Grateloupia* at 10% and 20%, with 20% of *Grateloupia* showing the highest, demonstrating potential for the prevention of carcinogenic events. Thus, *Grateloupia turuturu* and *Porphyra umbilicalis* collected from the Portuguese coast demonstrated their potential as functional food. Further studies are necessary for the remaining tested seaweeds.

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List of Abbreviations, Acronyms, and Symbols

€	Euros
χ^2	Chi-square
8-oxo-dG	8-hydroxydeoxyguanosine
B.C.	Before Christ
CAT	Catalase
Cco	Cytochrome c oxidase
CI	Confidence interval
df	Degrees of freedom
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DT75	Development time of 75% of population
EMS	Ethyl methane sulfonate
ETC	Electron transport chain
EU	European Union
f	Spot frequency per 10^4 cells
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSTs	Glutathione S-transferases
HIV	Human immunodeficiency virus
IMTA	Integrated multi-trophic aquaculture
IP	Inhibition percentage
LOH	Loss of heterozygosity
m	Multiplication factor
mtDNA	Mitochondrial deoxyribonucleic acid
n	Sample size
N	Population size
NADH	Nicotinamide adenine dinucleotide reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form

NC	Negative control
OK	Oregon-K
PBS	Phosphate buffered saline
PC	Positive control
ROS	Reactive oxygen species
SD	Standard deviation
SN	Streptonigrin
SOD	Superoxide dismutase
U.S.	United States
UK	United Kingdom
UN	United Nations
USA	United States of America
UVB	Ultraviolet B
w	*white*
WHO	World Health Organization
WoRMS	World Register of Marine Species
ω-3 PUFAs	Omega-3 polyunsaturated fatty acids

Chapter 1 - Introduction

1.1. Seaweeds

1.1.1. Potential of Marine Organisms

One of the most prominent features of our planet is water (H_2O). It covers approximately 70% of the Earth's surface (S.-K. Kim & Himaya, 2011; U.S. Department of the Interior & U.S. Geological Survey, 2016). The oceans represent about 96.5% of the world's H_2O and contain most of the living beings on Earth (U.S. Department of the Interior & U.S. Geological Survey, 2016). It is thought that about 80% of living organisms live in seawater masses of our planet (as many species are yet to be discovered) (Jha & Zi-rong, 2004; Lopes, 2014), with a current number of 239,094 marine species identified accordingly to the database WoRMS Editorial Board (2017).

The exploration of marine resources is emerging worldwide, especially since the last decades, revolutionising the scientific community and the societies (Figure 1.1). The ocean environment lays in extreme conditions since it covers a wide temperature variety, pressure range, nutrient range, oxygen (O_2) variable concentrations, salt variable concentrations, and it has extensive photic and non-photic zones (Alves, 2011; Jha & Zi-rong, 2004). This variability allowed the development of different phylogenetic levels, from microorganisms to mammals, developing defensive strategies that resulted in a significant level of structural and chemical diversity of compounds (Jha & Zi-rong, 2004). Therefore, they can produce a large number of secondary metabolites that are not found in terrestrial living beings (Rasmussen & Morrissey, 2007; Santos et al., 2015).

The researches in these area brought scientific knowledge which demonstrated that marine organisms can have several applications in areas such as food industry, agriculture, pharmaceutical industry, textile industry, fuel industry, among many others (Lopes, 2014; Mendis & Kim, 2011).

Many countries do not possess a territory that allows them to exploit these marine resources. On the other hand, the Portuguese territory displays an enormous potential for the exploration of marine resources since it has an exclusive economic zone in a pendant phase to be increased (Alves, 2011; Firmino, 2014). If this proposal is approved by UN, the territory reaches almost 4 million km^2, being that 97% of the territory will represent ocean territory (Firmino, 2014).

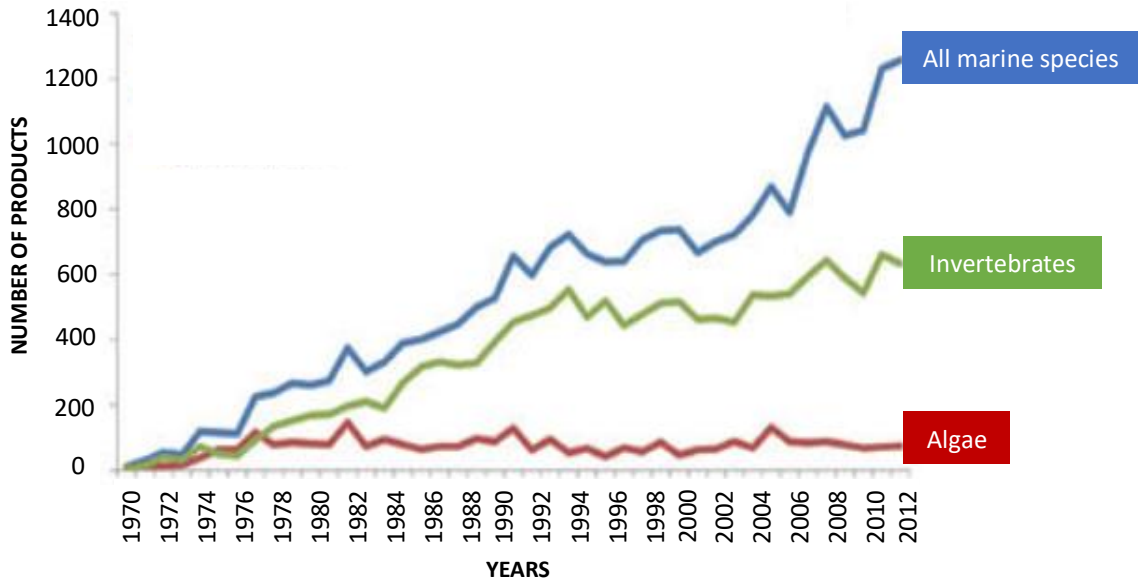

Figure 1.1 - Number of marine natural products discovered from 1970 to 2012 (non-cumulative). The blue line represents the products developed from all marine biota, while the other two lines focus on the two groups that contribute more for the products discovery: the green line focuses on products developed from invertebrates and the red line represents algae products (adapted from Dabb, Potter, White, Blunt, & Munro, 2016).

One of the best examples of a group of marine organisms that displays a large potential (in all aspects), wherein each year are obtained new natural products from them, which leads to the development thereof in aquaculture, culminating in millions of euros in income every single year worldwide in different industries... this group is known as marine macroalgae or seaweeds.

1.1.2. Study of Macroalgae

Macroalgae are defined as multicellular eukaryotic and macroscopic algae (Baweja, Kumar, Sahoo, & Levine, 2016). They are present in both seawater and freshwater ecosystems, as well as in brackish waters (mix of seawater and freshwater). In their natural environment macroalgae are usually benthic, i.e., they grow on substrates like hard rocks, shells, unconsolidated rocks, and others, and form stable multi-layered vegetation (Dawes, 2016; Levine, 2016).

Macroalgae's distribution and growth depends upon many environmental factors such as: physical (substrate, temperature, light quality and quantity, winds, and storms), chemical (pH, nutrients, gases, and pollution level), biological (herbivores, parasites, and diseases)… who also affect the chemical composition of macroalgae (Baweja et al., 2016; Lopes, 2014).

Relatively to macroalgae's chemical composition, the detailed chemical characterization is still largely unexplored in many species. In the last years, many scientific researches related to this area were released (Santos et al., 2015). This made it possible to understand which are

the main components of macroalgae, according to Holdt and Kraan (2011), Lopes (2014) and Mendis and Kim (2011), as presented in Figure 1.2.

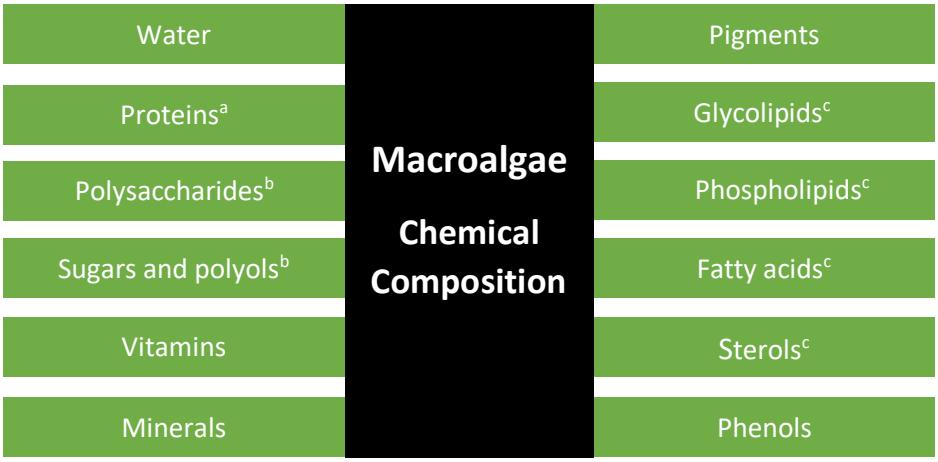

Figure 1.2 - Main groups of compounds found in macroalgae (chemical composition). [a] including peptides and amino acids; [b] main carbohydrates; [c] main lipids.

These organisms possess vast morphological types according to their complexity, structure, which resulted from their polyphyletic origin and environmental adaptations (Lopes, 2014; L. Pereira, 2015). Thus, macroalgae communities can be found in a wide variety of aquatic ecosystems through the year and through the world, being one of the most important organisms maintaining the ecosystem's stability since they are the primary producers in the aquatic ecosystems. They are the ones responsible for the production of the most important gas for life (for most living beings) in seawater, freshwater, and brackish water ecosystems, O_2 (Paiva, Lima, Patarra, Neto, & Baptista, 2014; Ramanan, Kim, Cho, Oh, & Kim, 2015). They are able to perform photosynthesis, using sunlight, carbon dioxide (CO_2), and inorganic substances present in H_2O, producing organic compounds for the food chain and O_2 for the respiratory chain. They provide, as well, shelter and reproductive sites for other living beings as mammals, birds, fish, and invertebrates. In fact, all algae are essential to life in all aquatic ecosystems (L. Pereira, 2015) and, therefore, deregulation in algae communities could have a domino effect on other communities of the same ecosystem. For this reason, macroalgae species are frequently used as indicators of ecosystem health (Baweja et al., 2016).

Focusing on Portugal, with regards to the diversity of macroalgae, it is located in a prime location demonstrating an interesting gradient of species along the coast. To the North, species are similar to those present on the coast of Central Europe, and in the South, a great influence of Mediterranean and African species prevails (Alves, 2011).

A separation within the macroalgae group can be made into three different groups taking into account the colour of algae's body: red, green, and brown algae for both seawater (including brackish water) and freshwater algae (Integrated Taxonomic Information System online database, 2016; WoRMS Editorial Board, 2017). Focusing on macroalgae living in seawater (including brackish water), they are usually designated as marine macroalgae or seaweeds and there are many inconsistencies in their taxonomic classification. Although, online databases gather the approved and most used seaweeds classification, that can be divided as (Figure 1.3) (Guiry, 2016a; Guiry, 2016b):

- Red algae (kingdom Plantae, phylum Rhodophyta),
- Green algae (kingdom Plantae, phylum Chlorophyta, classes Chlorophyceae, Dasycladophyceae, Siphoncladophyceae, and Ulvophyceae),
- Brown algae (kingdom Chromista, phylum Ochrophyta, class Phaeophyceae).

However, other authors propose different or more embracing taxonomic groups to be inserted in each one of the three groups of seaweeds, arising the controversy in algae classification.

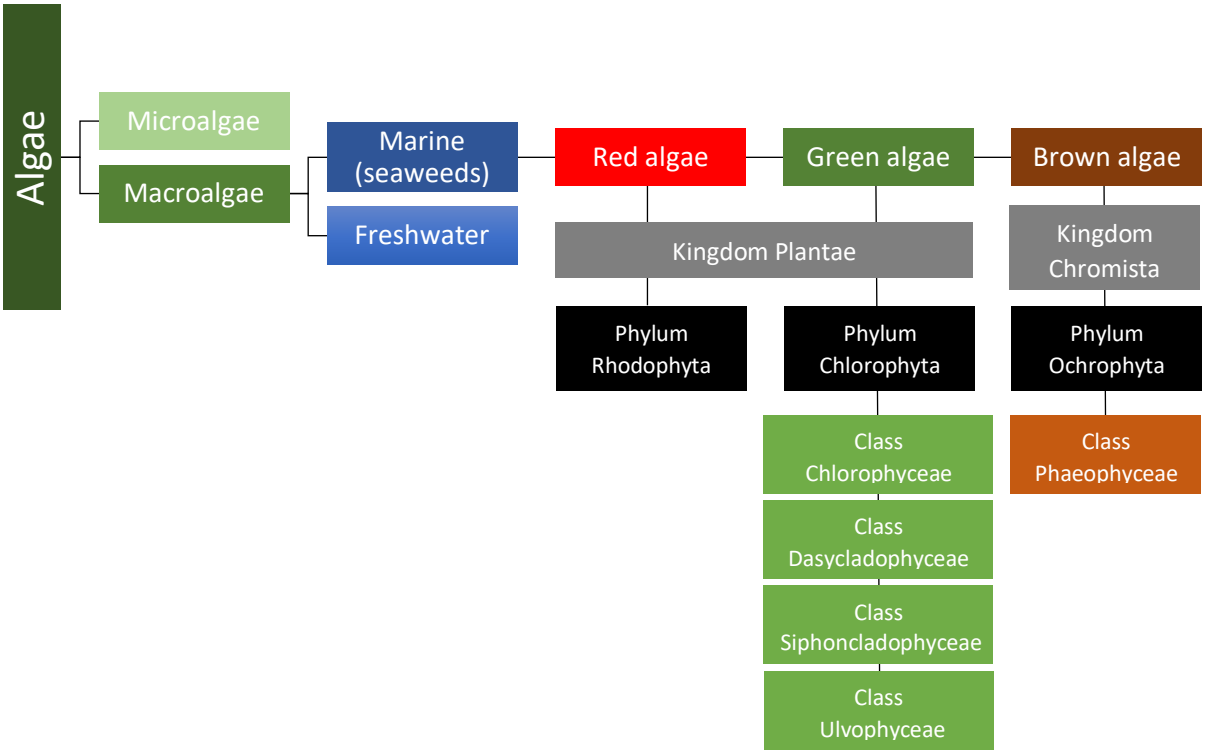

Figure 1.3 - Algae classification, focusing on seaweeds taxonomic differentiation.

Furthermore, seaweeds distinction in red, green, and brown algae involves more substantial differences than body pigmentation (Guiry, 2016a; WoRMS Editorial Board, 2017)…

1.1.3. Focusing on Seaweeds Phyla

Following, the pigments that define seaweeds' colour, the geological time in which they first appeared, characteristics, the habitat and global distribution, the number of species, and examples of genera and species present in the Atlantic European coast, are going to be displayed for the three seaweeds phyla.

The exemplified seaweeds were chosen taking into account different reasons shown along the subchapter and, therefore, they will be focused for the rest of the work. Chemical compositions from specimens of Atlantic European coast are presented. According to many authors such as Francavilla, Franchi, Monteleone, and Caroppo (2013), Kendel et al. (2013), and Schmid, Guihéneuf, and Stengel (2014), seasonal variation defines different environments and consequently seaweeds chemical composition is always changing, giving rise to different values and wide ranges for the same parameter.

Red Algae

Red algae (phylum Rhodophyta) are constituted by a variety of different photosynthetic pigments in their chloroplasts, that define their colour as red, such as: chlorophyll a, the pigment that lies in the other algae groups and even in the rest of kingdom Plantae, accessory pigments like carotenes (e.g. β-carotene), xanthophylls (e.g. lutein and zeaxanthin), phycobilins, and phycobiliproteins (e.g. R-phycocyanin and R-phycoerythrin) (Baweja et al., 2016; Guiry, 2016b; L. Pereira, 2015). R-phycoerythrin provides the red pigmentation to algae by reflecting red light and absorbing blue light, and whose activity masks the other pigments (Baweja et al., 2016; Dawes, 2016). On the other hand, with small concentrations of this pigment, red algae may appear more green, blue, as well as purple or brown (Guiry, 2016b; L. Pereira, 2015). Notably, the Rhodophyta seaweeds can tolerate a wider range of light than any other group of photosynthetic organisms because of their pigment constitution (Baweja et al., 2016).

Relatively to the geological time in which they first appeared, there is not a consensus since the fossil records are not clear. The oldest evidence of red algae that is convincing dates from between Mesoproterozoic and Neoproterozoic eras (600 to 1250 million years ago approximately) (Dawes, 2016; Levine, 2016). Thus, they represent an ancient photosynthetic lineage of eukaryotes, being the oldest group of eukaryotic algae together with green algae (Baweja et al., 2016; Guiry, 2016b). Evidences show as well that the precursors of aquatic plants may belong to this phylum (Baweja et al., 2016).

There are some distinctive characteristics in red algae in comparison to the other two groups: the reserves of energy are constituted by starch, although it is floridean and floridoside

starch, a different starch from higher plants and green algae (Guiry, 2016b; Lopes, 2014); the cell walls are made of cellulose and unique (only present in red seaweeds) sulphated polysaccharides such as agars, carrageenans, and porphyrans. Porphyrans are exclusive from *Porphyra* spp.; the main sugar/polyol is sorbitol; among others (Guiry, 2016a; Jiménez-Escrig, Gómez-Ordóñez, & Rupérez, 2011).

Agar and carrageenan arose further attention than the other red algae components, not only for their biological activities but also for their commercial applications. They are commercially known as phycocolloids. They form a stable viscous gel in water, and its primary functions are: binder, stabilizer, emulsifier and/or moulding agent (Baweja et al., 2016; Karleskint, Turner, & Small, 2010). The most used seaweeds for agar extraction are *Gracilaria* spp. and *Gelidium* spp. (Karleskint et al., 2010; L. Pereira, 2011). The red algae with the most carrageenans content are dominated by the warm water species *Kappaphycus alvarezii* and *Eucheuma denticulatum*, the most used ones for the extraction of this phycocolloid. *Chondrus crispus* and *Grateloupia* spp. have been documented as good sources too (Bixler & Porse, 2011; Sahoo, 2010).

Members of Rhodophyta can occur at all latitudes from tropical and temperate regions to colder seas and even in polar and sub-polar regions a few species can be found. Thanks to their photosynthetic pigments (specially R-phycoerythrin), unlike other algae, they have the ability to live in great depth in the ocean because they absorb light with high infiltration power (Baweja et al., 2016; Lopes, 2014).

Online database AlgaeBase (Guiry, 2016b) and Levine (2016) counts shows that there are about 10,000 species of seaweeds. From this, 6,500 are red seaweeds species (Guiry, 2016b).

Three examples of marine red algae genera are going to be described next.

Gracilaria Greville, 1830

- Common names: commonly known as graceful red weed, *ogonori* (Japanese), *cabelo-de-velha* (Portuguese), and others;
- Chemical composition: Table 1.1;
- Distribution: over 150 species are dispersed worldwide. Species from this genus are native in the Northwest Pacific Ocean. They were introduced in North American East and West coasts, Northeast Atlantic (including Portugal), and East Pacific. The species *Gracilaria gracilis* and *Gracilaria vermiculophylla* are the ones present in the Atlantic European coast (Figure 1.4) (Baweja et al., 2016; Dawes, 2016; L. Pereira, 2015).

Recently, molecular studies have discovered a number of cryptic species that have almost equal anatomy, morphology, and reproduction. Consequently, the species identification in this genus is difficult when using these physical traits, being necessary genetic analysis to avoid misidentification errors.

Table 1.1 - Main chemical composition of *Gracilaria* specimens from Atlantic European coast (France, Ireland, and Portugal).

***Gracilaria* chemical content**	
Water[1] (% of wet weight)	80-90%[a]
Minerals (% of dry weight)	29%[b] 34%[c]
Proteins (% of dry weight)	10.5%[c] 20.2%[b]
Carbohydrates (% of dry weight)	29.1%[c] 46.6%[b]
Agar[2] (% of dry weight)	8-18.7%[d]
Lipids (% of dry weight)	0.6%[b] 2.6%[c]
Fatty acids[3] (% of dry weight)	1.2%[b] 1.4%[e]
ω-3 PUFAs[4] (% of total fatty acids)	1.4%[b] 3.4%[e]
Phenols (% of dry weight)	2.3%[b]

[1] According to Borowitzka et al. (2009), water content of fresh seaweeds falls within (or next to) the range of 80 to 90% of wet weight; [2] included in polysaccharides; [3] included in lipids; [4] ω-3 PUFAs are omega-3 polyunsaturated fatty acids.
[a] Borowitzka et al. (2009); [b] D. Rodrigues et al. (2015) (Buarcos bay, Figueira da Foz, Portugal); [c] Jard et al. (2013) (France); [d] Mollet, Rahaoui, and Lemoine (1998) (France); [e] Schmid et al. (2014) (Ireland).

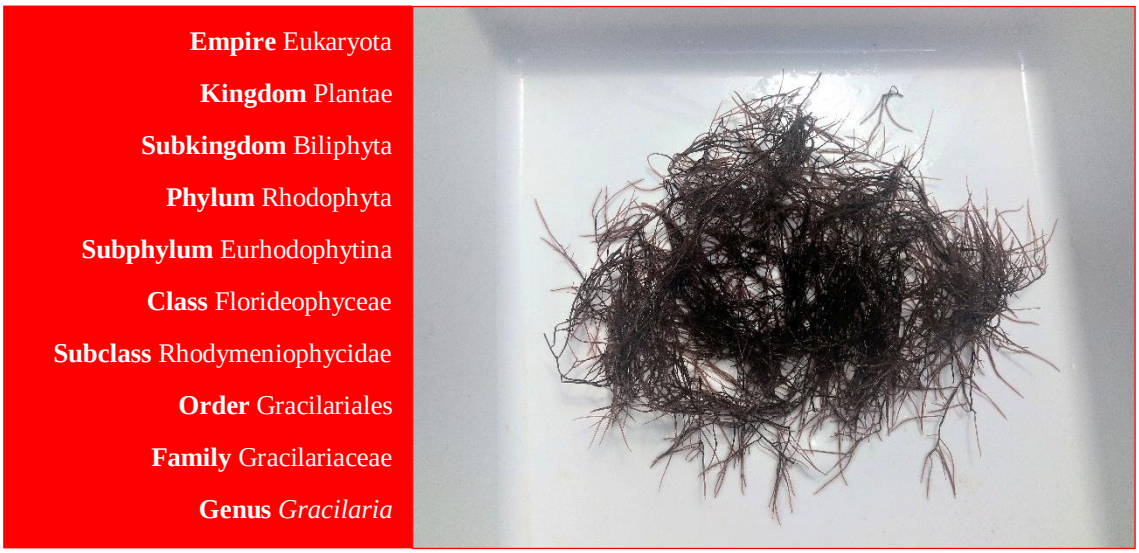

Figure 1.4 - *Gracilaria* taxonomic classification (Guiry, 2016a) and a section of a *Gracilaria* sp. specimen (pendent DNA confirmation between *Gracilaria gracilis* and *Gracilaria vermiculophylla*).

Grateloupia C.Agardh, 1822

- Common names: *tamba-nori* (Japanese), *ratanho* (Portuguese), and others. Specifically for *Grateloupia turuturu*, it is devil's tongue weed;

- Chemical composition:

Table 1.2 - Main chemical composition of *Grateloupia* specimens from Atlantic European coast (France and Portugal).

***Grateloupia* chemical content**	
Water[1] (% of wet weight)	80-90%[a]
Minerals (% of dry weight)	18.5%[b] 20.5%[c]
Proteins (% of dry weight)	16.2-21.8%[d] 22.5%[c] 22.9%[b]
Dietary fibres[2] (% of dry weight)	60.4%[b]
Lipids (% of dry weight)	2.2-2.6%[c] 3.6%[e] 2.8-5.4%[d]
Fatty acids[3] (% of dry weight)	2.1%[c]
ω-3 PUFAs[4] (% of total fatty acids)	12.8%[b] 14.4%[e] 31.6[c]
Glycolipids (% of total lipids)	45%[e]
Phospholipids (% of total lipids)	21.9%[e]
Phenols (% of dry weight)	2.1%[c]

[1] According to Borowitzka et al. (2009), water content of fresh seaweeds falls within (or next to) the range of 80 to 90% of wet weight; [2] according to Gidley (2015), dietary fibers include, principally, polysaccharides associated with cell walls; [3] included in lipids; [4] ω-3 PUFAs are omega-3 polyunsaturated fatty acids.
[a] Borowitzka et al. (2009); [b] Denis et al. (2010) (France); [c] D. Rodrigues et al. (2015) (Buarcos bay, Figueira da Foz, Portugal); [d] Munier, Dumay, Morançais, Jaouen, and Fleurence (2013) (France); [e] Kendel et al. (2013) (France).

- Distribution: distributed all over the world, with approximately 96 recorded species. Are considered native to Japan, China, and Korea, but have spread (introduced species) to the Northeast Atlantic (including Portugal), the Mediterranean, South America, Australia, and New Zealand. *Grateloupia turuturu* is the one located in the Atlantic European coast (Figure 1.5) (Baweja et al., 2016; Dawes, 2016; L. Pereira, 2015).

Empire Eukaryota
Kingdom Plantae
Subkingdom Biliphyta
Phylum Rhodophyta
Subphylum Eurhodophytina
Class Florideophyceae
Subclass Rhodymeniophycidae
Order Halymeniales
Family Halymeniaceae
Genus *Grateloupia*

Figure 1.5 - *Grateloupia* taxonomic classification (Guiry, 2016a) and a section of a *Grateloupia turuturu* specimen.

Porphyra C.Agardh , 1824

- Common names: purple laver, pink laver, laver, *nori* (Japanese), *erva-patinha* (Portuguese), and others. *Pyropia* is currently regarded as a taxonomic synonym;
- Chemical composition:

Table 1.3 - Main chemical composition of *Porphyra* specimens from Atlantic European coast (England, France, Ireland, Portugal, and Spain). Chemical composition data as well from Azores archipelago specimens.

***Porphyra* chemical content**	
Water (% of wet weight)	77.1%[a] 87.1%[b]
Minerals (% of dry weight)	9.3%[a] 19.1%[c] 20.6%[d] 21%[e] 28.2%[b]
Proteins (% of dry weight)	24.1%[c] 24.8%[b] 28.3-28.8%[e] 44%[a]
Carbohydrates (% of dry weight)	50-76%[f]
Lipids (% of dry weight)	0.7%[a] 1%[c] 0.1-2.5%[f] 3.3%[g] 3.4%[h] 8.8%[b]
Fatty acids[1] (% of dry weight)	0.8-1.7%[i]
ω-3 PUFAs[2] (% of total fatty acids)	7.2%[c] 9.8%[b] 16-24.4%[i]
Glycolipids (% of total lipids)	55.9%[j]
Phospholipids (% of total lipids)	25.2%[j]
Phenols (% of dry weight)	< 0.4%[e]

[1] included in lipids; [2] ω-3 PUFAs are omega-3 polyunsaturated fatty acids.
[a] Marsham, Scott, and Tobin (2007) (England); [b] Paiva et al. (2014) (Azores, Portugal); [c] Sánchez-Machado, López-Cervantes, López-Hernández, and Paseiro-Losada (2004) (Viana do Castelo, Portugal); [d] Rupérez (2002) (Spain); [e] Rupérez and Saura-Calixto (2001) (Spain); [f] Morrissey, Kraan, and Guiry (2001) (Ireland); [g] Patarra, Leite, Pereira, Baptista, and Neto (2013) (Azores, Portugal); [h] Kendel et al. (2013) (France); [i] Schmid et al. (2014) (Ireland); [j] Fleurence, Gutbier, Mabeau, and Leray (1994) (France).

- Distribution: has nearly 133 species distributed all over the world. One of the most abundant is *Porphyra umbilicalis* (Figure 1.6) that prefers temperate to colder waters. It occurs in the Northeast Atlantic, in Iceland and from Norway to Portugal, and in the Western Mediterranean (Baweja et al., 2016; Dawes, 2016; L. Pereira, 2015).

Empire Eukaryota
Kingdom Plantae
Subkingdom Biliphyta
Phylum Rhodophyta
Subphylum Eurhodophytina
Class Bangiophyceae
Subclass Bangiophycidae
Order Bangiales
Family Bangiaceae
Genus *Porphyra*

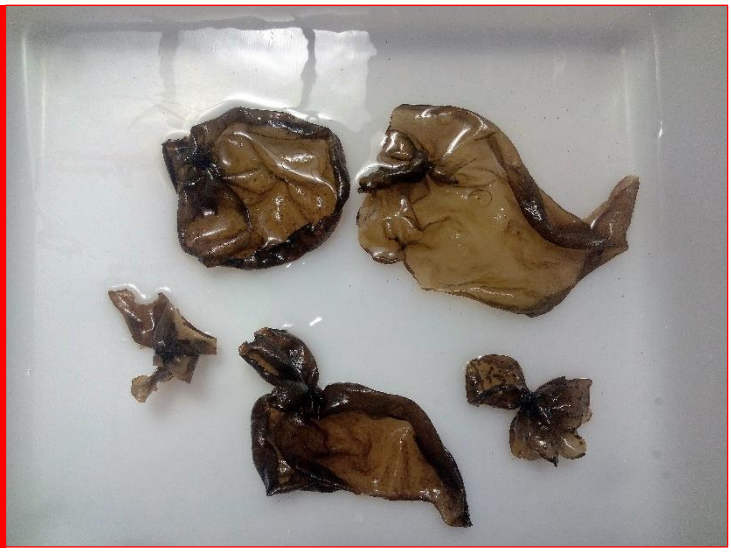

Figure 1.6 - *Porphyra* taxonomic classification (Guiry, 2016a) and sections of a *Porphyra umbilicalis* specimen.

Green Algae

The green algae, commonly called chlorophytes (phylum Chlorophyta), display a green colour similarly to terrestrial plants (Dawes, 2016). Relatively to photosynthetic pigments, they are constituted by chlorophyll a and b, β-carotene, and xanthophylls (the main is lutein) located in chloroplasts in a proportion resembling terrestrial plants (Guiry, 2016b; L. Pereira, 2015). Chlorophyll b is the most concentrated pigment in green algae, thus being responsible for their green colour (Lopes, 2014).

Just like red algae, green algae appeared between Mesoproterozoic and Neoproterozoic eras (Dawes, 2016; Levine, 2016). They are also predecessors of plants, more specifically the terrestrial ones (Guiry, 2016b). Although, there is currently some debate on this issue.

Green seaweeds species grow from a few centimeters to a few meters, being often smaller than red seaweeds (McHugh, 2003); the food reserves consist of starch, some fats, or oils, like higher plants (Guiry, 2016b). Concerning the location where the reserves of energy are, Chlorophyta are different from other seaweeds because the reserves accumulate in the pyrenoids (organelles within chloroplasts), rather than in the chloroplasts' cytoplasm; the cell walls are composed by cellulose, but xylans and mannans can substitute cellulose in some species (Lopes, 2014). In fact, when cellulose is present, green algae cell wall cellulose content is on the same order of magnitude as wood's; in Ulvales order there are present unique sulphated polysaccharides, ulvans; the main sugar/polyol is sucrose (Baweja et al., 2016).

The marine Chlorophyta are globally distributed from Northern to Southern hemispheres and, between each hemisphere, the species vary and the same species show different

characteristics (Lopes, 2014). In the ecosystems, green seaweeds often inhabit shallow coastal waters (Baweja et al., 2016).

In the online database AlgaeBase (Guiry, 2016b) approximately 1,500 species are classified as green seaweeds (800 species of class Chlorophyceae, 50 species of Dasycladophyceae, 400 Siphoncladophyceae, and 250 species from Ulvophyceae).

One example of a marine green algae genus is going to be described next.

Ulva Linnaeus, 1753

- Common names: sea lettuce or green laver, *awosa* or *aosa* (Japanese), *alface-do-mar* (Portuguese), and others;
- Chemical composition:

Table 1.4 - Main chemical composition of *Ulva* specimens from Atlantic European coast (Denmark, England, France, Ireland, Portugal, and Spain).

***Ulva* chemical content**	
Water (% of wet weight)	79.6%[a] 92%[b]
Minerals (% of dry weight)	17.8%[a] 17.9%[c]
Proteins (% of dry weight)	13.1%[c] 15-25%[d] 29%[a]
Carbohydrates (% of dry weight)	31.4%[c] 42-46%[d]
Lipids (% of dry weight)	0.6-1%[d] 1.6%[c] 2.6%[b]
Fatty acids[1] (% of dry weight)	0.5%[a] 1.6-1.9%[e]
ω-3 PUFAs[2] (% of total fatty acids)	18%[f] 23.9%[b] 17-24.7%[e]
Glycolipids (% of total lipids)	29.1%[b]
Phospholipids (% of total lipids)	15.3%[b]
Phenols (% of dry weight)	2.2%[g]

[1] included in lipids; [2] ω-3 PUFAs are omega-3 polyunsaturated fatty acids.
[a] Marsham et al. (2007) (England); [b] Kendel et al. (2015) (France); [c] Jard et al. (2013) (France); [d] Morrissey et al. (2001) (Ireland); [e] Schmid et al. (2014) (Ireland); [f] H. Pereira et al. (2012) (Algarve, Portugal); [g] Farvin and Jacobsen (2013) (Denmark).

- Distribution: there are more than 125 *Ulva* spp. currently accepted. It is a ubiquitous genus, occurring throughout the shorelines around the world since they can tolerate a wide range of temperatures. *Ulva lactuca* and *Ulva rigida* are located in Portuguese waters (Figure 1.7) (Baweja et al., 2016; L. Pereira, 2015; Silva, Vieira, Almeida, & Kijjoa, 2013).

The species identification is difficult as in *Gracilaria* genus. They face many taxonomic complications triggered by little morphological differences among species (Baweja et al., 2016).

Figure 1.7 - *Ulva* taxonomic classification (Guiry, 2016a) and a section of a *Ulva* sp. specimen (pendant DNA confirmation between *Ulva lactuca* and *Ulva rigida*).

Brown Algae

Brown seaweeds (class Phaeophyceae) are constituted by different photosynthetic pigments that define their colour as brown. Their main photosynthetic pigments consist of chlorophylls a and c, carotenes (e.g. β-carotene), and xanthophylls (e.g. diatoxanthin, fucoxanthin, and violaxanthin); the one responsible for the brown, sometimes yellowish colour, is fucoxanthin which dominance masks the activity of the other pigments (Baweja et al., 2016; Dawes, 2016). Phlorotannins (phenols) stored in vesicles designated as physodes, also promote the brown colour of these seaweeds (Schoenwaelder, 2008).

Brown algae appeared in a non-direct lineage that lead to the formation of red algae, green algae, and plants (Rice, 2007). Therefore, brown algae are classified into the kingdom Chromista (Guiry, 2016b). Fossils from brown algae have been found in rocks from Ordovician (488 to 443 million years ago approximately) (University of California Museum of Paleontology, 2016). Other fossil records have been found and nucleotide sequences suggest different outcomes, which raises many questions about the origin of these algae (Dawes, 2016).

The food reserves consist of polysaccharides (mainly laminarin), sugars, and higher alcohols; cell walls are mainly made of cellulose, fucoidans, and alginates; mannitol is the main sugar/polyol (Jiménez-Escrig et al., 2011).

Alginates are the other phycocolloid most recurrent for industrial purposes. Sodium and calcium alginates are the main forms of alginates extracted. Although alginates are present in the majority of brown algae, the most useful grow in cold turbulent waters in both the Northern and Southern hemispheres (McHugh, 2003). *Laminaria* spp. and *Lessonia* spp. are the most

used genera for alginates extraction. However, species from order Fucales are also used (Bixler & Porse, 2011; Guiry, 2016b).

Brown algae represent the largest algae group in terms of size (Baweja et al., 2016). They seem to be the most affected, amongst the three groups of seaweeds, by different environmental conditions. Depending on the geographic region, the same species exhibit very dissimilar morphological and chemical features (Baweja et al., 2016). They are most prevalent in colder to temperate waters, particularly in the Northern Hemisphere (Guiry, 2016b). They are lithophytes and can be free-floating (Dawes, 2016). So, they are present in a great variety of marine ecological niches (Lopes, 2014).

Accordingly to Baweja et al. (2016), Dawes (2016), and Guiry (2016b), approximately 2,000 species of marine macroalgae belong to the brown algae group.

One example of a marine brown algae genus is going to be described next.

Fucus Linnaeus, 1753

- Common names: wrack, *bodelha* or *fava-do-mar* (Portuguese), and others;
- Chemical composition:

Table 1.5 - Main chemical composition of *Fucus* specimens from Atlantic European coast (England, France, Ireland, and Spain). Chemical composition data as well from Azores archipelago specimens.

***Fucus* chemical content**	
Water (% of wet weight)	81%[a] 87.7%[b]
Minerals (% of dry weight)	19%[a] 22.3%[b] 30%[c] 30.1%[d]
Proteins (% of dry weight)	6.2-6.9%[c] 9.7%[b] 5-10%[e] 17%[a]
Carbohydrates (% of dry weight)	62-66%[e]
Alginates[1] (% of dry weight)	18-22%[e]
Lipids (% of dry weight)	0.5-2%[e] 5.2%[b]
Fatty acids[2] (% of dry weight)	3.1%[f] 2.7-4.6%[g]
ω-3 PUFAs[3] (% of total fatty acids)	14%[b] 11.5-24%[g]
Glycolipids (% of total lipids)	30.1-33.5%[h]
Phospholipids (% of total lipids)	2.7-4.7%[h]
Phenols (% of dry weight)	2-6%[i] 2.8-5.5%[j]

[1] included in polysaccharides; [2] included in lipids; [3] ω-3 PUFAs are omega-3 polyunsaturated fatty acids.
[a] Marsham et al. (2007) (England); [b] Paiva et al. (2014) (Azores, Portugal); [c] Rupérez and Saura-Calixto (2001) (Spain); [d] Rupérez (2002) (Spain); [e] Morrissey et al. (2001) (Ireland); [f] Herbreteau, Coiffard, Derrien, and Roeck-Holtzhauer (1997) (France); [g] Schmid et al. (2014) (Ireland); [h] Le Tutour et al. (1998) (France); [i] Connan, Goulard, Stiger, Deslandes, and Gall (2004) (France); [j] Zubia, Fabre, Kerjean, Lann, et al. (2009) (France).

- Distribution: *Fucus* is a common genus in cold and temperate waters and the most common species, *Fucus vesiculosus* (Figure 1.8), is found on the western Baltic Sea, and in the Atlantic and Pacific Oceans (Portugal included) (Dawes, 2016; Guiry, 2016b; L. Pereira, 2015).

Empire Eukaryota
Kingdom Chromista
Phylum Ochrophyta
Class Phaeophyceae
Subclass Fucophycidae
Order Fucales
Family Fucaceae
Genus *Fucus*

Figure 1.8 - *Fucus* taxonomic classification (Guiry, 2016a) and sections of a *Fucus vesiculosus* specimen.

1.1.4. Seaweeds in Food Industry

Seaweeds have been used by humans since Chinese started to adopt them as an herbal medicine in 3000 B.C. In Europe, years after, Mediterranean seaweeds were used in Greek and Roman empires as medicine, and Greeks even started to apply them as animal feeding (Levine, 2016). For many centuries, coastal populations have harvested and later cultivated a wide variety of seaweeds.

Knowing the chemical composition, explaining which component is useful in a specific industry and why, made it possible to use each seaweed focused previously and/or each chemical component in different applications nowadays, such as in: agriculture (Baweja et al., 2016; Guiry, 2016b), animal feed (L. Pereira, 2015), biofiltering (Silva et al., 2013), biofuel (Kraan, 2016), cosmetics and hygiene products (Couteau & Coiffard, 2016; Rupérez, Ahrazem, & Leal, 2002), the textile industry (Dawes, 2016; McHugh, 2003), thalassotherapy (Baweja et al., 2016; Borges, 2014), medical and medical associated industries (Guiry, 2016b; Holdt & Kraan, 2011; McHugh, 2003)… However, their greater use occurs in food industry, which will be described next.

Seaweeds as Food Additives and Dietary Supplements

Phycocolloids general functions display a great deal of applications. In food industry, the major applications of phycocolloids occur as food additives (Guiry, 2016b).

Due to its long and safe history of use, agars, carrageenans, and alginates are generally recognised as safe (GRAS) by experts from the U.S. Food and Drug Administration (FDA) and, consequently, are approved food additives (Holdt & Kraan, 2011; L. Pereira, 2011).

In Portugal and in the rest of the European Union, as well as in Switzerland, "E" numbers have been given to phycocolloids: E400 to E405 that represent alginates and derivatives (e.g. sodium alginates and calcium alginates), E406 for agar, and E407 represents carrageenans, as a way of identifying them as food additives belonging to the group of thickeners, stabilizers, and emulsifiers (Guiry, 2016b; McHugh, 2003; L. Pereira, 2011).

Accordingly to Bixler and Porse (2011), 80% of agar produced globally (about 7,680 t in 2009) is for food applications. *Gracilaria* spp. are the main source of large scale production of food grade agar, being commercially cultivated (main source) in Indonesia, Chile, and others such as Portugal (Delaney, Frangoudes, & Ii, 2016; J.-I. Yang et al., 2012). It should be noted that Portugal is one of the major agar producers in the world, although many of seaweeds raw material is imported (Delaney et al., 2016).

Relatively to carrageenans, the main seaweed species raw material comes from aquaculture in Philippines and Indonesia (Bixler & Porse, 2011; L. Pereira, 2015). Carrageenans are the most important phycocolloid in food industry, with about 45,000 t produced in 2009 for food industry (Bixler & Porse, 2011; L. Pereira, 2011).

Alginates production relays mainly on China's production from cultivated *Laminaria japonica*. In 2009, the production of alginates reached 26,500 t and is thought that a great share of it was for food industry (Bixler & Porse, 2011; Guiry, 2016b).

Processed foods such as dairy products (chocolate milk, yoghurts, cheeses, puddings, frozen desserts, ice creams, among others) have one or more of this "E" numbers to prevent fractionation of milk constituents; in processed meat and seafood, phycocolloids serve for binding and shaping them to resemble usual cuts of meat, such as nuggets, roasts, and meat loaves; in pre-cooked products a phycocolloid is important to avoid H_2O loss during cooking; sauces, syrups, jellies, marmalades, health drinks, and even beers and wines contain these sulphated polysaccharides; calcium alginate coatings have been used to help preserve frozen fish (Fleurence, 2016; Guiry, 2016b; Holdt & Kraan, 2011).

Other compound of seaweeds, mannitol, is used as a sweetener in the production of diabetic food, chewing gum, among others (Baweja et al., 2016); phycobiliproteins are mainly used in the production of gums, sorbets, ice-cream, candies, soft beverages, wasabi, and dairy products, to provide pigmentation (Fleurence, 2016).

Seaweeds are also used as dietary supplements. FDA (2016, June 1) stated that "Some supplements can help assure that you get enough of the vital substances the body needs to function; others may help reduce the risk of disease. But supplements should not replace

complete meals which are necessary for a healthful diet…". Being so, seaweeds compounds such as alginates, fucoidans, ω-3 PUFAs, vitamins, and others are extracted from seaweeds and sold as dietary supplements. A few grinded whole seaweeds are also being sold as dietary supplements (Jiménez-Escrig et al., 2011; Škrovánková, 2011).

Seaweeds Full Intake

Besides the utilization of seaweeds components as food additives and dietary supplements, seaweeds can, and should, be consumed in its whole (full intake). Seaweeds that can be safely eaten by humans are known as edible seaweeds and can be eaten fresh, dried, cooked, and among these they can be in powder, flakes, as well as many other combinations. They are often used as sushi wrappings (*Porphyra* spp.) (Figure 1.9 A), seasonings and vegetables for rice, noodles, soups, salads, desserts (e.g. *Gracilaria* spp., *Grateloupia* spp., *Porphyra* spp., *Ulva* spp. and *Fucus* spp. are used)… Hence, the majority of these applications occur in East Asian countries like Japan, China, and South Korea (their biggest consumers). However, many of these applications, especially sushi, are becoming available all over the world much due to the migration of people from the previous countries and reinvention of world cuisine (globalization) (FAO, 2016; Guiry, 2016b; Yuan & Walsh, 2006). Also, many seaweeds are being sold dehydrated, in packages, for later moisturizing at home for their use as vegetables and related products. Products such as these are available in European trade, including in Portugal (Figure 1.9 B).

Santos et al. (2016) stated that more than 100 edible seaweeds are consumed worldwide. Hence, European regulation only considers 22 edible seaweeds species (Abreu, Pereira, & Sassi, 2014). As the world's biggest seaweeds consumers (whole seaweed), Japanese eat between 10% to 25% of seaweeds relatively to their total food intake (Collins, Fitzgerald, Stanton, & Ross, 2016; Peinado, Girón, Koutsidis, & Ames, 2014). Consumption varies widely among individuals, and accordingly to Gomez-Gutierrez, Guerra-Rivas, Soria-Mercado, and Ayala-Sánchez (2011), and Fleurence (2016), the daily amount of seaweeds eaten in Japan is estimated at about 4 to 8 g of seaweeds dry weight per person. Other authors refer to the annual seaweeds intake in Japan, such as Cornish and Garbary (2010) and S.-K. Kim and Pangestuti (2011), referring that on average 1.4 to 1.6 kg of seaweeds (dry weight) is eaten per person. In Europe, seaweeds trace quantities are consumed per person, being that many Europeans never even tasted them (Fleurence, 2016).

Figure 1.9 - Applications of *Porphyra* spp. in food industry. (A) Use of *Porphyra* leaves in the making (wrapping) of sushi; (B) Package with whole dried *Porphyra* spp. inside, developed by a Portuguese aquaculture seaweeds company (ALGAplus) under the brand Tok de Mar®.

Seaweeds became appealing for the current world when chemical composition was checked: richness in proteins, low content in lipids (low calories), high concentration of fibers, minerals, pigments, PUFAs, vitamins, phenolic compounds, among others (Zubia, Fabre, Kerjean, Lann, et al., 2009).

Nowadays, the search for foods with high proteins content and with few fats is a reality, since the current standards of beauty demand it. Red and green seaweeds protein levels (generally smaller in brown seaweed) are comparable and even higher to those found in high protein cereals and vegetables, essential for the "gym-generation" for muscle growth (Dumay & Morançais, 2016); even though the carbohydrate content is high in seaweeds (higher than most vegetables), a good share corresponds to insoluble dietary fibers not taken up by the human body. So, they make an excellent intestinal environment. Soluble fibers (higher content than insoluble) dissolve in H_2O, forming a gel when they reach the stomach and decrease the appetite; mineral content is very high in seaweeds. As a comparison, spinach has a known exceptionally high mineral content of 20% of dry weight, while *Gracilaria* spp., as shown previously, reaches 34% of dry weight. Seaweeds are rich in boron (B), calcium (Ca), iodine (I), selenium (Se), among many others. *Ulva lactuca* has more Ca than most cheeses (Gomez-Gutierrez et al., 2011) and *Porphyra* spp. are one of the major natural sources of I (Brown et

al., 2014); chlorophylls and fucoxanthin, β-carotene, and lutein (carotenoids) are the major sources of pigments in seaweeds which, depending from seaweed to seaweed, have similar quantities relative to vegetables (Škrovánková, 2011); ω-3 PUFAs present in human metabolism derive from marine products. They are accumulated in fish and other marine animals that consume algae and get passed on to other species through food chain, ending up in humans' body (Holdt & Kraan, 2011; Silva et al., 2013); seaweeds vitamins comprise at least vitamins A, B1, B3, B12, C, and E. Vitamin C is present in large amounts in *Ulva* spp. and *Gracilaria* spp. (Škrovánková, 2011). Species from *Ulva* and some *Porphyra* spp. are one of the few non-animal sources of vitamin B12, which may provide an alternative source for vegetarians and vegans (MacArtain, Gill, Brooks, Campbell, & Rowland, 2007); phenols, such as polyphenols (e.g. phlorotannins), are present in higher concentrations in brown seaweeds compared with the other two (Machu et al., 2015).

1.1.5. Beneficial Effects on (Human) Health

In human nutrition, there was always a demand for natural products or natural compounds with beneficial effects for human health.

Since the last decades, seaweeds have been explored for their bioactive compounds, i.e., seaweeds components that promote beneficial effects on living organisms such as the human beings. Consequently, seaweeds bioactive compounds are the main "ingredients" in the previously referred dietary supplements and, together with edible seaweeds, belong to the group of nutraceuticals (Biesalski et al., 2009; MacArtain et al., 2007).

Edible seaweeds have been given the label as functional food, which is reserved for food that have been demonstrated to provide specific health benefits beyond basic nutrition for the intended population. In the case of seaweeds, many bioactive compounds are present in their constitution, which are either missing in the analogous conventional food or present at lower concentrations, such as in vegetables. Seaweeds, as functional food, promote optimal health by using its nutritional potential to reduce the risk of chronic diseases and enhancing the ability to manage chronic diseases, thus improving quality of life (Holdt & Kraan, 2011; Mendis & Kim, 2011; Zubia, Fabre, Kerjean, Lann, et al., 2009).

The following beneficial properties justify the designation of bioactive compounds to the components of seaweeds and their use in medicine and related areas, while still receiving the dietary supplements and functional food "labels" in food market. They refer to submitted scientific researches that measured effects from specific compounds or extracts from seaweeds.

Among the main potential beneficial effects of seaweeds and their chemical compounds, some preventive and therapeutic actions can be pointed out in general: antibiotic (against *Escherichia coli*, *Staphylococcus* spp., among others), antifungal, antiviral (against HIV, herpes, among others), and vermicide; anti-inflammatory, antiallergic, and immunomodulatory; anticoagulant; antidiabetic; antinociceptive (analgesic); anti-obesity, antihypertension, antihyperlipidemic, and cardiovascular diseases preventive and therapeutic; digestive system regulator (acting as a prebiotic, among others); neuroprotective (preventive against Alzheimer's, Parkinson's, among others); organ or tissue regeneration, repair, and protection (antiosteoporosis, anti-hyperoxaluria, ophthalmological diseases preventive, hepatoprotective, wound healing, among others); among many others (Déléris, Nazih, & Bard, 2016; Mohamed, Hashim, & Rahman, 2012; Vonthron-Sénécheau, 2016).

The beneficial effects focused next were highlighted because they are intimately linked to this work.

Antioxidant Protection and Other Genome Protective Actions

Oxidative stress is defined as the oversized formation of reactive oxygen species (ROS) in living beings, that exceeds the removal availability of antioxidants, as a result of endogenous and exogenous factors. There are several ROS directly involved in the establishment of oxidative stress, whose activity can directly damage macromolecules. Thus, oxidative stress is associated with about 200 diseases of which can be highlighted: cardiovascular diseases, cancer, atherosclerosis, hypertension, ischemia (such as brain ischemia), diabetes mellitus, hyperoxaluria, neurodegenerative diseases (Alzheimer's and Parkinson's), depression, chronic inflammation, rheumatoid arthritis… culminating with a quicker aging (less longevity) (S.-I. Kim, Jung, Ahn, Restifo, & Kwon, 2011; S.-K. Kim & Himaya, 2011; Machu et al., 2015).

Antioxidants are chemical compounds that interact with and neutralize ROS, thus preventing them from causing damage to cells. Consequently, preventing associated diseases and slowing down aging can be achieved, in a certain degree, since they have other influences for their arising and development. The body produces some of the antioxidants, however it relies on the diet to obtain the rest of the antioxidants it needs. The intake of dietary antioxidants help to maintain an adequate antioxidant status, as proven scientifically. Antiaging (or longevity enhancing) properties also reflect antioxidant protection (Déléris et al., 2016; NCI, 2014).

Focusing on the protection of the nucleic acids, linked to oxidative stress inhibition as well, both of the following protective actions are difficult to separate and in many cases authors

attribute both terms to a certain compound or extract of seaweeds: antigenotoxic, prevents against diversified alterations at the genome level caused by endogenous or/and exogenous causes; antimutagenic, defines the protection directly against mutations in the DNA caused by internal or/and external sources. Both antigenotoxic and antimutagenic act as preventers of some carcinogenic events (Mesáro et al., 2014; Panieri & Santoro, 2016).

Further information about oxidative stress, antioxidant protection, and other genome protective actions is going to be described further in the chapter.

Recent studies showed that phycobiliproteins, such as phycoerythrin, display antioxidant properties, which could be beneficial in the prevention or treatment of neurodegenerative diseases caused by oxidative stress (Baweja et al., 2016). In rat astrocytes, a phycoerythrin derived from a *Grateloupia* sp. reduced ROS induced by hydrogen peroxide (H_2O_2) (Jung et al., 2016). In Sonani et al. (2014), phycoerythrin was used as a dietary supplement in *Caenorhabditis elegans*, which extended its longevity.

Sulphated polysaccharides have demonstrated their antioxidant potential *in vitro* and *in vivo*, being considered as cancer preventers (Jiménez-Escrig et al., 2011; Silva et al., 2013). Sulphated polysaccharides from a *Grateloupia* sp. demonstrated its potential in scavenging free radicals *in vitro* (Ye et al., 2015). Malondialdehyde, a marker of endogenous lipid peroxidation (caused by oxidative stress), was significantly reduced in the liver, heart, and brain of aging mammals by porphyrans from *Porphyra* spp. (Mohamed et al., 2012). Porphyrans from a *Porphyra haitanensis* also displayed antiaging effects in *Drosophila melanogaster* (Zhao, Zhang, Qi, & Li, 2007; Zhao, Zhang, Qi, Liu, & Li, 2008). Ulvans (from *Ulva* spp.) were found to have antioxidant activity as well (Silva et al., 2013). Sulphated polysaccharides' extracts from a *Fucus vesiculosus* exhibited antioxidant potential *in vitro* (Rupérez et al., 2002). Agar has been reported to affect the absorption of ultraviolet rays, thus protecting cells against genotoxic events, and alginates protect against potential carcinogens by clearing the digestive system and protecting the membranes of the stomach and intestine (Holdt & Kraan, 2011).

Epidemiological studies suggest that high mineral content in seaweeds must have accounted for the low prevalence of cancer in some countries of Asia (Mendis & Kim, 2011). It was proposed that I may act as an antioxidant (Brown et al., 2014). B displayed its antigenotoxic potential against ethyl methane sulfonate (EMS) in *Drosophila melanogaster* (Sarıkaya et al., 2016), and Se, presented antigenotoxic properties against potassium dichromate in *Drosophila melanogaster* as well (Rizki, Amrani, Creus, Xamena, & Marcos, 2001).

Fucoxanthin, β-carotene, and lutein found in seaweeds exhibit powerful antioxidant properties (Baweja et al., 2016; Škrovánková, 2011). The protective role of carotenoids is based on the effective supressing and prevention of ROS. Studies have shown the correlation between a diet rich in carotenoids and a diminishing risk of lung cancer (Holdt & Kraan, 2011; Mendis & Kim, 2011). β-carotene exhibited protective effects against the genotoxicity of doxorubicin (chemotherapy drug) in *Drosophila melanogaster* (Dias, Araújo, Dutra, & Nepomuceno, 2009). In *Drosophila melanogaster*, antiaging effects of fucoxanthin, β-carotene (Ekaterina et al., 2015), and lutein (Zhang, Han, Wang, & Wang, 2013) were exhibited. Antigenotoxic activities of chlorophyll a and chlorophyll b (in separate) against acrolein and malondialdehyde (in separate) were proved in *Drosophila melanogaster* (Esref Demir, Kaya, & Cenkci, 2013), and may play a significant role in cancer prevention (Mendis & Kim, 2011).

A study with diabetic rats administrated with ω-3 PUFAs revealed decreased oxidative stress in the cerebral cortex (Pan, Lai, Tsai, Wu, & Ho, 2012). Many studies have shown a reduction in the risk of developing prostate cancer when administrating ω-3 PUFAs (Haas-Haseman, 2015). Toorang, Djazayery, and Djalali (2016) stated that whether ω-3 PUFAs can reduce the oxidative damage is not clearly understood as well as their longevity enhancing activity.

Vitamin B12 prevented DNA damage induced by paclitaxel (anticancer drug) in human blood lymphocytes (Alzoubi, Khabour, Khader, Mhaidat, & Al-Azzam, 2014). It is believed that vitamin C prevents cancer by neutralizing free radicals before they can damage DNA and initiate tumor growth (Škrovánková, 2011). Antigenotoxic activities of vitamin C, in *Drosophila melanogaster*, were showed against acrolein, malondialdehyde (Esref Demir et al., 2013), γ-rays, chromium oxide (Olvera, Zimmering, Arceo, Guzman, & Rosa, 1995), and methyl urea plus sodium nitrite (Graf, Abraham, Guzman-Rincon, & Würgler, 1998). Vitamin C also regenerates vitamin E, which inhibits the oxidation of low density lipoproteins. High content of Vitamin E in a *Ulva rigida* proved its role in the prevention of cancer (Yildiz, Celikler, Vatan, & Dere, 2012). Vitamins B12 and C are particularly recommended for slowing down the effects of ageing (L. Pereira, 2011). In a study in *Drosophila melanogaster*, vitamin E displayed its capability to enhance longevity as well (Driver & Georgeou, 2013).

A great number of studies have shown the ROS scavenging potential of phenols, specially phlorotannins isolated from brown seaweeds (S.-K. Kim & Himaya, 2011). Therefore, they are as well associated with antiaging properties (Pan et al., 2012). Phlorotannins can absorb at short wavelengths and display a photoprotective role against UVB radiation (S.-K. Kim & Himaya,

2011; Zubia, Fabre, Kerjean, Lann, et al., 2009). Quercetin, a flavonoid present in seaweeds, demonstrated its antigenotoxic potential in *Drosophila melanogaster* against paraquat (Sotibrán, Ordaz-Téllez, & Rodríguez-Arnaiz, 2011).

Other studies showed antioxidant and other genome protective properties of seaweeds crude extracts. Different crude extracts from a *Gracilaria vermiculophylla* demonstrated antimutagenic activity in bacteria cultures (Osuna-Ruiz et al., 2016). Crude extracts from *Gracilaria gracilis* and *Grateloupia* sp. showed antioxidant potential *in vitro* (Jiang et al., 2013; Zubia, Fabre, Kerjean, & Deslandes, 2009). In zebrafish embryos, a *Porphyra umbilicalis* extract and a *Fucus vesiculosus* extract demonstrated UVB photoprotective activity (Guinea, Franco, Araujo-Bazán, Rodríguez-Martín, & González, 2012). Water extracts and solvent fractions from *Ulva rigida* showed remarkable antioxidant capacities *in vitro* in Yildiz et al. (2012) and Chernane, Mansori, Latique, and Kaoua (2014). Antigenotoxic (and antimutagenic) activity of crude ethanolic extracts of a *Ulva rigida* in human lymphocyte cell culture was proved against the effects of the chemotherapeutic agent mitomycine-C (Celikler, Yildiz, Vatan, & Bilaloglu, 2008). A high antioxidant activity of a extract of a *Fucus vesiculosus* was proven in an *in vitro* study (Peinado et al., 2014), and for other two extracts from a *Fucus vesiculosus* was proven the same in a cellular system and in mice (Zaragozá et al., 2008). The antigenotoxic potential of a *Fucus vesiculosus* aqueous extract in cultured human lymphocytes was proven against doxorubicin induced DNA damage (Leite-Silva, Gusmão, & Takahashi, 2007). In *Drosophila melanogaster*, a mixture of seaweeds containing a *Fucus vesiculosus* demonstrated antigenotoxic potential against the spontaneous genotoxic agents of *Drosophila melanogaster* and against a genotoxic, streptonigrin (SN) (Borges, 2014; Valente, 2014). In the same studies, the mixture of seaweeds containing *Fucus vesiculosus* also displayed antiaging effects.

1.1.6. Market of Wild Seaweeds vs. Aquaculture Seaweeds

In the last decades, the growing demand by commercial sector for big quantities of contaminant-free seaweeds has changed the industry (Cottier-Cook et al., 2016). The main techniques for obtaining seaweeds were summarized in wild harvesting but, with the need to fulfil the market needs, the main production of seaweeds started to occur in aquaculture (Holdt & Kraan, 2011; Lopes, 2014). In addition, some commercial seaweeds have been genetically engineered to produce disease-resistant strains in aquaculture (Cottier-Cook et al., 2016).

Currently, seaweeds are largely produced in IMTA (integrated multi-trophic aquaculture). IMTA can be defined as a system where effluents from intensive production of animals (e.g. fish or shrimps), potentially threatening environment, become resources for organisms that filter (e.g. mussels or oysters) or absorb (e.g. seaweeds) the nutrients (Cottier-Cook et al., 2016). According to Helena Abreu in *Público* journal relatively to IMTA: "One organism recycles the waste produced by another and transforms it into a product of value", being that the product of value can be seaweeds (Ferreira, 2014).

IMTA allows ecosystem service in the form of bioremediation and higher seaweeds yields (Abreu et al., 2015; Cottier-Cook et al., 2016). Another advantage of IMTA focuses on the human consumption of IMTA animals. E.g., ω-3 PUFAs are present in fish as a result of consuming primary producers like seaweeds (high concentrations of ω-3 PUFAs in IMTA fish), which are then passed to humans in the trophic chain (Kumari, Kumar, Gupta, Reddy, & Jha, 2010).

Accordingly to the global statistical collection from FAO (FAO, 2016), aquaculture production of seaweeds in the world had a significant increase from 2000 to 2014, contrarily to seaweeds wild harvest (Table 1.6). From all the (almost) 27 million t of seaweeds produced in aquaculture in 2014 (Table 1.6), there was a revenue of more than 5 thousand million €. Relatively to wild harvest, less than 1 million t of seaweeds were harvested in 2014 (Table 1.6) (FAO, 2016).

Table 1.6 - Differences between production values (in t) of seaweeds produced in aquaculture and wild harvested seaweeds (including marine areas and brackish waters) from the different continents and in the world in 2000, 2010, and 2014 (most recent data). The values were withdrawn from FAO's global statistical collections (FAO, 2016).

	AQUACULTURE QUANTITY (in t)			WILD QUANTITY (in t)		
LAND AREA	YEAR			YEAR		
	2000	2010	2014	2000	2010	2014
Africa	51 462	136 662	148 218	39 408	20 313	17 216
Americas	33 577	12 907	13 563	358 809	399 534	474 239
Asia	6 315 770	15 605 028	26 582 906	221 627	85 727	143 953
Europe	6 040	1 617	2 591	385 716	239 922	271 665
Portugal*	0	0	3	1 224	498	782
Oceania	16 424	13 705	25 133	14 594	2 332	2 679
Worldwide	6 423 453[a]	15 769 919	26 772 408	1 020 154	747 828	909 752

0 - data not available or a null value; * values included in Europe; [a] FAO estimate.

In 2014, more than 96% of seaweeds were produced in aquaculture worldwide, leaving the rest for wild harvested seaweeds. However, in Europe, more than 99% of marketed

seaweeds were harvested from natural resources (Table 1.6) (FAO, 2016; Fleurence, 2016). Currently, this percentage seems somewhat outdated due to the progression of aquaculture EU directives, which provide for the need to expand aquaculture production in European countries (Ferreira, 2014).

Asian countries were responsible for more than 99% of seaweeds global aquaculture production in 2014. Countries such as China, Indonesia, South Korea, Philippines, and Japan are producing the vast majority of seaweeds in aquaculture (Cottier-Cook et al., 2016; FAO, 2016). Accordingly to FAO (2016) and DLG Benelux (2016), Portugal produced about 3 t of aquaculture seaweeds in 2014, being *Ulva* spp. the major ones produced, as well as a few kilograms from red seaweeds.

In wild harvesting data from 2014, surprisingly, American continent dominated the market (Table 1.6). Chile was the one with more quantity of seaweeds captured, followed by China, Norway, Japan, and Indonesia (FAO, 2016). Europe was the second continent with more harvested seaweeds in 2014 (Table 1.6) (FAO, 2016). Norway, France, and Ireland dominate the European seaweeds harvest market, although Spain, Portugal, the UK, and others, also participate in this market (Peinado et al., 2014).

In 2014, Portugal's seaweeds wild harvest quantity (Table 1.6) was due (mainly) to the wild harvest of red algae (such as *Gracilaria* spp., *Gelidium* spp., and *Pterocladia* spp.) as raw material for agar production (DLG Benelux, 2016; FAO, 2016).

It was estimated that in 2012 about 9 million t of aquaculture seaweeds were used for direct human consumption (of about 25 million t of aquaculture seaweeds), mostly in East Asia, in product forms recognizable as seaweeds by consumers (not accounting for phycocolloids industry) (FAO, 2014). In Europe, on the other hand, seaweeds were primarily wild harvested for food industry. It is thought that about 90% of seaweeds (cultivated and wild) are used for all applications of food industry (FAO, 2014).

Gracilaria spp. and *Porphyra* spp. are included in the main five aquaculture seaweeds produced in 2014, with about 20% of total production derived from these seaweeds. Even though, *Grateloupia* spp., *Ulva* spp., and *Fucus* spp. were also produced in IMTA.

In Ria de Aveiro, only in 2012, the first and currently the only seaweeds aquaculture company in Portugal based on a IMTA system appeared under the name of ALGAplus (Abreu, Sousa, & Pereira, n.d.; Ferreira, 2014). The seaweeds *Gracilaria* (pendent DNA confirmation between *Gracilaria gracilis* and *Gracilaria vermiculophylla*), *Grateloupia turuturu*, *Porphyra umbilicalis*, *Ulva rigida*, and *Fucus vesiculosus* are produced in ALGAplus facilities. They

have some commercial brands available in Portugal which are associated with products containing seaweeds and seaweeds sold alone (e.g. Tok de mar® in Figure 1.9 B), as a measure of implementing seaweeds in the Portuguese diet.

1.2. Model Organism: *Drosophila melanogaster*

1.2.1. Main Characteristics

Drosophila melanogaster is an insect commonly found inhabiting rotten fruit, being its most well-known feature and primary food source in nature. Because of that, it is commonly known as fruit fly (Ong, Yung, Cai, Bay, & Baeg, 2014).

The fruit fly undergoes complete metamorphosis to reach adulthood (holometabolism), passing from an egg, through three larval stages (known as instars), to pupa, and ecloding as an adult fruit fly (or imago) (Figure 1.10) (Sandhyarani, 2016; Tyler, 2010). In laboratory (at 25 ºC), development time (time spent from egg to adult eclosion) has a duration of about 9 to 12 days (Figure 1.10) and mean longevity about 40 to 60 days, being dependent of other conditions as well: strain, humidity, light cycles, food constitution and quantity, diseases, among others (Jennings, 2011; Ong et al., 2014; Pandey & Nichols, 2011). Continuous exposure to temperatures above 25 ºC results in a quicker life cycle (high metabolism; decreased development time and longevity). Although, exposure to temperatures above 30 ºC may result in sterilization or even death. Contrarily, at temperatures lower than 25 ºC life cycle is prolonged (low metabolism; increased development time and longevity), but when temperature is too low (less than 16 ºC) the viability of flies could be impaired as well (Deepa, Akshaya, & Solomon, 2009; Dillon, Wang, Garrity, & Huey, 2009).

As other insects, *Drosophila melanogaster* remains immersed within the food source and feeds constantly during most of the larval phase, displaying what is known as foraging behaviour. Adults do not increase in size (less eating) since growth occurs almost exclusively during the larval development. During the third and final instar, larvae enter the designated wandering stage, characterized by cessation of eating and exiting the food to search for a suitable pupation site (Ainsley, Kim, Wegman, Pettus, & Johnson, 2008).

The amount and quality of nutrients consumed during developmental periods by fruit flies have a particular impact on life history traits. Therefore, the most important and often limiting nutrients for *Drosophila melanogaster* larvae nutrition are proteins (mainly yeasts) and carbohydrates (mainly sugars), which are the principle nutrients of rotten fruit. But lipids,

vitamins, minerals, and others, also play their part in enhancing fruit flies' health (Güler, Ayhan, Koşukcu, & Önder, 2015; Schwarz, Durisko, & Dukas, 2014).

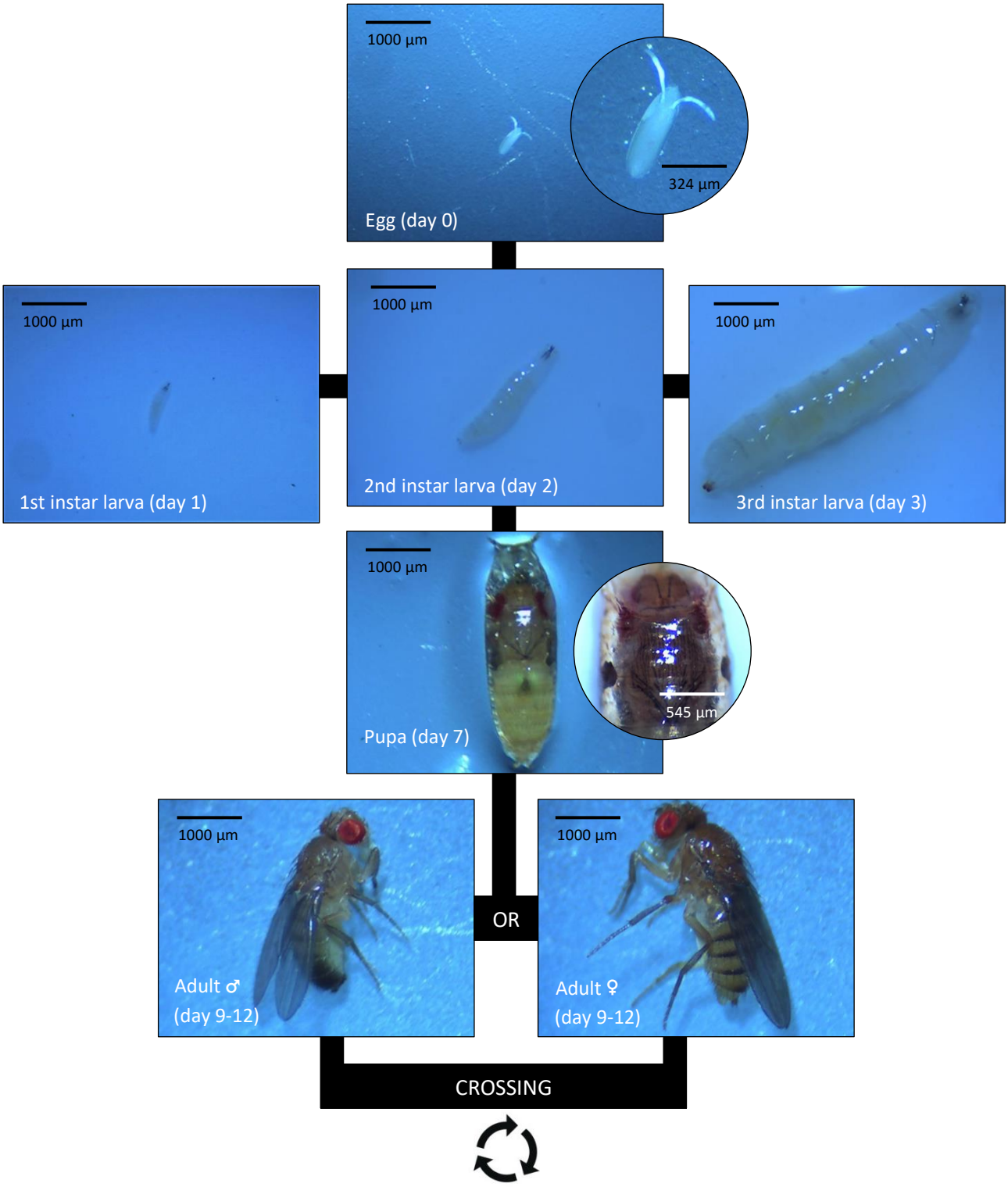

Figure 1.10 - *Drosophila melanogaster* life cycle at 25 ºC. The day off eggs laying is considered day 0 and the days at each stage (instars, pupa, and adult) represent the number of days since eggs were laid. After adults crossing the cycle restarts.

Why *Drosophila melanogaster* as a model organism?

Fruit flies have been used for over a century in research to explore behaviour, development and evolution, diseases, learning, nutrition… focusing largely on genetics, towards the extrapolation of results to superior animals and ultimately, humans (Sandhyarani, 2016). Being one of the favourites to be used as a model organism in diversified areas of scientific knowledge, the reasons for which *Drosophila melanogaster* has acquired this status are:

- It is easily cultured, being able to produce a large number of offspring per day (about 100 eggs per female) (Sandhyarani, 2016; The Berg Lab, n.d.);
- It has a quick developmental time and mean longevity, i.e., a short life cycle (Figure 1.10) (Gonzalez, 2013; Ong et al., 2014). Thus, a celerity of studies comparatively with other model organisms is verified (Pandey & Nichols, 2011; Sandhyarani, 2016);
- The small size allows it to be handled and studied with limited space conditions, being easily anaesthetised, and manipulated individually with unsophisticated equipment in a great battery of tests. In other words, easy and inexpensive laboratory maintenance (Flagg, 2005; Murillo-Maldonado & Riesgo-Escovar, 2016). Although, sophisticated techniques (more expensive) can be applied in *Drosophila melanogaster* experiments, including high-resolution microscopy of living cells and organs, and the inactivation or misexpression of almost any gene in a timely and tissue-specific or even cell-specific manner (Gonzalez, 2013);
- They exhibit sexual dimorphism and, in addition, virgin flies are physically distinct from mature adults, making it easy to separate them. Thereby, despite the small size, each individual is large enough for rapid notation of sex and sexual maturation with low magnification or even with the naked eye (Deepa et al., 2009; Flagg, 2005);
- *Drosophila melanogaster*'s genome (about 120 Mb) has been completely sequenced and published in Adams et al. (2000). It encodes about 13,600 genes on four chromosomes, only three of which carry the bulk of the genome (more than 95%). It is very small compared to human's genome (about 3008 Mb), making it easy to manipulate (International Human Genome Sequencing Consortium, 2004; Ong et al., 2014). Furthermore, several public-domain websites feature the complete genome of fruit flies for researchers to explore (Pandey & Nichols, 2011; Rand, 2010; Sandhyarani, 2016);
- *Drosophila melanogaster* and mammals have evolutionarily conserved genes, genetic pathways, and biochemical processes (Murillo-Maldonado & Riesgo-Escovar, 2016). Nearly 75% of human disease-causing genes have a functional homolog in the fruit fly (Gosslau, 2016;

Lyer et al., 2016). This allowed the understanding of the behaviour of many homologous genes and coded proteins (Sandhyarani, 2016);

- There are different phenotypes available for *Drosophila melanogaster* apart from wild-type specimens, including mutants for eyes, antennae, bristles, body colour, wings, among others. Taking into account the previous points, genomic manipulation with gene alteration makes it possible to generate diabetic fruit flies, with Alzheimer's, larger sized, more resistant to certain bacteria... repositories harbour mutant flies for almost every gene (Flagg, 2005; Murillo-Maldonado & Riesgo-Escovar, 2016);
- The use of insects does not entail ethical issues. Many individuals can be used in a single experiment contrary to, e.g., rodents and fish (Mishra, Srivastava, Agrawal, & Tewari, 2016). So, fruit flies minimise the use of higher animals (Stamenković-Radak & Andjelković, 2016).

1.2.2. Focusing on Eyes Development and Structure

The genetic conservation between humans and fruit flies starts in the genes that determine the body plan and development (Rajan & Perrimon, 2013). About two-thirds of the vital genes in *Drosophila melanogaster* have been estimated to be necessary for eye development. However, some genes are likely to be specific to eye development, other expressed genes in the eye are probably required for general cellular processes as well (Lyer et al., 2016). Therefore, a complex interaction of gene pathways is necessary for eye development and formation, in the way that, taking into account the nature of the research, only a simplistic version of the eye formation is going to be presented.

Many of the molecular events critical for *Drosophila melanogaster* embryogenesis occur during oogenesis. During oogenesis, maternal genes are responsible for the initiation of anterior-posterior and dorsal-ventral polarity. After fertilization, the axes of the embryo are specified, followed by the division of the early embryo into a repeating series of segmental primordia along the anterior-posterior axis. After the segmental boundaries are set, by the end of blastoderm stage, specification of the characteristic structures of each segment takes place. In the late embryogenesis, larval structures are set and groups of diploid cells of undifferentiated epithelium, imaginal discs, are formed in the embryo (Gilbert & Barresi, 2016; Ong et al., 2014). Imaginal discs start out as epidermal thickenings that then invaginate to become vesicles, but they never separate entirely from the epidermis (Tyler, 2010). In total, there are 19 discs in the larva, with 9 bilateral pairs that will form epidermal structures and a genital medial disc. The eye-antennal imaginal discs (a pair) will give rise to the compound eyes and the antennas.

In a newly hatched first instar larva the eye-antennal discs contain between 20 to 70 cells each. By mid-to-late first instar, disc cells resume mitosis and continue dividing exponentially during second and third instar stages. Thus, imaginal discs grow considerably during the larval stages (Gilbert & Barresi, 2016; Ong et al., 2014). During the third instar, a furrow, designated as morphogenetic furrow, initiates at the posterior end of the disc in direction to the anterior end, being responsible for sorting cell clusters in a pattern (in rows) and starting to differentiate them (Aydemir, Sevim, Celikler, Vatan, & Bilaloglu, 2009; Kumar, 2012). Then, it is in pupa that metamorphosis takes place. Initiation of metamorphosis is regulated by systemic hormonal signals, which are responsible to start the larval organs self-destructing (histolysis; most of the larval structures) and continuation of differentiation of eye units known as ommatidia (or facets), finishing with the differentiation into two eyes presented symmetrically in the head (Aydemir et al., 2009; Kumar, 2012; Tyler, 2010).

The adult eye of *Drosophila melanogaster*, together with adult eye structure information, is presented in Figure 1.11.

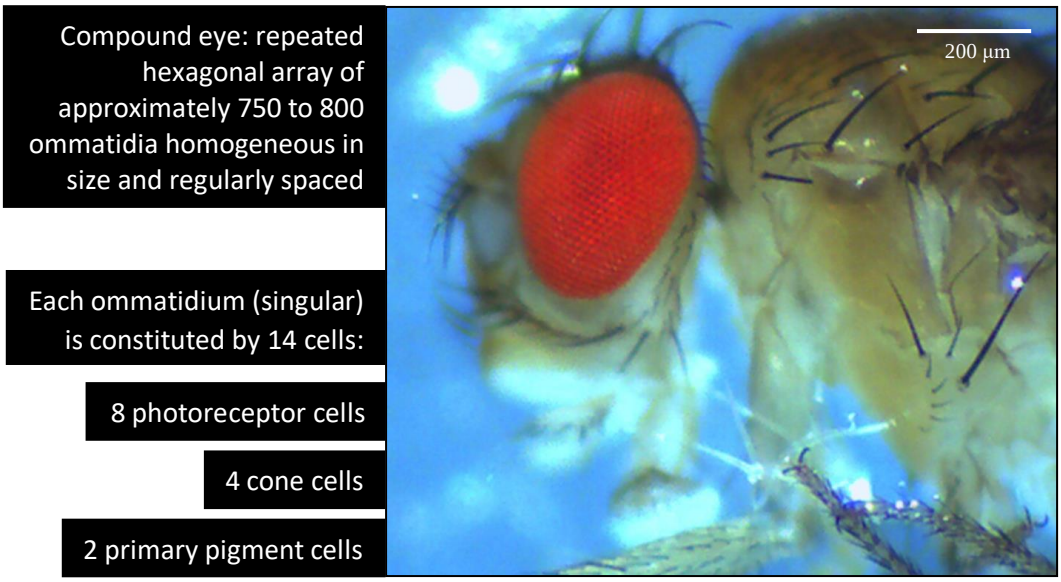

Figure 1.11 - *Drosophila melanogaster* wild-type eye (red eye). Each ommatidium is constituted by 14 cells, but shares 6 secondary pigment cells: 3 tertiary pigment cells and 3 mechano-sensory bristle complexes with its surrounding ommatidia. Information from Ong et al. (2014) and Lyer et al. (2016).

The adult eye is particularly used in toxicological assays because subtle defects in ommatidia development are amplified several hundred times in the entire eye (by mitosis) (Gonzalez, 2013). Thus, it is very easy to detect genetic alterations that slightly alter its structure or pigmentation (Nichols, 2006). Special mention must be done to the use of the developing

eyes of *Drosophila melanogaster* to identify genetic interactions in cancer-relevant genes (Gonzalez, 2013).

1.2.3. *Drosophila melanogaster* as a Model Organism for Toxicological Assays

The essential goal of modern medicine and public health is to prevent harm before it happens. This goal is only fully achieved with primary prevention, which requires harms identification prior to exposing. One of the options for achieving that is through toxicology. Toxicology is responsible for studying the effects of chemical compounds, drugs, food, herbicides… as well as physical factors such as radiation or temperature, in order not to compromise human health in the first place and secondly reducing the (negative) impact in other living beings (Mandrioli & Silbergeld, 2016; Mishra et al., 2016).

The main focused areas by toxicology are medical (and associated industries) and food industry, being that many chemical compounds, drugs, foods, and others can be tested for their toxicity. Although, toxicology is not only based in the search of harmful effects for health caused by toxics. Many substances have the opposite effect, they are beneficial for health and for that they, for the rest of the work, are going to be designated as antitoxics. Furthermore, the concentration is an essential parameter since antitoxics can become toxic with an excessive dosage. When the concentration of a substance is too low, there are not effects and an increase is needed to verify the toxic or antitoxic effects (Gosslau, 2016).

Toxicology can be divided in different subgroups. One of them is genotoxicology (or genetic toxicology) that is responsible for the toxicological impacts directly into the genome. Like the large group where it is inserted, genotoxicology is not used only to detect the defects caused by a substance in DNA, that is responsible for developmental defects, cancer initiation… but is also applied in the genome protective effects that a substance can have. Harmful impacts in the genome are caused by genotoxics, contrarily to antigenotoxics that, in some degree, benefit the individual by protecting its genome, as shown in the previous subchapter. Genotoxics and antigenotoxics can be, as well as toxics and antitoxics, drugs, food, their chemical compounds, and others (Lombardot et al., 2015; Mishra et al., 2016).

Toxic side effects, including genotoxic ones, are a major reason for the delay or termination of drug development, food supplements development, among others, as well as reducing sales or even prohibiting the marketing of food, food supplements, drugs, and others that exhibit the genotoxicity. In contrary, when antitoxic effects (including antigenotoxic) are proved, products in development get to be commercialized and the ones already in the market

with these characteristics are much more required. I.e., to commercialize these products, toxicological assays must be done (Gosslau, 2016; Lombardot et al., 2015).

Drosophila melanogaster is currently being used as one of the preferable model organisms for toxicological studies, specifically in genotoxic ones (Mishra et al., 2016). In these studies, it is emphasized the high level of conservation between *Drosophila melanogaster* and humans in many aspects, not only in individual domains and proteins, but also in entire complexes and multistep pathways. Specially, metabolic pathways responsible for dietary input are conserved between humans and fruit flies. Many of the analogous organ systems that control nutrient uptake, digestion, absorption, storage, and metabolism in humans are present in fruit flies (Lemaitre & Miguel-Aliaga, 2013; Zhang et al., 2013).

Drosophila melanogaster applications in Toxicology and Genotoxicology

When *Drosophila melanogaster* is exposed to toxics, life traits are perturbed negatively. Although, antitoxics consumed during developmental periods have a positive effect in the organism. Thus, parameters such as development time, survival (number of ecloded individuals), sex ratio (ratio between the number of ecloded individuals of each sex), adult body size, and others, can be assessed in *Drosophila melanogaster* as a way of measuring the non-specific toxicity/antitoxicity of a drug, food, chemical compound, or other, in the development phase (Güler et al., 2015; Neethu, Babu, & Harini, 2013).

In recent years, researches started to use *Drosophila melanogaster* to understand the relationships between food metabolism and developing of diseases and ageing, specifically, how food can prevent them or, at least, delay them (Fernández-Bedmar et al., 2011; Murillo-Maldonado & Riesgo-Escovar, 2016).

Specific damage to the genetic material is often the cause of various diseases such as cancer. Antigenotoxicity researches in *Drosophila melanogaster* with bioactive compounds capable of preventing and/or in some degree help in cancer therapeutics are rising (Ong et al., 2014; Prakash, Hosetti, & Dhananjaya, 2014). Genotoxicological studies with *Drosophila melanogaster* deal with the assessment of alterations in genetic material through various assays like: germ line mutation assays, somatic mutation assays, chromosomal aberration assay, micronucleus assay, comet assay, and other DNA sequence based assays. In particular, somatic mutation and recombination test (SMART) has proven to be a good tool for detecting a broad range of genetic alterations quickly and inexpensively (Eşref Demir, Kaya, Marcos, Cenkci, & Çetin, 2013; Stamenković-Radak & Andjelković, 2016).

Originally, SMART was assayed with four different tests, but only two of them made it through to the present days: the wing-spot test and the eye-spot test (Marcos, Sierra, & Gaivão, 2014). The wing-spot test was first described by Graf et al. (1984) and eye-spot test by Vogel and Zijlstra (1987). Both have shown to be assays with high values of sensitivity, specificity, and accuracy.

The imaginal discs are exposed to genotoxic agents, if one genetic alteration occurs in one of these discs, this alteration will be present in the daughter cells and will form a group of mutant cells. Then, they can be phenotypically manifested in the adults in structures such as the wings and the eyes, which can be assessed according to the wing-spot test and the eye-spot test, respectively. This can be accomplished when using genetic markers (for eyes or for wings), by detecting in heterozygous individuals the loss of heterozygosity (LOH) for those markers in the respective adult tissue (Marcos et al., 2014; Prakash et al., 2014; Vlastos et al., 2015).

Drosophila melanogaster longevity assays are a common direct method to measure the rate of aging. The main objective of this assay is to check if the effect of a certain substance (e.g. food) causes negative (toxic) or positive (antitoxic) responses of the organism throughout its life, being able to reduce longevity or enhance longevity, respectively. The effects of genotoxicity/antigenotoxicity are also reflected in the longevity of individuals (Güler et al., 2015; Neethu et al., 2013; Zhao et al., 2007).

1.3. Oxidative Stress in (Geno)Toxicology

As stated previously, oxidative stress is defined as the overproduction of ROS in an organism, being a consequence from endogenous and exogenous factors, that exceeds the removal availability of antioxidants. Although, other reactive species such as reactive nitrogen species (RNS) can, as well, be capable of acting together with ROS and oxidizing all major biomolecules. Together they are known as electrophilic species (ES), but ROS are the major ones responsible for the damage manifested in cells (Burton & Jauniaux, 2011; Gosslau, 2016).

ROS are produced in the reduction of O_2 to H_2O. ROS is a collective term that includes both oxygen radicals (free radicals), such as superoxide anion radicals (O_2^-) and hydroxyl radicals (OH) and some nonradicals that are oxidizing agents and/or are easily converted into radicals, such as H_2O_2 (Burton & Jauniaux, 2011; Gosslau, 2016).

The first ROS member produced after an assimilation of an electron (e^-) by O_2 is O_2^-. The reduction of O_2^- origins a fairly stable ROS member, H_2O_2. Addition of two more e^- to H_2O_2 produces H_2O. The major problem appears when O_2^- and H_2O_2 react with one another by Haber-

Weiss reaction resulting in the highly toxic OH radical, producing as well an hydroxide ion (OH^-) and O_2:

$$O_2^- + H_2O_2 \rightarrow OH + OH^- + O_2$$

Additionally, heavy metals such as iron (Fe; major one), copper (Cu), zinc (Zn), and others, can generate OH radical through Fenton reaction by adding a single e^- to H_2O_2. E.g., ferrous Fe (Fe^{2+}) adds an e^- to H_2O_2, resulting in OH, OH^-, and ferric Fe (Fe^{3+}):

$$H_2O_2 + Fe^{2+} \rightarrow OH + OH^- + Fe^{3+}$$

OH is the most genotoxic species, being the major responsible for the damages on macromolecules. Therefore, the amount of OH radicals formed in a cell depends on ROS generation (Haber-Weiss reaction), but also on the amounts of reduced metal ions for the Fenton reaction to occur (Figure 1.12) (Gosslau, 2016; Luisa & Giorgio, 2015; Nimse & Pal, 2015).

Through the reading of the previous subchapter, it was possible to infer that ROS is capable of causing changes to all humans' macromolecules (as well as in *Drosophila melanogaster*'s): proteins, carbohydrates, lipids, and nucleic acids.

The oxidation of amino acid residues, the subsequent formation of protein aggregates by crosslinking, and the production of protein fragments may result in loss of activity and inactivation of enzymes and metabolic pathways, finally ending up with cell death. All ROS-induced damages may cause malfunctioning of enzymes, transporters, signal transducers, or structural proteins. Lipid peroxidation of cell structures containing lipids can lead to the generation of different toxic products, including alcohols, alkanes, aldehydes, ethers, and ketones which have the potential to contribute to cell damage, necrosis, or apoptosis. Nucleic acids are delicate targets of ROS leading to genetical alterations. Damage induced by ROS may result in single and double strand breaks, DNA-DNA or DNA-proteins crosslinks, or numerous base modifications such as 8-hydroxydeoxyguanosine (8-oxo-dG), 5-hydroxymethyluracil (5-HmU), 8-hydroxydeoxyadenosine (8-oxo-dA), and thymine glycol. When ROS-induced DNA damage is to high leads to p53 activation, growth arrest, and apoptosis. The mitochondrial DNA (mtDNA) is particularly susceptible to oxidative damage because of the absence of associated histones, an incomplete mitochondrial DNA repair system, and when mtDNA is already damaged by ROS, the generation of more ROS through electron leakage from the electron transport chain (ETC) takes place. Furthermore, carbohydrates oxidation may also be involved in DNA damage. Oxidation and fragmentation of deoxyribose is believed to play a major role

in mutations by blocking the action of DNA polymerase and DNA ligase (Gosslau, 2016; S.-I. Kim et al., 2011; Rizzo et al., 2010).

With time, unrepaired oxidative damage is expected to accumulate and put at risk the homeostasis in the organism, i.e., to affect development, to provoke diseases (such as cancer), triggering cell death and aging, culminating in a precocious death (Bourg, 2001).

In general, ROS is generated from mitochondria, peroxisomes, and cytoplasmic enzymes (e.g. NADPH oxidases), as a result of intracellular metabolism (endogenous causes). Among these, ETC has been considered as the main source of ROS (Cui, Kong, & Zhang, 2012; Pan et al., 2012).

Numerous studies showed that increased ROS production occurs after the metabolism of exogenous toxic substances by the organism (Ong et al., 2014). Although the mechanisms leading to toxic effects in humans and *Drosophila melanogaster* are multifactorial, the majority of toxic effects appear to converge on the generation of ROS. Whereas a variety of toxics generate ROS directly, others induce a secondary response leading indirectly to generation of ROS by immunocompetent leukocytes (chronic inflammation). These and other observations strengthen the hypothesis that toxics leading to oxidative stress play central roles in generalised toxicity, non-hereditary cancers, and aging (Gosslau, 2016).

1.3.1. Impact of Oxidative Stress in Carcinogenesis and Aging

Carcinogenesis is a multi-step process responsible for the formation of cancer. Simplistically, cancer arises due to a series of somatic mutations that accumulate within the nucleus of a cell which alters the genome integrity, enabling the cell to proliferate in an unregulated manner (Gosslau, 2016; Hyndman, 2016).

According to several researches, including in *Drosophila melanogaster*, oxidative stress can be the major cause of mutations occurred in genes regulating cell cycle, resulting in cancerous cells (Gosslau, 2016; Schumacker, 2015). Continuous cellular damage induced by ROS can promote carcinogenesis directly, as well as indirectly by promoting chronic inflammation that triggers a cascade of oncogenic events (Kakehashi, Wei, Fukushima, & Wanibuchi, 2013; Panieri & Santoro, 2016). Oxidative stress can also affect the phenotypic behaviour of the (already) cancerous cells and compromise their responsiveness to therapeutic interventions (Schumacker, 2015). Furthermore, tumor cells display genetic alterations that promote a continuous and elevated production of ROS. Whereas such oxidative stress conditions would be harmful to normal cells, they facilitate tumor growth in multiple ways by

causing DNA damage and genomic instability, and ultimately, reprogramming cancer cell metabolism (Panieri & Santoro, 2016).

As stated before, the ETC is the major contributor in the organism for ROS production. In various types of human cancers, a growing body of evidence has demonstrated the presence of both somatic and germline mutations in mtDNA as a leading cause for cancer initiation. These studies suggest that one of the major DNA base modifications in mtDNA, 8-oxo-dG, causes problems in the assembly or either the function of the ETC, which increases the amounts of O_2^- and/or H_2O_2 by electron leakage in cells, which triggers more damage to mtDNA. It becomes a cycle with increasing damage to mtDNA and more ROS present in cells which, subsequently, can lead to the development of carcinogenic events (Cui et al., 2012; Peng, Chan, Huang, Yu, & Chen, 2011).

Progressive accumulation of damaged and defective cellular components, loss of cell and organ physiological function, failure of physical activity, among others, occur during aging process (Huangfu et al., 2013; Pan et al., 2012). There are many theories of aging that have been proposed to explain the phenomenon. However, no single theory is able to account for all parameters of aging (Pan et al., 2012).

Aging research has advanced rapidly by using model organisms such as *Drosophila melanogaster*. Thus, genes displaying a profound influence in aging have been successfully identified. The interesting aspect is that those longevity genes identified in *Drosophila melanogaster* are also found in humans' homologs (Luisa & Giorgio, 2015; Pan et al., 2012).

Although genetics has its share of responsibility for ageing, oxidative damage is believed to be a major contributor to the functional decline associated with aging, accordingly to free radical and mitochondrial theories of aging. ROS causes nuclear DNA damage (including damage to "longevity genes") that trigger DNA-damage response through p53-dependent pathways that leads to cell cycle arrest, apoptosis, and cellular aging. Another ROS effect is irreversible protein oxidation and aggregation. The accumulation of oxidative intracellular protein aggregates is known to result in loss of cellular function associated with aging pathology in many organisms. The oxidation of lipids and carbohydrates also contributes to aging process (Luisa & Giorgio, 2015; Peng, Chan, et al., 2011).

A strong negative correlation of longevity with the rate of mitochondrial ROS generation is an aspect that relates aging to ROS undoubtedly (Luisa & Giorgio, 2015). Several mitochondrial alterations have been described with aging, including reduced synthesis of mitochondrial proteins, reduced activity of oxidative enzymes, and lower mitochondrial mass.

So, it is widely accepted that mitochondrial respiratory capacity declines during the aging process. In agreement with the free radical theory of aging, increased 8-oxo-dG content in mtDNA and increased ROS production are frequently detected in aged tissues, suggesting that progressive accumulation of oxidative DNA damage is a contributory factor to the aging process. Furthermore, the terminal oxidoreductase of ETC, cytochrome c oxidase (CcO), shows an age-related decline in *Drosophila melanogaster* but also in vertebrates such as rats. CcO deficiency leads to the reduction of total ETC activity, resulting in increased amounts of either O_2^- and/or H_2O_2 in mitochondria, that ultimately causes cell death and ageing of the organism (Ghosh et al., 2011; Peng, Chan, et al., 2011; Peng, Zuo, et al., 2011).

1.3.2. Antioxidant Defence

As the proven idea that oxidative stress initiates toxic events such as cancer and drives its progression, reduces the longevity by a quicker ageing ratio, and so, fuelled a long-standing interest of discovering new and better antioxidants, antigenotoxics, antimutagenics... When a toxic substance hits the organism, in a more direct or less direct way, the major function is to defend the organism from ROS damaging to cells (Schumacker, 2015).

Counteracting measures were developed by the organism to act at the different stages of ROS formation and propagation. To avoid ROS high concentrations, an organism responds by increasing the activity of its antioxidant defence system, which comprises both enzymatic and non-enzymatic sources. Despite different mechanisms of action, the principal antioxidant mechanism is based in the preventive action that can supress ROS formation and propagation by scavenging ROS and eliminate them (Aguiar, Figueira, Gottschalk, & Rosa, 2016; Held, 2015; Panieri & Santoro, 2016).

Reduction of O_2, passing through the intermediate forms, to H_2O, can be done spontaneously in ETC. However, certain enzymes can enhance the speed of the transformations many times (Cui et al., 2012; Lushchak, 2011). All the enzymatic defences have a transition metal at their core capable of taking on different valences as they transfer e^- to ROS during the detoxification process (Burton & Jauniaux, 2011). The most relevant antioxidant scavenging enzymes include: superoxide dismutase (SOD), catalase (CAT), peroxidases such as glutathione S-transferases (GSTs), glutathione reductase (GR), and glutathione peroxidase (GPx). In insects, although GR and GPx are absent, GSTs have been suggested to compensate for it (Kakehashi et al., 2013). Furthermore, the enzymatic detoxification systems of toxic

substances are similar between *Drosophila melanogaster* and mammals (Gaivão, 1999). Their functions as antioxidants are (Figure 1.12) (Lü, Lin, Yao, & Chen, 2010; Nimse & Pal, 2015):

- SOD performs a protective function in the cell by scavenging O_2^- and catalyse it to H_2O_2 and O_2:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

- CAT converts H_2O_2 to H_2O and O_2, and thus completes the detoxification initiated by SOD:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

- Glutathione peroxidases such as GSTs, GR, and GPx scavenge and catalyse the degradation of H_2O_2 using the reducing power derived from glutathione (GSH), a tripeptide, producing oxidized GSH (GSSG) and H_2O:

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

It is clear that the antioxidant enzymes play a major role in the prevention of oxidative damage (Nimse & Pal, 2015). Even though, living beings have not developed specific enzymatic systems for OH detoxification, probably because OH is short-lived and, therefore, has a short diffusion distance. One way of limiting its formation is by scavenging O_2^- and H_2O_2 and catalyse their transformation into H_2O so that Haber-Weiss and Fenton reactions do not occur (Bourg, 2001; Lü et al., 2010; Pan et al., 2012).

Antioxidant enzymes can be a good source to prevent carcinogenesis. Stimulation of the genes that produce these scavenging enzymes is a crucial action for cells' health. However, after cancerous cells are settle in, the action of the "antioxidant genes" is no longer so simple because studies showed that an increased antioxidant ability occurs in cancer cells. Completely contradictory this increase, can promote tumor growth by reprogramming metabolism. Further studies are needed to understand this relationship (Panieri & Santoro, 2016).

Longevity depends upon "longevity genes" regulation as well as in the expression of enzymes involved in antioxidant activity (Paaby & Schmidt, 2009). In accordance with the free radical theory, increasing or decreasing the activity of these enzymes should increase or decrease longevity, respectively (Bourg, 2001).

Endogenous non-enzymatic antioxidants also play an important role in antioxidant defence (Panieri & Santoro, 2016). They include GSH, coenzyme Q10, alpha lipoic acid, ferritin, lactoferrin, uric acid, bilirubin, metallothionein, L-carnitine, melatonin, and others, to maintain physiological function (Rizzo et al., 2010). One of the most important is GSH since

its primary function is to support enzymes that directly scavenge H_2O_2, such as GSTs, GR, and GPx (Held, 2015; Panieri & Santoro, 2016). GSH and others (including dietary antioxidants) can directly scavenge and neutralize OH, but also O_2^- and H_2O_2 (Lü et al., 2010; Lushchak, 2011). GSH scavenging activity can directly expedite the repair of ROS-induced damage (Nimse & Pal, 2015; Schumacker, 2015). Using metal ion chelators to prevent Fenton reaction to occur is another efficient way to protect cells against deleterious OH effects. As examples, ferritin and lactoferrin are proteins that chelate Fe, and metallothionein is an important storage protein for Cu and Zn (Figure 1.12) (Lü et al., 2010; Lushchak, 2011).

Natural dietary antioxidants, antigenotoxics, and so (such as those present in seaweeds), are of particular interest for humans since they are only obtained from food and may be potential agents for human cancer prevention. Cancer prevention is regarded as a promising avenue for cancer control. This strategy is based on the reduction of cancer incidence level by increasing the public consumption of antioxidants, antigenotoxics, antimutagens, and others that limit ROS accumulation. The most common antioxidant mechanisms of dietary compounds are scavenging and giving e^- or protons (H^+) for ROS conversion, and chelating metal ions responsible for Fenton reaction (metal chelators) (Mezzoug, Abrini, Serano, Alonso-Moraga, & Idaomar, 2006; Panieri & Santoro, 2016). Antioxidants consumption plays, as well, a crucial role in modulating the longevity of an organism. Various animal studies with vertebrate and invertebrate models, such as *Drosophila melanogaster*, suggested that supplementing the regular diet with health-promoting bioactive compounds through supplements and functional foods, can promote the delay of the aging process by modulation of enzyme activity and direct antioxidant scavenging activity, and avoid certain age-related diseases (Figure 1.12) (Galenza, Hutchinson, Campbell, Hazes, & Foley, 2016; May, Doroszuk, & Zwaan, 2015). On the other hand, calorie restriction has been shown to extend longevity in various model animals (Peng, Chan, et al., 2011). Many data demonstrate that calorie restriction is stimulated by restricting specific nutrients and consume others (such as antioxidants) instead of limiting total energy intake (Tatar, Post, & Yu, 2014). Over the past century, due to the improvement of the nutrition quality, and the advancements in medicine, the life expectancy of humans has significantly increased (Pan et al., 2012).

It is expected that the formation rate and accumulation of damage does not depend only on the activities of the different protective mechanisms naturally present in cells, but also on the capacity of cells to repair damage (Figure 1.12) (Gaivão & Comendador, 1996; Galenza et al., 2016). Endogenous and exogenous antioxidant systems work in parallel to terminate the

propagation of ROS reactions, limit the formation of new ROS, and stimulating repair mechanisms, in order to prevent (some) cancer events and other diseases influenced by oxidative stress, and slowing down the aging process (Zhang et al., 2013).

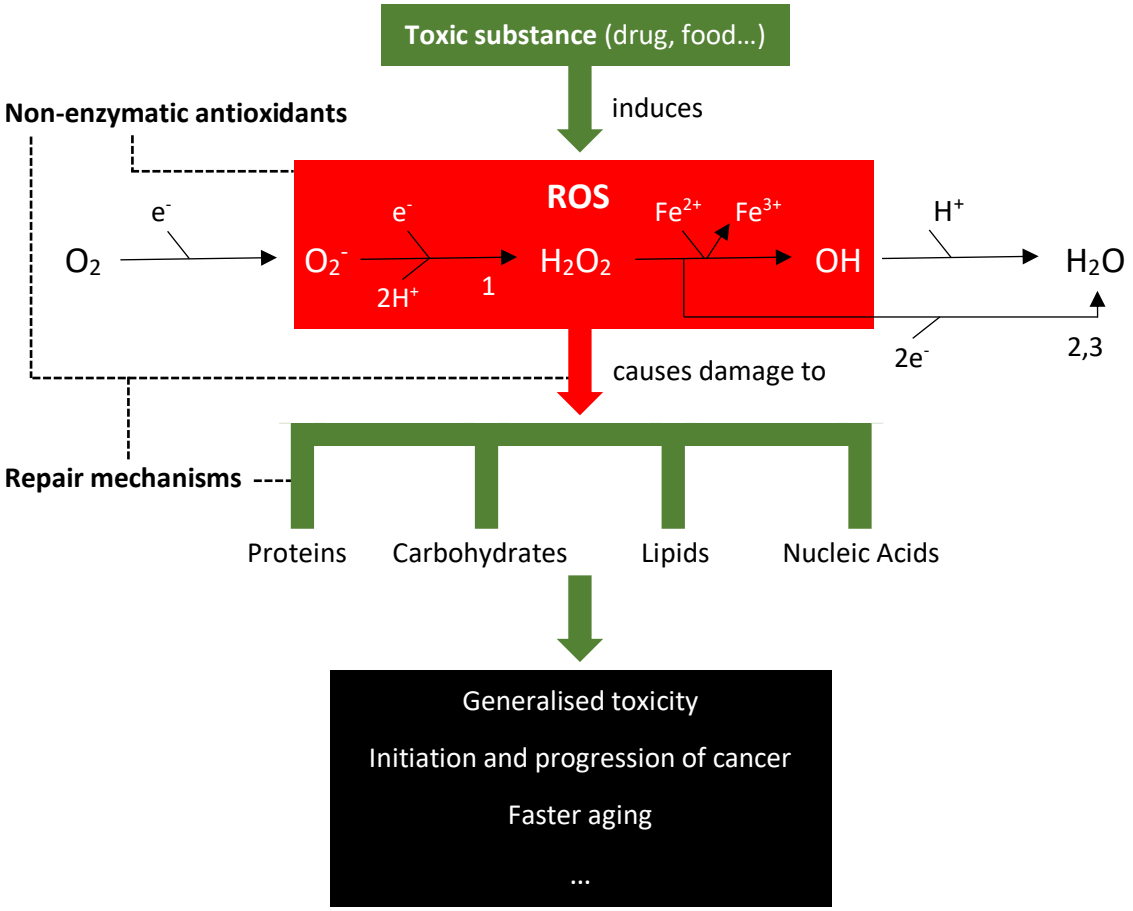

Figure 1.12 - Simplistic summary of the formation and impact of excessive ROS in cells induced by a toxic substance. In this case, the antioxidants (endogenous and exogenous) are not capable of avoiding excessive ROS formation and progression, as well as communicating to repair mechanisms for repairing all damages in macromolecules. Thus, the damages to macromolecules can promote: generalised toxicity, as in *Drosophila melanogaster* with the increase of development time or even kill developing larvae; cancer initiation and progression with all the damaging mutations; enhance the rate of aging, which culminates in a precocious death (less longevity); other impacts include developing of other oxidative stress related diseases.
1 - SOD; 2 - CAT; 3 - Glutathione peroxidases.

1.3.3. Streptonigrin: A Genotoxic Agent

SN is a chemical compound ($C_{25}H_{22}N_4O_8$) with the CAS number 3930-19-6, isolated from *Streptomyces flocculus*, a species of bacteria. It was firstly extracted in 1959 and, during 1960s and 1970s a broad-spectrum of anticancer activities from SN was discovered. In the late 1970s, reached phase II clinical trials, which proved to be on the right track for the medical application as an anticancer antibiotic, a type of anticancer drug that blocks cell growth by interfering with DNA (Donohoe, Jones, & Barbosa, 2011; Donohoe et al., 2013). SN showed

anticancer activity against a wide range of cancers including breast, lung, head, neck, and skin (Bolzán & Bianchi, 2001). However, allied to the great potential as a chemotherapeutic drug, was an unavoidably high degree of toxicity to the healthy cells, which caused the cessation of the trials. Its most common toxicity is by genotoxicity events (Deepa, Akshaya, & Solomon, 2011; Donohoe et al., 2013). With regards to the damages that could be caused to the remaining macromolecules, its potential has never been much explored.

Genotoxic effects of SN include the formation of DNA adducts. In the presence of certain metal cations (Zn^{2+}, Cu^{2+}, Fe^{2+}, manganese - Mn^{2+}, cadmium - Cd^{2+} and gold - Au^{2+}), SN binds to DNA forming SN-metal-DNA complexes (DNA adducts). The reduction of the quinone of SN occurs, via one or two e^- with NADH as a cofactor, forming a semiquinone or hydroquinone intermediate, respectively. Semiquinone reacting with O_2 produces O_2^- and regenerates quinone. Relatively to hydroquinone, it can give rise to H_2O_2, while quinone is regenerated (Figure 1.13). Then, H_2O_2 through a Fenton-type reaction catalysed by the metal cation produces OH. O_2^- can react with H_2O_2 and generate OH as well (Bolzán & Bianchi, 2001; Donohoe et al., 2013; Troxell, Xu, & Yang, 2012). SN multiple metal complexation sites and structural elements responsible for the toxic activity include the two pyridyl nitrogen in rings B and C, the C ring carboxylic acid, and the 7-aminoquinoline-5,8-dione in AB ring system (Bolzán & Bianchi, 2001; Donohoe et al., 2013).

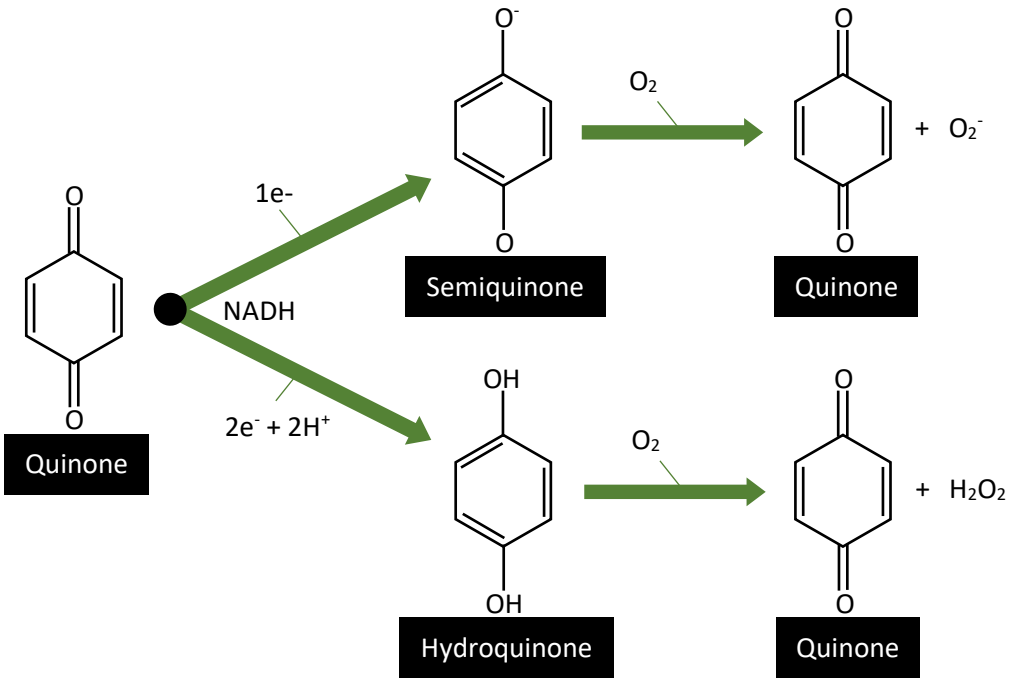

Figure 1.13 - Quinone class from SN and its reduction to semiquinone or hydroquinone (with the respective chemical structures) that upon reacting with O_2 leads to quinone regeneration and ROS production.

The prolonged SN linkage to DNA itself and ROS continuous production (derived from the linkage) can inhibit the synthesis of DNA and RNA, induce unscheduled DNA synthesis, and promote DNA strand breaks. Additionally, SN inhibits topoisomerase II by stabilizing cleavable complexes (Deepa et al., 2011; Gaivão, Sierra, & Comendador, 1999). Topoisomerase II is an essential enzyme evolutionary conserved in eukaryotes and essential for chromosome segregation during mitosis (Bolzán & Bianchi, 2001; Nature, n.d.; Pommier, Sun, Huang, & Nitiss, 2016). These genotoxic events of SN can lead, as several studies showed, to mutations that can give rise to large-scaled chromosomal aberrations (such as mitotic recombination). These actions create genomic instability in cells and, consequently, may develop into carcinogenic events (Bolzán & Bianchi, 2001), counteracting its potential as an anticancer drug.

In *Drosophila melanogaster*, the genotoxic effects of SN where first proved by Gaivão et al. (1999) in SMART assay with seven other ROS-inducer compounds (menadione, plumbagin, juglone, paraquat, diquat, tert-butyl hydroperoxide, and 4-nitroquinoline 1-oxide), where SN showed the greatest genotoxic activity with the lowest lethal toxicity.

Prevention of SN-induced DNA Damage

As SN imposes an oxidative stress in cells, which leads to the emergence of genome damage, theoretically and as showed before, endogenous and exogenous antioxidants should supress the genotoxic activity of SN. In a research from Testoni, Bolzán, Bianchi, and Bianchi (1997), the addition of enzymatic antioxidants SOD and CAT, and a dietary antioxidant (mannitol) in Chinese hamster ovaries, decreased the genotoxic capacity of SN. This research showed the power of ROS inhibition in the decreasing of DNA damaged by SN, confirming that oxidative stress is the major responsible for SN-induced DNA damage (Gaivão et al., 1999).

As stated previously, SOD applies its protective effect on DNA by eliminating the O_2^- generated during SN reduction. CAT by removing H_2O_2 avoids the Fenton reaction and OH production which are responsible for the major damage to DNA from SN (Troxell et al., 2012).

Metal chelators were also found to prevent DNA damage by preventing the SN-metal-DNA association (Bolzán & Bianchi, 2001; Ming, 2003). The toxicity of SN can be dramatically reduced when a Fe^{2+} chelator is added to cells, which supports the involvement of metal ions in the action of SN (Ming, 2003; Troxell et al., 2012).

In Borges (2014) and Valente (2014), the antigenotoxicity of products used in thalassotherapy were shown against SN in *Drosophila melanogaster*.

Chapter 2 - Objectives

The potential of seaweeds is still underexploited worldwide. More studies are needed to better understand the biomedical and nutritional potential of seaweeds, so that their use in food industry becomes more frequent among western countries.

There are a few published studies focusing seaweeds beneficial effects, but are carried out mainly *in vitro*. With special attention to their toxicological effects, including the protective actions such as antioxidant, antigenotoxic, antimutagenic, among others, the number of studies is more restricted. Some of these researches are based on the evaluation of specific compounds of those seaweeds. Although necessary for the discovery of the effects caused by each compound, they may not represent the actual effect when administered together with the other chemical compounds of a seaweed. Extracts are also widely used, and often "try" to represent the seaweed in its whole or part of it, however, quantitative and qualitative loss of compounds may occur. Concerning toxicological researches focusing on using seaweeds in its whole (full intake), no published studies were found.

Thus, it was decided to take advantage of the gap regarding to *in vivo* evaluation directed to the impacts of the full intake of seaweeds, to test the toxicological effects of these aquatic resources. A *Gracilaria* sp. (pendent DNA confirmation between *Gracilaria gracilis* and *Gracilaria vermiculophylla*), a *Grateloupia turuturu*, a *Porphyra umbilicalis*, a *Ulva* sp. (pendent DNA confirmation between *Ulva lactuca* and *Ulva rigida*), and a *Fucus vesiculosus* were collected from the Portuguese coast and tested in *Drosophila melanogaster*, a model organism whose application in the toxicological area is largely described and approved but very little explored concerning seaweeds.

Assuming the use of the five wild seaweeds and the evaluation of their toxicological effects on *Drosophila melanogaster* with the purpose of demonstrating their potential as functional food as the main objective of this work, it was necessary to consider the following specific objectives:

1) Evaluate the toxicological effects of the five seaweeds in an initial toxicological screening through the verification of *Drosophila melanogaster*'s development time, survival, and sex ratio;

2) Determine the impact on *Drosophila melanogaster*'s longevity when exposure to the two wild seaweeds with the most promising initial screening results;

3) Demonstrate the genotoxicological effects of the two wild seaweeds with the most promising results from initial screening (but only the two best concentrations per seaweed) in order to understand seaweeds function in cancer prevention.

Chapter 3 - Materials and Methods

3.1. Seaweeds

3.1.1. Seaweeds Harvest

Five wild seaweeds were harvested in the Portuguese coast, namely:

- A *Gracilaria* sp. (pendent DNA confirmation between *Gracilaria gracilis* and *Gracilaria vermiculophylla*),
- A *Grateloupia turuturu*,
- A *Porphyra umbilicalis*,
- A *Ulva* sp. (pendent DNA confirmation between *Ulva lactuca* and *Ulva rigida*),
- A *Fucus vesiculosus*.

The sites of predominance of each seaweed and the identification of each one was only possible with the accompaniment and advice of members of the company ALGAplus. Thus, the red seaweed *Porphyra umbilicalis*, the green seaweed *Ulva* sp., and the brown *Fucus vesiculosus*, were all identified and harvested from Mindelo beach (41º18'36.8"N 8º44'25.9"W), Vila do Conde, Porto. Relatively to the other two red seaweeds, the *Gracilaria* sp. was identified and collected from Torrão do Lameiro (Ria de Aveiro; 40º49'33.1"N 8º39'58.2"W), Ovar, Aveiro, and the *Grateloupia turuturu* was from Aguda beach (41º02'53.7"N 8º39'14.5"W), Gaia, Porto. Seaweeds were collected between the end of September and the beginning of October of 2015.

As mentioned so far, these were the seaweeds chosen for this work since: they are present in the Portuguese coast, are edible seaweeds, are produced in aquaculture (cultivated in Portugal by ALGAplus), are used in different industrial applications (including food industry), but are still underexploited in terms of their biomedical and nutritional potential.

3.1.2. Seaweeds Preparation

Seaweeds were taken to the premises of ALGAplus, where they were dried (Figure 3.1). Seaweeds drying occurred at 25 ºC and left drying with 10 to 12% humidity in their constitution. They were packed and sent to the Genotoxicity Laboratory, Genetics and Biotechnology Department, Veterinary Science Building - Laboratory Blocks I from University of Trás-os-Montes e Alto Douro (UTAD). All the following procedures were performed in this laboratory.

The first step was to grind the dried seaweeds in a coffee grinder (Figure 3.2). This step was very important in order to, in the assays, weigh seaweeds and put them in the media mixed

with other components, facilitating the ingestion by *Drosophila melanogaster*. The grinded seaweeds were stored into zip food bags in a dark dried place.

Figure 3.1 - Samples of the dried: (A) *Gracilaria* sp., (B) *Grateloupia turuturu*, (C) *Porphyra umbilicalis*, (D) *Ulva* sp., and (E) *Fucus vesiculosus*.

Figure 3.2 - Dried and grinded samples of, from left to right: *Gracilaria* sp., *Grateloupia turuturu*, *Porphyra umbilicalis*, *Ulva* sp., and *Fucus vesiculosus*.

3.2. *Drosophila melanogaster*

3.2.1. Strain, Genotypes, and Phenotypes Used

Vogel, Nivard, and Zijlstra (1991) developed six strains with different metabolic activities. One of these strains, Oregon-K **(**OK), was chosen to be used throughout the present work since a study from Gaivão and Comendador (1996) proved, between the same six strains, that OK is one of the best strains to be used in genotoxicological assays. OK presented the highest capacity to induce ROS and the lowest activity of antioxidant enzymes in a biochemical characterisation in Gaivão and Comendador (1996).

Two distinct phenotypes were used for eye colour: a wild-type strain (dominant) with red eyes, homozygous for the wild-type allele of the *white* (*w*) gene for females (w^+/w^+ genotype), and w^+/Y for males; a mutant strain (recessive) homozygous for the mutant allele of the *w* gene for females (*w/w*) and *w*/Y for males (phenotypically with white eyes - without pigmentation) (Figure 3.3). *w* gene is located at position 1.5 of *Drosophila melanogaster*'s X chromosome (Marcos et al., 2014).

Figure 3.3 - Difference in eye colour between a OK wild-type *Drosophila melanogaster* (red eyes) and a OK *w* mutant *Drosophila melanogaster* (white eyes).

Other genotype is possible for females, w^{+}/w, which will appear later in the crossings performed. And, since the wild-type allele is dominant relative to the mutant, these fruit flies develop red eyes.

The OK strain with the two different phenotypes for eye colour was kindly provided by Prof. E. W. Vogel (Leiden, The Netherlands).

3.2.2. Sexual Distinction and Sexual Maturation Distinction in Adults

The sexual distinction (or sexing) is easy to perform in *Drosophila melanogaster* since it is a species that presents sexual dimorphism. Therefore, the main differentiating characteristics between the two sexes are (Figure 3.4) (Deepa et al., 2009; Flagg, 2005; Ong et al., 2014):

- Body size - on average, females have 3 mm in length and 1.4 mg in weight, while males are only 2 mm accompanied by 0.8 mg in weight;
- Shape of abdomen - females are easily distinguishable by the larger and sharper abdomen than males (which is smaller and rounder);
- Bands on the abdomen - dark bands are easily visible on the dorsal surface of the females' abdomen. The last few bands on the tip of males' dorsal surface of the abdomen display a uniform dark pigmentation (segments are fused);
- Sternites - on the ventral part of abdomen, females have more sternites (ventral subunits of the abdomen) than males;
- External genitalia on abdomen - located at the ventral posterior part of the abdomen, the vaginal plate of females is pointed and light coloured. The male genital arch is more rounded and is surrounded by heavy dark bristles;
- Sex combs - only males have sex combs, about ten black bristles that are present in the articulation of the superior tarso of the first pair of legs.

Important physical features allow the distinction of virgins relatively to sexual matured individuals: a lighter body colour (still to gain pigmentation), curled and wet wings, and meconium, a dark greenish spot located on the right ventral part of the abdomen representing the remains of the last meal before pupating (Deepa et al., 2009; The Berg Lab, n.d.). Within 8 to 12 h of eclosion, fruit flies become sexually mature, i.e., they cease to exhibit these characteristics (Sandhyarani, 2016; The Berg Lab, n.d.).

In practice, fruit flies were anaesthetised (see 3.2.4), placed on a piece of paper, and separated by sex with the help of a thin paint brush, with the naked eye. For sexual maturation

and sexing of the virgin fruit flies, the same was done but at the stereoscopic microscope with about 10 to 12x magnification.

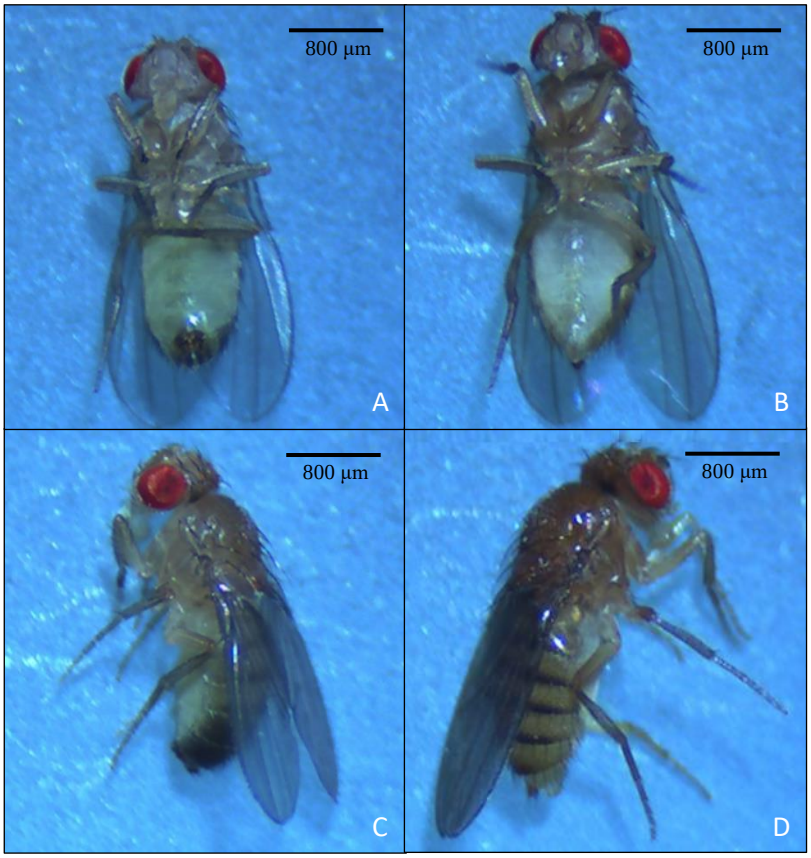

Figure 3.4 - Physical comparison between a male and a female OK wild-type. Comparison of the male (A) and the female (B) in the ventro-dorsal position, where are visible the differences in body size, shape of abdomen, external genitalia, and the presence of sex combs; comparison of the male (C) and the female (D) in lateral position, in order to focus on the differences of the bands on the abdomen.

3.2.3. Culture Medium: Cultures Maintenance

For the healthy maintenance of the strains of *Drosophila melanogaster* used for the assays it was necessary to make culture medium able to nourish fruit flies and maintain them without contaminations. The culture medium used needs to be cooked at high temperatures, at about 85 to 90 ºC, being designated by normal medium, and is composed, for 1000 ml of distilled H_2O (dH_2O), by:

- 100 g of sucrose (industrial sugar),
- 100 g of yeasts (baker's yeast),
- 12 g of agar,
- 1 coffee spoon (about 5.4 g) of sodium chloride (NaCl; cooking salt),
- 5 ml of propionic acid.

Depending on the amount of culture medium that was required, a lower (or higher) amount could be made by using less (or more) dH_2O and maintaining the proportion of the constituents of the medium.

Sucrose and yeasts are essential in *Drosophila melanogaster*'s diet, since they are also the main constituents of their food of choice in nature, rotten fruit. Agar is necessary to promote the solidification of the medium. NaCl is used to make the medium more appealing in terms of flavour (gustatory reinforce), and as a source of sodium (Na) and chlorine (Cl) (Russell, Wessnitzer, Young, Armstrong, & Webb, 2011). Propionic acid inhibits the growth of mold and some bacteria, so as to avoid destruction of cultures, i.e., functions as a preservative (Haque, Chowdhury, Islam, & Akbar, 2009; The Dow Chemical Company, 2015). And dH_2O acts on the mixture of the components of the medium and on the hydration of fruit flies.

Bottles of 200 ml capacity were used in the maintenance of the *Drosophila melanogaster* cultures with normal medium. Although, normal medium was also inserted in vials (17.7 ml capacity) to be used in the assays (described posteriorly).

Drosophila melanogaster's cultures were kept in an incubator at 24 ± 1 ºC.

3.2.4. Method of Anaesthesia and *Drosophila melanogaster* Disposal

For the successful accomplishment of virgin separation and/or separation of sex, and their placement in bottles (or vials) with new medium, it was necessary to use anaesthesia because without it would be impossible to perform these procedures.

The anaesthetic administered to anaesthetise fruit flies was diethyl ether (or simply ether). It is a highly volatile organic compound, $(C_2H_5)_2O$, capable of etherize *Drosophila melanogaster* after inhalation for about 20 to 30 s (dependent on the quantity used) (Deepa et al., 2009; Flagg, 2005). Exposure time was taken into account in the etherisation of fruit flies, as prolonged exposure may lead to sterility or even death, usually more than 1 min. Depending on the amount of ether used, the fruit flies remained etherised for 5 to 10 min.

The fruit flies were etherised in the etheriser, a plastic funnel with a net covering its most tapered end, which was inserted in a laboratory glass bottle with a cotton soaked in ether in the interior.

In order to eliminate fruit flies, they were anaesthetised and poured directly from the funnel into a soapy water jar, "the morgue", where they drowned while anaesthetised.

3.3. *Drosophila melanogaster* Toxicological Assays with Seaweeds

3.3.1. Initial Toxicological Screening

Initial toxicological screening tested the incorporation of seaweeds in *Drosophila melanogaster*'s diet, in the larval development phase, functioning as an assay of toxicological estimation for the tested seaweeds. It also allowed the choice of the seaweeds, and later the concentrations of the same, for the next assays of the present work.

Four major steps were required to perform the initial toxicological screening:

1st Making the Media with Seaweeds

For toxicological researches with *Drosophila melanogaster*, the use of a medium which does not need to be cooked is preferable to the normal culture medium. Formula 4-24® Instant *Drosophila* Medium from Carolina Biological Supply Company (referred just as instant medium for the rest of the work), seaweeds, and dH_2O, were used to make the media for this assay.

Five concentrations of the harvested seaweeds were chosen (the concentrations were the same for all seaweeds):

1.25%, 2.5%, 5%, 10%, and 20% (percentage relative to dry weight).

With the use of vials (17.7 ml capacity each) in this assay, in each vial was placed:

2.5 ml (instant medium + seaweed) + 2.8 ml dH_2O.

Different conditions were tested taking into account that five seaweeds were used, separately, in five concentrations for each one. In addition to these conditions, negative controls (without seaweed) were also constructed for each seaweed (five negative controls). For replication purposes, duplicates were performed for all conditions with seaweed and for controls (from each seaweed), totalling 12 vials for each seaweed, i.e., 60 vials total were used to perform this screening.

The seaweeds concentrations were expressed in seaweed mass in relation to the mass of the instant medium. In Table 3.1 it is displayed the constitution of the treated media used.

Table 3.1 - Constitution of the media used for the initial toxicological screening according to the concentrations of seaweeds used.

C (in %)	**Instant medium** (in g)	**Seaweed** (in g)*	**dH_2O** (in ml)
0	0.7500	0	2.8
1.25	0.7406	0.0094	2.8
2.5	0.7313	0.0188	2.8
5	0.7125	0.0375	2.8
10	0.6750	0.0750	2.8
20	0.6000	0.1500	2.8

C - seaweeds concentrations; * equal for all seaweeds.

For the construction of the treated media was necessary:

- 42.0940 g of instant medium,
- 0.5813 g of the dried and grinded *Gracilaria* sp.,
- 0.5813 g of the dried and grinded *Grateloupia turuturu*,
- 0.5813 g of the dried and grinded *Porphyra umbilicalis*,
- 0.5813 g of the dried and grinded *Ulva* sp.,
- 0.5813 g of the dried and grinded *Fucus vesiculosus*,
- 168 ml of dH_2O,
- 60 vials,
- 1 permanent marker,
- 1 laboratory spoon,
- 1 weight boat (7 ml),
- 1 analytical balance,
- 1 plastic funnel,
- paper (for cleaning; the necessary),
- carded cotton (the necessary),
- 1 beaker (600 ml),
- 1 micropipette of 5 ml (plus tip).

In terms of procedure:

- The vials were identified with a permanent marker, so that the exact amounts of each component of the media were placed in the correct vials. The first component to be added was instant medium. The instant medium flakes were withdrawn from the package with the aid of a spoon, and placed on a weight boat on top of the analytical balance. The instant medium was weighed for the different conditions (according to Table 3.1). The instant medium, with the desired mass in the weight boat, was poured into each of the vials with the aid of a funnel;
- Then, the same procedure was performed for seaweeds. After cleaning the weight boat, the spoon, and the funnel, from the feeding bags with zip (with the seaweeds) were withdrawn the necessary quantities of each seaweed to complete the media (according to Table 3.1);
- The media were then hydrated with dH_2O. Before placing dH_2O in the media, media were homogenized. The dH_2O was previously placed on a beaker so as to facilitate the removal of dH_2O with a micropipette for the vials. As the instant medium causes the medium to solidify

rapidly after hydration, a slight pendulum movement was performed shortly after the addition of dH_2O to smooth the surface of the medium and carded cotton was put in all vials.

The hydration was only carried out on the day of transferring fruit flies to the treated media vials, being that vials with media (without dH_2O) were sealed with carded cotton and stored in a dark dried place until hydration.

2nd The pre-crossing

The second step involved the preparation of fruit flies for the transfer to the previously made media. Before the fruit flies were placed in the treated media for eggs laying, a pre-crossing was performed. The pre-crossing consisted in putting fruit flies in vials with normal medium for 48 h, so that females when placed in the treated media, were in the maximum eggs laying and stayed, therefore, the shortest time possible feeding from the media destined for their offspring (those who were evaluated in the assay).

Cultures' fruit flies were etherised, separated by sex, and placed in vials with normal medium. Ten couples with about 3 to 4 days of life were placed in each vial, being that 60 vials were needed, i.e., 600 couples total. OK wild-type and OK *w* mutant fruit flies were randomly mixed in vials since it is a parameter that does not interfere with the assay; then, they were kept in the incubator at 24 ± 1 ºC during 48 h.

3rd Transfer of Fruit Flies to Treated Media (and removal)

After 48 h of pre-crossing, fruit flies were passed from the vials with normal media to the vials with the treated media. The treated media were hydrated just before putting the fruit flies in their interior. The fruit flies were transferred without anaesthesia, since they were already separated and counted in each vial with normal medium.

The fruit flies already in the treated media laid their eggs, being then eliminated in the morgue after 24 h of the transfer to these same media. It was assumed that the number of eggs laid in each vial with treated medium was very similar, since the fruit flies had similar ages between themselves and were subjected to similar settings of temperature (24 ± 1 ºC) in culture bottles, pre-crossing vials, and in each vial with treated medium.

4th Development of Offspring

After the removal of fruit flies from the vials with treated media, the offspring developed in the same media. The offspring developed through the ingestion of the treated media, at a temperature of 24 ± 1 ºC, being exposed from the day they were laid in the media (as eggs) until their eclosion into adults, i.e., they were subjected to a chronic treatment.

Day zero of the offspring development began at the time the parents were placed in the treated media (eggs laying occurred). The follow up and registration of the number of eclosions in each treated medium and the passage to vials with normal medium occurred daily until the 17th day after eggs laying (including). The passage to vials with normal medium was made with etherisation of fruit flies.

Data Evaluation and Statistical Analysis

The following parameters were evaluated for the ecloded fruit flies:

- Development time - the time spent (days after eggs laying) to the eclosion of individuals,
- Survival - the total number of ecloded individuals,
- Sex ratio - the ratio between the total number of ecloded individuals of each sex.

In order that results from development time, survival, and sex ratio obtained for the fruit flies exposed to the media with seaweeds had meaning, were compared with the results of the controls to obtain semi-quantitative estimates of the toxicological effects of the seaweeds.

Taking into account that duplicates were made for the conditions with seaweed (small sample sizes (*n*s)), non-parametric statistical tests were performed (Altman & Bland, 2009; Frost, 2015). Microsoft Office Excel 2016 (Microsoft Corporation, USA) was used to perform the statistical tests as well as to present the results in the form of graphs.

For each of the parameters evaluated, the following was performed in terms of data evaluation and statistical analysis:

<u>Development Time</u>

- For each day of eclosions and for each condition with seaweed, replicates were summed relative to the number of eclosions;
- The mean of the 10 controls was performed for each day and, these values, were multiplied by two;
- For comparing the control with the different conditions with seaweed, a parameter was created, the development time of 75% of population (DT75). DT75 represented the time it took to eclode at least 75% of the individuals in a seaweed condition. When at least one of the concentrations of a seaweed reached greater than or equal to 75% of eclosions, that day was chosen as the reference for that seaweed and the percentage of eclosions was calculated for all concentrations of the same seaweed and for the control in the same day. However, the total eclosions of this chosen concentration had to be greater than the total eclosions of control.

Survival

- For the total number of ecloded individuals, chi-square (χ^2) goodness of fit tests between the replicates for each condition with seaweed were performed and then were summed;
- A χ^2 goodness of fit test was performed for the total number of eclosions of the 10 controls, as well as a mean of all controls and a duplication of the mean. Differences were considered significant when χ^2 value(9) > 16.92, $p < 0.05^*$;
- The comparison of the total number of control eclosions with the total number of ecloded individuals for each condition with seaweed was possible with χ^2 goodness of fit tests;
- To estimate the degree of association between the number of ecloded individuals and the tested concentrations of each seaweed (plus control), Spearman's rank-order correlation coefficient was applied.

Sex Ratio

- χ^2 goodness of fit tests were performed between the numbers of males and females of each replicate of each condition with seaweed. Males and females of each duplicate were then summed;
- For the controls, χ^2 goodness of fit tests were performed between the numbers of males and females of each control and, then, a mean was performed for males of all controls and the same for females, being then duplicated both means;
- χ^2 goodness of fit tests were performed between the numbers of males and females for control (after means and duplications) and for each condition with seaweed (after the sums).

χ^2 goodness of fit tests were significant when χ^2 value(1) > 3.84, $p < 0.05$, except for *.

3.3.2. Longevity Assay

The longevity assay allowed to evaluate whether the antitoxic effects of seaweeds on initial toxicological screening had an influence on *Drosophila melanogaster*'s aging.

After the passage of the ecloded individuals from the treated medium vials to the normal medium vials in initial toxicological screening, the fruit flies exposed to the two seaweeds with the strongest positive correlations were selected for longevity assay: *Porphyra umbilicalis* and *Grateloupia turuturu*, according to the results to be presented later in Chapter 4. The longevity of the fruit flies of the 10 controls was followed as well. The remaining fruit flies were eliminated in the morgue.

In the 17th day after eggs laying, after the introduction of all the ecloded individuals into each vial with normal medium, the first transfer to vials with new normal medium was made. This passage was performed using anaesthesia, with sexing and counting of the individuals in each vial in order to verify the mortality of males and females.

Once a week, the normal media were renewed, and the numbers of dead males and females from each vial were registered the same way as for the first week, until all individuals were dead.

Data Evaluation and Statistical Analysis

Duplicates were made for the conditions with seaweed (small *n*s). Thus, non-parametric statistical tests were performed. Statistical analyses of the data were performed using OASIS: online application for survival analysis (J.-S. Yang et al., 2011) and OASIS 2: online application for survival analysis 2 (Han et al., 2016). The survival curves were graphed with OASIS.

In practical terms, for the data evaluation and statistical analysis, it was carried out:

- Using Kaplan-Meier survival analysis (Kaplan & Meier, 1958) it was obtained mean, median, and maximum longevities for males and females of the different replicates of the conditions previously exposed to seaweed. Mantel-Cox tests were used to assess the statistical significance of differences in mean longevity (Mantel, 1966) and Wang-Allison tests to assess the statistical significance of differences in median and maximum longevities (Wang, Li, Redden, Weindruch, & Allison, 2004). Replicates were summed for males and for females in terms of survivors per day for each condition with seaweed;
- For the 10 controls, Kaplan-Meier survival analysis were performed to obtain the same parameters as above. For each sex, Mantel-Cox and Wang-Allison tests were also used. A mean of the survivors per each day from all controls was performed for each sex. The mean of the survivors per day for both males and females was duplicated for each day. Control's males were then compared with control's females with Mantel-Cox and Wang-Allison tests;
- After, Mantel-Cox and Wang-Allison tests were used to compare control's males to males of each of the seaweeds conditions, as well as compare control's females to females of each condition of the two seaweeds. Further comparisons were made.

Differences were considered significant when χ^2 value(1) > 3.84, $p < 0.05$.

3.3.3. SMART

SMART was used for the evaluation of the genotoxicological potential of the seaweeds when ingested by fruit flies in the larval development phase (chronic treatment).

From among the two types of SMART currently used, in practical terms, the eye-spot test has an advantage over the wing-spot test since it is faster in detecting LOH for the chosen markers. As such, it is indicated when intended to evaluate a large number of variables (Gaivão et al., 1999) and, therefore, was the test used in the present work.

The eye-spot test uses the *w* gene as a recessive marker to monitor, on wild-type eyes, the presence of mutant ommatidia (or spots or clones) that indicate the occurrence of LOH by deletions, point mutations, mitotic recombination (the most frequent), or nondisjunction in the somatic cells of the developing *Drosophila melanogaster* females (Figure 3.5). A mutant ommatidium can be generated by more than one of the previous genetic alterations (Amkiss, Dallouh, Idaomar, & Amkiss, 2013; Marcos et al., 2014; Mishra et al., 2016).

In other words, when wild-type females (w^+/w^+) are crossed with white eyed males (w/Y), or vice versa (w/w with w^+/Y), a heterozygous offspring is developed for females (w^+/w) and, in its development period, genotoxic agents can trigger genotoxic events that can cause the formation of white phenotype spots in the red eyes (Figure 3.5). Thus, the assay is also designated as w/w^+ SMART (Gaivão & Comendador, 1996), among other equivalent designations. Further on the subsubchapter, Figure 3.6 displays examples of red eyes with white ommatidia.

The methodology used in the present work was followed as described by Vogel and Nivard (1993).

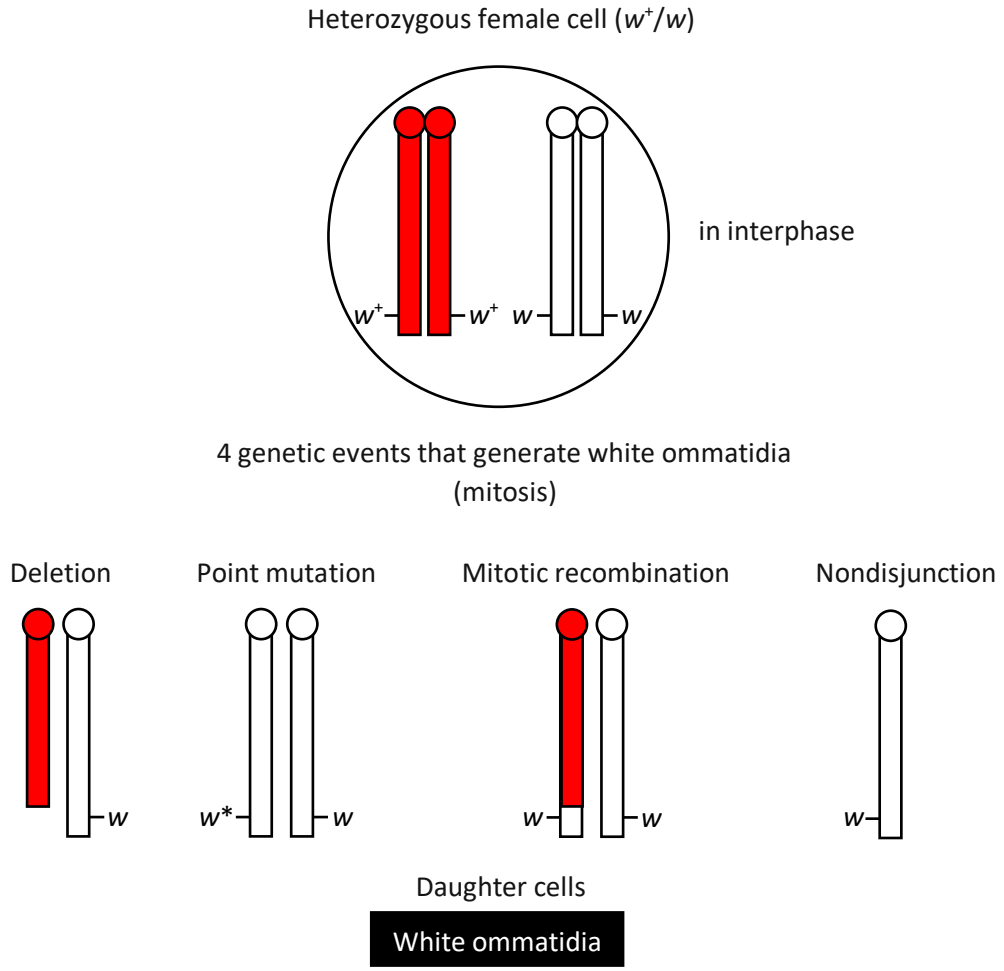

Figure 3.5 - Scheme of the possible four types of genetic alterations that generate white ommatidia in a heterozygous *Drosophila melanogaster* female for the *w* gene. In the scheme, the heterozygous female cell has two X chromosomes with two chromatids each (duplicated DNA in interphase) and the daughter cells have two X chromosomes but only one chromatid each (except for nondisjunction). The X chromosomes in red indicate that they are carriers of the w^+ allele and those in white indicate that they carry the *w* allele, however there are a few exceptions that will be described below. The localization of the alleles in X chromosomes is represented in a purely illustrative, non-exact way.
In the development of a heterozygous female for the *w* gene (w^+/w), different genetic alterations may occur in the mitosis of heterozygous cells of the future ommatidia cells (imaginal discs), and produce daughter cells that develop white phenotype ommatidia. The genetic alterations that cause mutant phenotypes can be: deletion in one X chromosome including the *white* locus (in the wild-type allele); a point mutation in the wild-type allele by substitution, insertion, or deletion. *w** is a mutated wild-type expressing white phenotype; mitotic recombination between chromatids of the homologous X chromosomes that substitute the wild-type locus by a mutant locus; nondisjunction that causes the loss of the chromosome with the wild-type allele. Information from Amkiss et al. (2013), Marcos et al. (2014), and Mishra et al. (2016).

Four major steps were required to perform SMART:

1st Making the Media with Seaweeds

So as for longevity assay, only two seaweeds of the initial toxicological screening were chosen for SMART. However, of these two seaweeds, only two concentrations of each were tested. The two lowest concentrations for each of the two seaweeds with survivals significantly

higher than the control's survival and with a DT75 inferior than control's DT75 were selected. According to the results to be presented in Chapter 4, the two seaweeds with their respective two concentrations that were chosen for SMART were: 5% of *Porphyra umbilicalis* (5%P), 10% of *Porphyra umbilicalis* (10%P), 10% of *Grateloupia turuturu* (10%G), and 20% of *Grateloupia turuturu* (20%G) (relatively to dry weight).

The instant medium was used again to made media. However, for media hydration, PBS was used instead of dH_2O, taking into account that: the genotoxic agent SN was added in some media, which is more stable in this buffer than in dH_2O; the survival and sex ratio were tested again and PBS was used to check if there were any differences in results regarding dH_2O used in initial screening.

Taking into account that bottles (200 ml capacity) were used in this assay, the following 10 conditions (10 bottles) were delineated in triplicate for each condition (30 bottles total):

- 1 condition: 20 ml instant medium + 20 ml PBS (negative control - NC),
- 4 conditions: 20 ml (instant medium + seaweed*) + 20 ml PBS,
- 1 condition: 20 ml instant medium + 20 ml (PBS + 20 μM SN) (positive control - PC),
- 4 conditions: 20 ml (instant medium + seaweed*) + 20 ml (PBS + 20 μM SN).

* seaweed varies between 5%P, 10%P, 10%G, and 20%G.

The required seaweed mass for each concentration was calculated relative to instant medium. PBS was used with 0.2 M and pH 6.8. The genotoxic effects of SN in *Drosophila melanogaster* where first proved by Gaivão et al. (1999) in *w*/*w*$^+$ SMART, where SN showed the greatest genotoxic activity with the lowest lethal toxicity (as stated in Chapter 1) at 20 μM. Thus, SN was used at a concentration of 20 μM in PC and in the 4 conditions with seaweed, being dissolved in PBS before placing it in the media. In Table 3.2 it is displayed the constitution of the media used.

Table 3.2 - Constitution of the conditions used for SMART. The percentage of seaweed in CONDITIONS refers to each one of the conditions with 20 ml (instant medium + seaweed) + 20 ml PBS. The percentage of seaweed + SN in CONDITIONS refers to each one of the conditions with 20 ml (instant medium + seaweed) + 20 ml (PBS + 20 μM SN).

CONDITIONS	Instant medium (in g)	Seaweed (in g)	PBS (in ml)
NC	6	-	20
5%P	5.7	0.3	20
10%P	5.4	0.6	20
10%G	5.4	0.6	20
20%G	4.8	1.2	20
PC	6	-	20 (PBS + SN)
5%P + SN	5.7	0.3	20 (PBS + SN)
10%P + SN	5.4	0.6	20 (PBS + SN)
10%G + SN	5.4	0.6	20 (PBS + SN)
20%G + SN	4.8	1.2	20 (PBS + SN)

For the construction of the treated media was necessary:

- 163.8 g of instant medium,
- 5.4 g of the dried and grinded *Porphyra umbilicalis*,
- 10.8 g of the dried and grinded *Grateloupia turuturu*,
- 600 ml of PBS,
- 3 mg of SN,
- 30 bottles,
- 1 permanent marker,
- 1 laboratory spoon,
- 1 weight boat (200 ml),
- 1 analytical balance,
- 1 plastic funnel,
- paper (for cleaning; the necessary),
- carded cotton (the necessary),
- 2 beakers (600 ml each),
- 1 stir bar and retriever,
- 1 hot plate stirrer,
- 2 graduated cylinders (20 ml each).

A similar procedure to the one performed in the initial toxicological screening was followed:

- The empty bottles were identified with a permanent marker. The instant medium was withdrawn from the package with the aid of a laboratory spoon and placed on a weight boat on top of the analytical balance. The instant medium was weighed for the 30 bottles, and was poured in to each one of the bottles with the aid of a funnel (according to Table 3.2);
- The same was performed for seaweeds. After cleaning the weight boat, the spoon, and the funnel, from the feeding bags with zip with the stored *Grateloupia* and *Porphyra* were withdrawn the necessary quantities of seaweed to complete the media according to Table 3.2;
- Half of the media (15 bottles) were hydrated with PBS (according to Table 3.2). Before placing the PBS in the media, media were homogenized. The PBS located in a laboratory glass

bottle was previously put into a beaker to facilitate the transfer of it to a graduated cylinder and, from this, to each one of the bottles (20 ml of PBS to each one). A slight pendulum movement was performed shortly after the addition of PBS to smooth the surface of the medium and the bottles were capped with carded cotton;

- The remaining 15 bottles were hydrated with PBS + SN (according to Table 3.2). Thus, 300 ml of PBS plus 3 mg of SN were placed into a beaker and, this one (with a stir bar inside), was placed in a hot plate stirrer only with the stirrer function at an intermediate velocity for 15 min. After 15 min, it was found that there were still some SN grains to dissolve and, therefore, the stir bar extractor was used for extracting the stir bar and homogenize the final solution. From this step, the same procedure was followed as for the media with PBS without SN.

The hydration was only carried out on the day of transferring fruit flies to the treated media bottles, being that bottles with media were sealed with carded cotton and stored in a dark dried place until hydration.

2nd Virgin Females and Pre-crossing

The second step of SMART involved the separation of w^+/w^+ virgins and *w*/Y (non-virgins or virgins) from culture bottles, so that they were placed in a 48 h pre-crossing.

The reason why virgin females were used was to ensure that females did not have fertilized eggs from males with wild-type eyes before setting up the crosses in treated media bottles.

In terms of materials and procedure:

- Firstly, w^+/w^+ virgins were etherised and separated from culture bottles. They started to be collected at the beginning of the week so that males' collection and pre-crossing could occur at the end of the week. Throughout the week, virgins were stored in vials with normal medium at 24 ± 1 ºC, being set 20 virgins per vial, requiring a total of 600 virgin females (30 vials total);
- After collecting virgins, it was necessary to collect 600 *w*/Y. One to two days old males were collected from the culture bottles, being etherised and separated for bottles with normal medium. Twenty males were set per bottle, 600 for 30 bottles, during the same day;
- Then, on the same day of males' collection, five vials with the female virgins (one from each day of collection) were anaesthetised and mixed. From those, 20 anaesthetised virgins were poured into a bottle already with 20 awaked males. The same was done for the remaining females of the 5 vials and was repeated for the remaining 25 vials and for the bottles with males.

Thus, 20 w^+/w^+ virgins and 20 *w*/Y were crossed in each bottle with normal medium and kept in the incubator at 24 ± 1 ºC for 48 h.

The mixture of virgins reduced the effect of age on eggs laying, i.e., the fruit flies caught on the day before pre-crossing would not yet have the laying ability matured compared to the ones collected at the beginning of the week. However, pre-crossing also served to reduce this effect.

3rd Transfer of Fruit Flies to Treated Media (and removal)

The fruit flies of the normal medium bottles after 48 h of pre-crossing were transferred to the bottles with treated media. As in the initial screening, it was only at this time that the media were hydrated and the fruit flies were transferred without anaesthesia.

The crossing occurred for 72 h. After this time, the fruit flies were removed with anaesthesia and eliminated in the morgue. It was also assumed that the number of eggs laid in each bottle with the different treated media was very similar since: the virgins collected on the different days of the week were all mixed in the pre-crossing bottles, males had a similar age between themselves, and all the fruit flies were subjected to equal environments of temperature (24 ± 1 ºC) in culture bottles, pre-crossing bottles, and in each bottle with treated medium.

4th Development of Offspring and Observation of Females Eyes

The offspring developed in a chronic treatment at 24 ± 1 ºC. The number of individuals ecloded on each bottle was registered, and the females were separated for eyes observation.

Since the removal of the fruit flies ecloded in the treated media to the observation of their eyes, different materials were required:

- 30 bottles with treated media and ecloded fruit flies,
- 1 etheriser,
- 1 piece of paper,
- 2 thin paint brushes,
- Sheets (the necessary) and 1 pencil,
- Microcentrifuge tubes (the necessary),
- 1 permanent marker,
- 1 freezer,
- 1 small glass Petri dish,
- dH_2O (the necessary),
- 96% ethanol (the necessary),
- Triton™ X-100 (the necessary),
- 1 glass dropping bottle (30 ml),
- 2 laboratory glass bottles (100 ml each),
- 1 plastic Pasteur pipette (3 ml),
- 1 stereoscopic microscope,
- 1 black stage plaque,
- 1 cold white light source with two foci,
- 2 dissection needles,
- paper (for cleaning; the necessary),
- 1 morgue.

In terms of procedure:

- First, it was necessary to prepare two solutions to be used in the eyes observation at the stereoscopic microscope, according to Marcos et al. (2014):

SMART solution 1, for 100 ml:
90 ml of 96% ethanol,
9 ml of dH_2O,
1 ml of Triton^{TM} X-100.

SMART solution 2, for 100 ml:
10 ml of SMART solution 1,
90 ml of dH_2O.

The solutions were put in laboratory glass bottles, however, SMART solution 2 was passed to the glass dropping bottle to be accessible faster. The solutions were prepared again when needed;

- The first fruit flies ecloded in the bottles with the treated media were etherised, set on a piece of paper for sexing, and registered the number of ecloded individuals. Males were placed in microcentrifuge tubes and frozen for posterior assays (not included in the present work). Females were set in the Petri dish (without covering);
- SMART solution 1 was added with the plastic Pasteur pipette to the Petri dish so that females were submerged in the solution. The Petri dish was then placed on top of a pencil so that the solution 1 and the females concentrated only on a confined space of the dish;
- About 10 females were removed from the Petri dish with a thin brush and were deposited on the black stage plate placed on the stereoscopic microscope. In the black stage plate with the females it was necessary to put SMART solution 2, keeping the females together near one end of the plate;
- It was necessary a cold white light source with two foci for the illumination of the eyes and two dissection needles to position the females and their eyes to be visualized. The observation occurred with a magnification of about 40x, however, by using the zoom knobs of the stereoscopic microscope, the magnification was increased and decreased in order to validate the presence or absence of white spots in each red eye. For a more accurate observation, the image was focused and blurred (with focus knobs) and the eyes were constantly moved with the aid of the needles;

- After both eyes were visualized in the search for mutant spots, each female was put on one of the opposing ends of the black plate in relation to the females not yet analysed. The same procedure was repeated for all fruit flies present on the plate and, during this procedure, when necessary (evaporation occurred rapidly), solution 2 was added to the females on the plate to keep them "coated" by this solution, as well as in the Petri Dish after evaporation of solution 1. Then, they were eliminated in the morgue and the black plate was cleaned and more females of the Petri dish were positioned in the same way as the previous ones. The number of mutant spots observed and their size (how many ommatidia were together affected) were registered;
- The procedure was performed for all bottles to obtain 400 eyes analysed by condition. In other words, for each triplicate, eye observation only ended when two replicates had 134 eyes observed each and one replicate had 132 eyes. Coming to the number of females analysed by bottle, the remaining females not observed in the stereoscopic microscope were put together with males in the freezer. To complete all conditions (the 30 bottles) it took 12 days.

Some important points were taken into account in the observation of the eyes: SMART solutions had the function of avoiding the light reflection in the females eyes, which would disturb the visualization of the same; at the stereoscopic microscope the white mutant spots are seen as dark spots when surrounded by red ommatidia (pigmented cells), but when they are in the border of the eye they appear as white (Figure 3.6); when at least one replicate did not have the enough number of females to be observed, it had to be compensated with another replicate of the same condition; the stage plate used was black instead of the usual white or with frosted glass for contrasting with the spots observed in the border of the eyes.

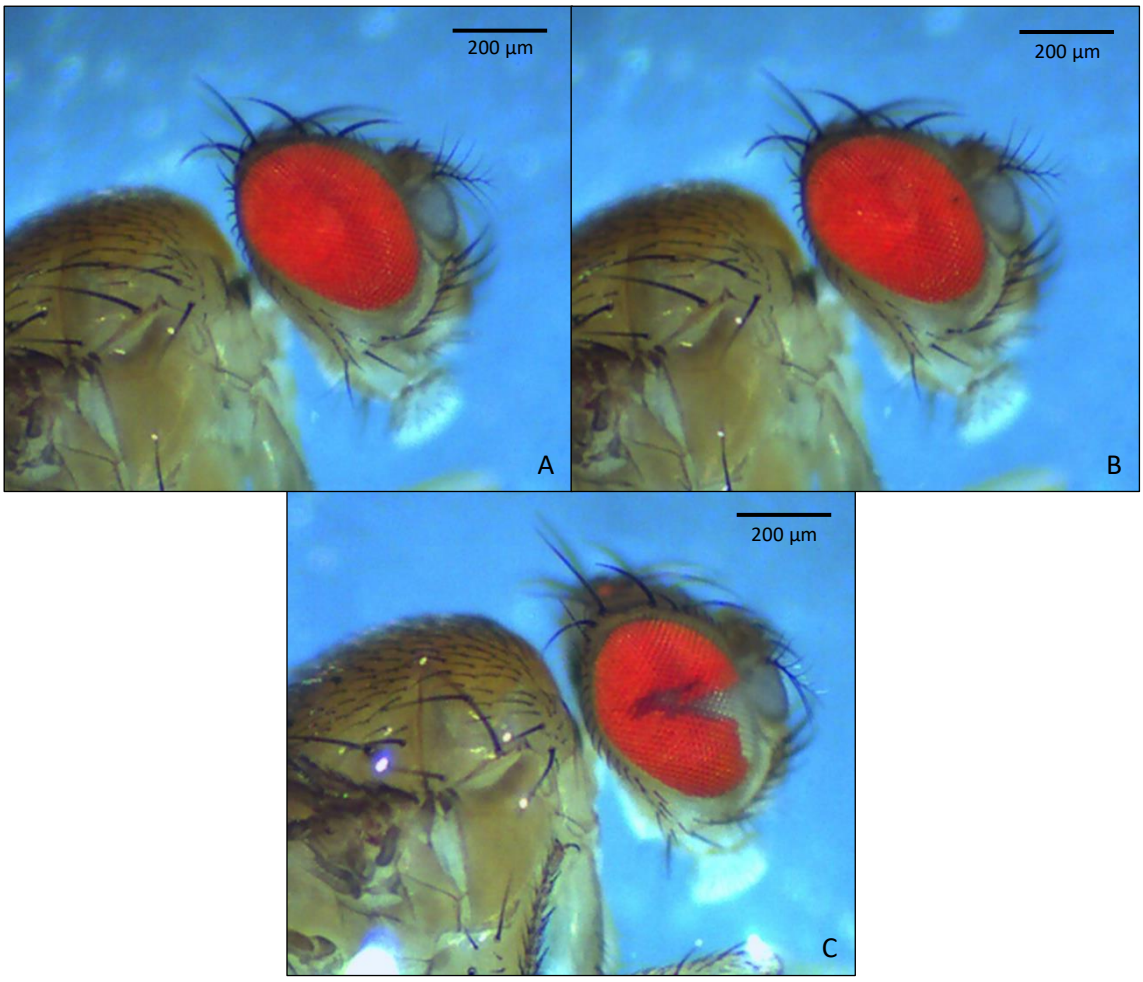

Figure 3.6 - Wild-type eyes of *Drosophila melanogaster*'s females at the stereoscopic microscope. (A) An eye without mutant spots, (B) an eye with a dark spot affecting one to two ommatidium(a), and (C) an eye with a spot that affected innumerable ommatidia. The females were not located in the black stage plate as a matter of lighting for the capture of the photographies.

Data Evaluation and Statistical Analysis

Since only triplicates were made for the conditions (small *n*s), non-parametric statistical tests were performed. Microsoft Office Excel 2016 (Microsoft Corporation, USA) was used to perform the statistical tests as well as to present the results in the form of graphs.

After the registration of the number of ecloded individuals in each one of the bottles with treated medium, two parameters were evaluated as in initial toxicological screening:

Survival

- χ^2 goodness of fit tests were performed between replicates for each condition, in terms of ecloded individuals. Replicates were then summed for each condition. χ^2 goodness of fit tests were significant when χ^2 value(2) > 5.99, $p < 0.05$;

- χ^2 goodness of fit tests were performed to compare the conditions with seaweed with the respective controls. Further comparisons were made. χ^2 goodness of fit tests were significant when χ^2 value(1) > 3.84, $p < 0.05$.

Sex Ratio

- χ^2 goodness of fit tests were performed to compare the number of ecloded males with the ecloded females in each treated medium. The replicates of the same condition were summed for males and females;
- χ^2 goodness of fit tests were performed between males and females for the conditions with the already summed males and females from the replicates.

χ^2 goodness of fit tests were significant when χ^2 value(1) > 3.84, $p < 0.05$.

After observing the eyes of *Drosophila melanogaster* and register the number of spots, as well as their separation in different sizes, it was possible to calculate some parameters:

Spots per 400 eyes

- The number of spots per 100 eyes was calculated for each of the replicates of each condition. χ^2 goodness of fit tests were performed between the replicates for each condition. The replicates were then summed in terms of spots, giving rise to x spots per 400 eyes for each condition. χ^2 goodness of fit tests were significant when χ^2 value(2) > 5.99, $p < 0.05$;
- For comparing the values of the spots per 400 eyes of each one of the conditions with seaweed with the respective controls, as well as other comparisons, the procedure used by Frei and Würgler (1988) for mutagenicity data was followed. Considering the formulation of two hypotheses, an H_0 which states that the quantity of mutant spots is equal between two conditions tested, and an H_1 that states that the mutant spots quantity of a certain condition is m (multiplication factor) times the mutant spots of another condition, it was possible to obtain four diagnostics in the comparison of two conditions through a multiple-decision procedure: positive, weak positive, negative, or inconclusive (Selby & Olson, 1981). To test the hypotheses a χ^2 test of homogeneity for proportions was applied. For each one of the hypothesis, when χ^2 value < 3.84 the hypothesis was accepted, but when χ^2 value > 3.84 the hypothesis was rejected. The ms used were 0.5 and 5;
- A inconclusive result was analysed with the one-tailed Mann-Whitney *U* test (Mann & Whitney, 1947), where spots per 100 eyes were used in order to verify if the medians were

equal or different between the two conditions compared. *U* test differences were significant when $z < -1.645$, $p < 0.05$;

Inhibition Percentage (IP)

- The IP (Abraham, 1994) is defined, adapted to this work, as the percentage of inhibition of SN activity when exposed to different conditions with seaweed, in comparison to PC. For each condition with seaweed plus SN, the formula was applied:

$$IP = \frac{\text{genotoxin alone - genotoxin plus seaweed}}{\text{genotoxin alone}} \text{ x } 100$$

However, by shaping the formula to the conditions without SN and to NC, an adaptation of the IP was also performed for these conditions.

Average Clone Size

- The average clone size (Szabad, Soós, Polgár, & Héjja, 1983) was utilised to know the mean size of spots in each condition, considering the registration of the spots in classes with different sizes;
- A χ^2 goodness of fit test was performed between all values of average clone size. Differences were considered significant at χ^2 value(9) > 16.92, $p < 0.05$.

Spots per 10^4 cells

- Taking into account Szabad et al. (1983) and the previous parameter, the spot frequency per 10^4 cells/ommatidia (f) was also calculated for each of the conditions according to the following formula:

$$f = \frac{2nm}{NC}$$

2 is the correction factor, n is the number of spots, m is the average clone size, N is the number of eyes analysed, and C is the number of ommatidia per eye (considered as 800);

- χ^2 goodness of fit tests were performed for comparing the fs of the conditions with seaweed with the respective controls. χ^2 goodness of fit tests were considered significant when χ^2 value(1) > 3.84, $p < 0.05$.

Chapter 4 - Results

4.1. Initial Toxicological Screening

In order to facilitate writing and reading of this subchapter (and the referent in Chapter 5), when using the following abbreviations for each of the seaweeds concentrations, it is intended to refer to the conditions of the initial toxicological screening with the concentrations of seaweed, as well with instant medium and dH_2O:

- 1.25% of *Gracilaria* sp. (1.25%Gr), 2.5% of *Gracilaria* sp. (2.5%Gr), 5% of *Gracilaria* sp. (5%Gr), 10% of *Gracilaria* sp. (10%Gr), and 20% of *Gracilaria* sp. (20%Gr);
- 1.25% of *Grateloupia turuturu* (1.25%G), 2.5% of *Grateloupia turuturu* (2.5%G), 5% of *Grateloupia turuturu* (5%G), 10% of *Grateloupia turuturu* (10%G), and 20% of *Grateloupia turuturu* (20%G);
- 1.25% of *Porphyra umbilicalis* (1.25%P), 2.5% of *Porphyra umbilicalis* (2.5%P), 5% of *Porphyra umbilicalis* (5%P), 10% of *Porphyra umbilicalis* (10%P), and 20% of *Porphyra umbilicalis* (20%P);
- 1.25% of *Ulva* sp. (1.25%U), 2.5% of *Ulva* sp. (2.5%U), 5% of *Ulva* sp. (5%U), 10% of *Ulva* sp. (10%U), and 20% of *Ulva* sp. (20%U);
- 1.25% of *Fucus vesiculosus* (1.25%F), 2.5% of *Fucus vesiculosus* (2.5%F), 5% of *Fucus vesiculosus* (5%F), 10% of *Fucus vesiculosus* (10%F), and 20% of *Fucus vesiculosus* (20%F).

When using (1) after the previous abbreviations refers to a replicate of a condition, and when using (2) is intended to refer to the other replicate.

4.1.1. Development Time

Conditions with *Gracilaria* sp.

Taking into account Figure 4.1, it was possible to verify that the fruit flies began to eclode earlier in control (between days 10 and 11) that in the conditions with *Gracilaria* sp. However, for 1.25%Gr, after the beginning of eclosions between days 11 and 12 (3 eclosions), the eclosions started to occur on a big scale, exceeding between days 14 and 15 (60 eclosions) the total number of control eclosions (50 eclosions). With respect to the remaining conditions with *Gracilaria*, the eclosion of individuals was shown to be late and in low number relative to the control and to 1.25%Gr. In 2.5%Gr the eclosions started only between days 15 and 16, in 5%Gr

between days 12 and 13, in 10%Gr again between days 15 and 16, and in 20%Gr not a single fruit fly ecloded until the 17th day after eggs laying.

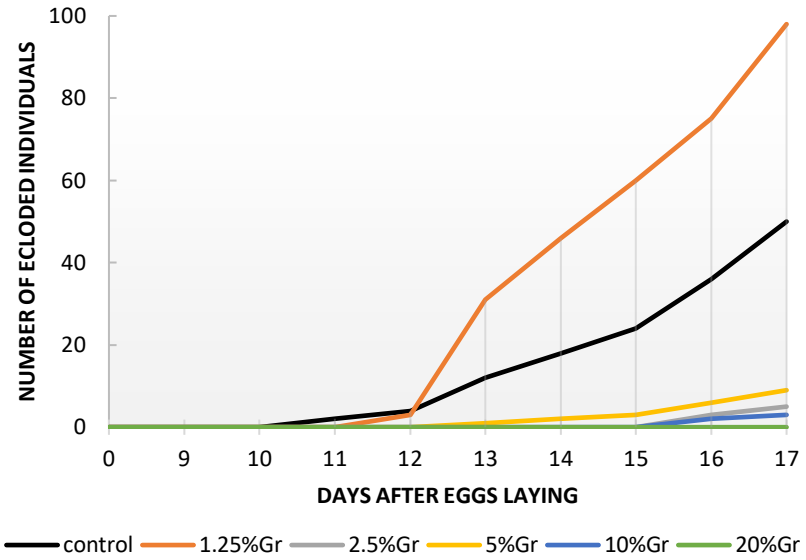

Figure 4.1 - Graph of the number of ecloded individuals in the control and in the conditions with *Gracilaria* sp. throughout the days after eggs laying (cumulative number of ecloded individuals).

For 20%Gr(1), a eclosion (1 male) happened 20 days after eggs laying, although it was not considered for the analysis since for the remaining conditions the count had ended in the 17th day after eggs laying.

Analysing data for DT75, 1.25%Gr in the 16th day after eggs laying reached at least 75% of total eclosions in first place between the control and the conditions with *Gracilaria* sp., with total eclosions higher than control's (Table 4.1). Taking into account the 16th day after eggs laying for all conditions, the percentages of eclosions were very similar, except for 20%Gr with no individuals. Although, clearly that 1.25%Gr stands out since the number of total eclosions for that day was much higher than in control and much more relative to the remaining conditions with *Gracilaria* sp.

Table 4.1 - Number of ecloded individuals in the control and in the different conditions with *Gracilaria* sp. on the 16th and 17th (total eclosions) days after eggs laying, and the percentages of eclosions in day 16 after eggs laying relative to total eclosions (cumulative number of ecloded individuals).

DAYS AFTER EGGS LAYING	CONDITIONS WITH *Gracilaria* sp.					
	control	1.25%Gr	2.5%Gr	5%Gr	10%Gr	20%Gr
16	36	75	3	6	2	0
17	50	98	5	9	3	0
Percentage	72%	77%	60%	67%	67%	0%

Conditions with *Grateloupia turuturu*

A eclosion (1 individual) occurred in 10%G between days 10 and 11 after eggs laying, the same period of eclosions of the first individuals in control (2 individuals). The remaining conditions presented fruit flies with later development times, being that in 1.25%G and 2.5%G the first eclosions occurred between days 13 and 14. For 5%G and 20%G eclosions began to occur between days 12 and 13 and in large numbers, with 28 and 40 eclosions respectively (Figure 4.2).

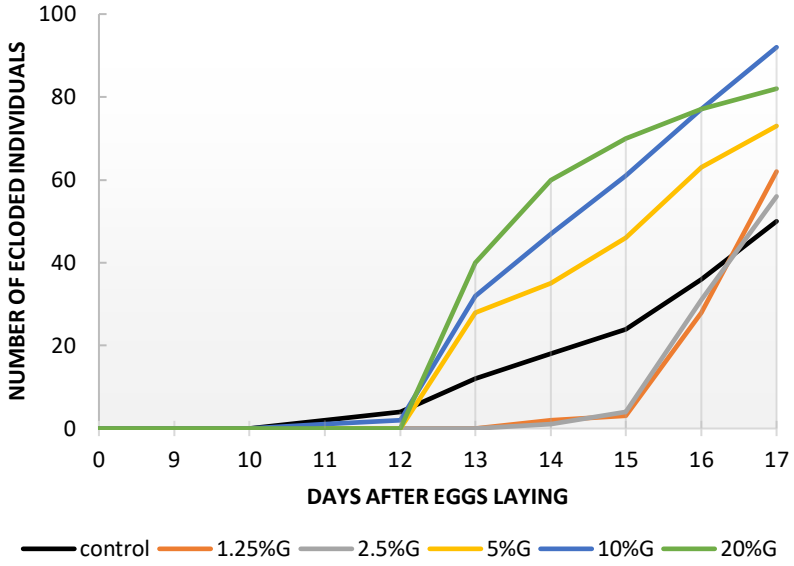

Figure 4.2 - Graph of the number of ecloded individuals in the control and in the conditions with *Grateloupia turuturu* throughout the days after eggs laying (cumulative number of ecloded individuals).

For the DT75 parameter, 20%G in the 15th day after laying eggs was the one that reached at least 75% of total eclosions in first place between the control and the conditions with *Grateloupia turuturu*, with total eclosions superior to those of the control (Table 4.2). Comparatively to control, in the 15th day after eggs laying the percentage of eclosions of 20%G was much higher, from 48% to 85%, as well as the number of eclosions for that day (24 in the control and 70 in 20%G). At 1.25%G and 2.5%G, as previously demonstrated, as eclosions occurred later in relation to control and the other conditions with *Grateloupia*, the percentages of eclosions for that day were very low with 5% for 1.25%G and 7% for 2.5%G, as well as the number of eclosions for the same day, 3 and 4 eclosions respectively. For 5%G and 10%G, a behaviour closer to 20%G was observed, with the number of eclosions for that day of 46 flies in 5%G and 61 flies in 10%G, although unable to reach the 75% of eclosions required for this chosen day.

Table 4.2 - Number of ecloded individuals in the control and in the different conditions with *Grateloupia turuturu* on the 15th and 17th (total eclosions) days after eggs laying, and the percentages of eclosions in day 15 after eggs laying relative to total eclosions (cumulative number of ecloded individuals).

DAYS AFTER EGGS LAYING	CONDITIONS WITH *Grateloupia turuturu*					
	control	1.25%G	2.5%G	5%G	10%G	20%G
15	24	3	4	46	61	70
17	50	62	56	73	92	82
Percentage	48%	5%	7%	63%	66%	85%

Conditions with *Porphyra umbilicalis*

As can be seen in Figure 4.3, in 5%P, 10%P, and 20%P the first eclosions occurred between days 9 and 10, i.e., earlier than in the control. For 5%P and 20%P, on the first day of eclosions, considerable numbers of individuals went from pupae to adult, 17 and 37 respectively. In relation to the lowest concentrations, 1.25%P and 2.5%P, there was an increase in the development time of the fruit flies.

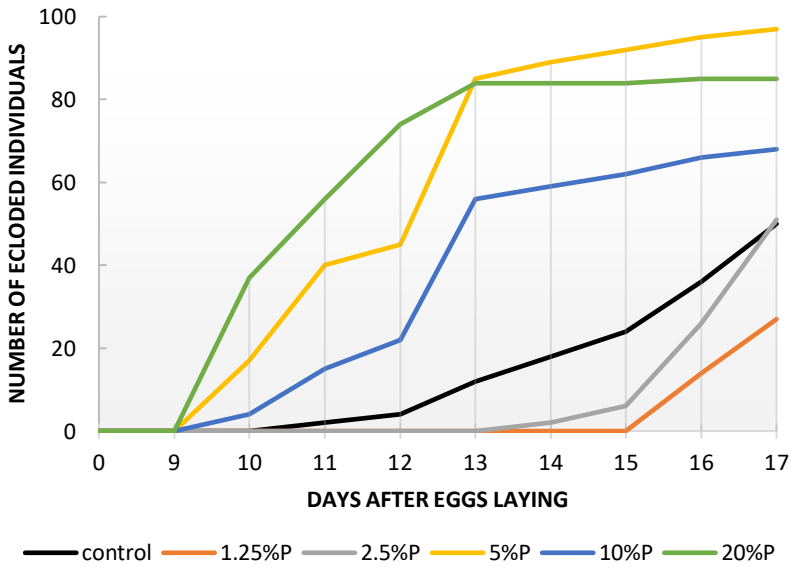

Figure 4.3 - Graph of the number of ecloded individuals in the control and in the conditions with *Porphyra umbilicalis* throughout the days after eggs laying (cumulative number of ecloded individuals).

For the DT75 parameter, it was 20%P which in the 12th day after eggs laying had at least 75% of total eclosions in first place between control and the conditions with *Porphyra umbilicalis*, with a total of eclosions higher than control's (Table 4.3). The percentage of eclosions in this concentration relative to control was much higher (8% in control and 87% in 20%P), as well as the number of eclosions for day 12 after eggs laying (4 in control and 74 in 20%P). For 1.25%P and 2.5%G, on the reference day for the DT75, there was not any emergence of individuals. At 5%P and 10%P the percentage of eclosions was much lower than

75%, although with considerable numbers of eclosions for the 12th day after eggs laying (45 and 22 respectively).

Table 4.3 - Number of ecloded individuals in the control and in the different conditions with *Porphyra umbilicalis* on the 12th and 17th (total eclosions) days after eggs laying, and the percentages of eclosions in day 12 after eggs laying relative to total eclosions (cumulative number of ecloded individuals).

DAYS AFTER EGGS LAYING	CONDITIONS WITH *Porphyra umbilicalis*					
	control	1.25%P	2.5%P	5%P	10%P	20%P
12	4	0	0	45	22	74
17	50	27	51	97	68	85
Percentage	8%	0%	0%	46%	32%	87%

Conditions with *Ulva* sp.

In 2.5%U and 5%U, it was observed that the beginning of eclosions occurred faster than in the control, i.e., individuals started to emerge between days 9 and 10 (in contrast to days 10 and 11 in control), and in large numbers (43 in 2.5%U and 37 in 5%U). For the remaining conditions, 1.25%U, 10%U, and 20%U, the first arisings occurred between days 10 and 11, as in the control, although all of them with a much higher number of individuals. For days 10 to 11, in 1.25%U 24 eclosions occurred, 55 in 10%U, and 19 in 20%U, contrasting with only 2 in control (Figure 4.4).

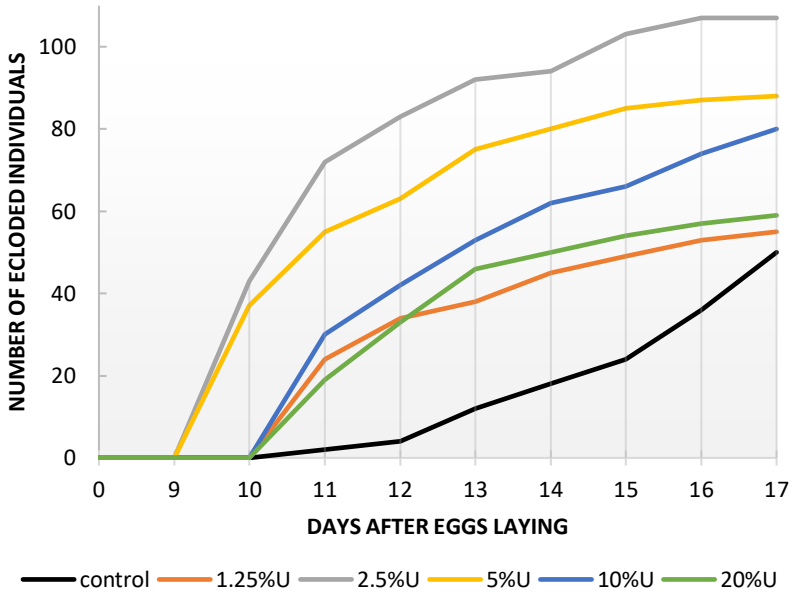

Figure 4.4 - Graph of the number of ecloded individuals in the control and in the conditions with *Ulva* sp. throughout the days after eggs laying (cumulative number of ecloded individuals).

The 2.5%U in the 12th day after eggs laying reached at least 75% of total eclosions in first place between control and the remaining conditions with *Ulva* sp., with a total of eclosions

higher than the control's (Table 4.4). The percentages of eclosions of the conditions with *Ulva* sp. were very similar among them, all being much higher than the 8% of the control. However, the number of adults arising for the 12th day after eggs laying of 2.5%U (83 individuals) stood out in relation to the rest.

Table 4.4 - Number of ecloded individuals in the control and in the different conditions with *Ulva* sp. on the 12th and 17th (total eclosions) days after eggs laying, and the percentages of eclosions in day 12 after eggs laying relative to total eclosions (cumulative number of ecloded individuals).

DAYS AFTER EGGS LAYING	CONDITIONS WITH *Ulva* sp.					
	control	1.25%U	2.5%U	5%U	10%U	20%U
12	4	34	83	63	42	33
17	50	55	107	88	80	59
Percentage	8%	62%	78%	72%	53%	56%

Conditions with *Fucus vesiculosus*

According to Figure 4.5, in 5%F individuals started to emerge between days 9 and 10 after eggs laying (earlier than in control) and in large numbers (21 fruit flies). For 1.25%F, the first emergence of individuals occurred between days 10 and 11 (as in the control), although in greater numbers (2 in control and 9 in 1.25%F). For 2.5%F, adults emergence began only on days 13 and 14, with a low number of individuals until day 15, but then experiencing a practically linear increase up to the 17th day after eggs laying. For 10%F and 20%F, the eclosions started between days 12 and 13.

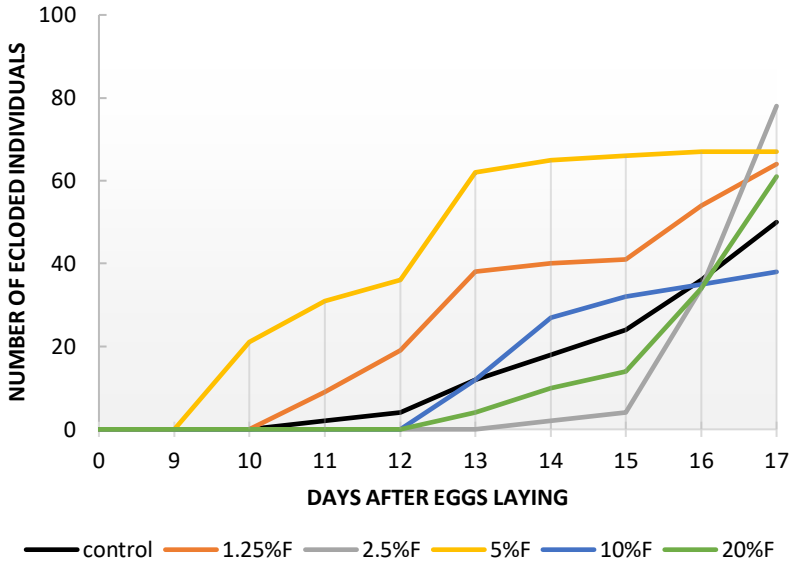

Figure 4.5 - Graph of the number of ecloded individuals in the control and in the conditions with *Fucus vesiculosus* throughout the days after eggs laying (cumulative number of ecloded individuals).

It was 5%F that in the 13th day after laying eggs reached at least 75% of total eclosions in first place between control and the *Fucus vesiculosus* conditions, with a total of eclosions higher than the control's (Table 4.5). For day 13 after eggs laying, it was verified that in 2.5%F there was not a single individual and even in 20%F the number and the percentage of eclosions was lower than those of the control. In 1.25%F and in 10%F, the eclosions for the 13th day were higher than those of the control, as well as the percentages of eclosions, although they had not reached 75%. The 5%F condition was chosen, reaching 93% of the total of eclosions in the 13th day after eggs laying with 62 emerged individuals for that day, a number well above the control and the remaining conditions with *Fucus*.

Table 4.5 - Number of ecloded individuals in the control and in the different conditions with *Fucus vesiculosus* on the 13th and 17th (total eclosions) days after eggs laying, and the percentages of eclosions in day 13 after eggs laying relative to total eclosions (cumulative number of ecloded individuals).

DAYS AFTER EGGS LAYING	CONDITIONS WITH *Fucus vesiculosus*					
	control	1.25%F	2.5%F	5%F	10%F	20%F
13	12	38	0	62	12	4
17	50	64	78	67	38	61
Percentage	24%	59%	0%	93%	32%	7%

4.1.2. Survival

Conditions with *Gracilaria* sp.

For the χ^2 goodness of fit tests performed for comparing the replicates for each condition with *Gracilaria* sp. in terms of total number of eclosions, non-significant results were obtained for all comparisons, χ^2 values(1) < 3.84, *p*s > 0.05. However, in 20%Gr there were not any eclosions, so this concentration did not undergo statistical treatment. In this way, the replicates were summed for each condition with *Gracilaria* sp. in terms of total eclosions.

In Figure 4.6 it is possible to observe the number of total ecloded individuals for the control (50 individuals) and for the conditions with *Gracilaria* sp., 1.25%Gr (98 individuals), 2.5%Gr (5 individuals), 5%Gr (9 individuals), 10%Gr (3 individuals), and 20%Gr (0 individuals). For each condition, the comparison with the control gave statistically significant results with χ^2 tests: control vs. 1.25%Gr, χ^2 value(1, N = 148) = 46.08, p = 1.14E-11; control vs. 2.5%Gr, χ^2 value(1, N = 55) = 40.50, p = 1.97E-10; control vs. 5%Gr, χ^2 value(1, N = 59) = 33.62, p = 6.70E-9; control vs. 10%Gr, χ^2 value(1, N = 53) = 44.18, p = 3.00E-11.

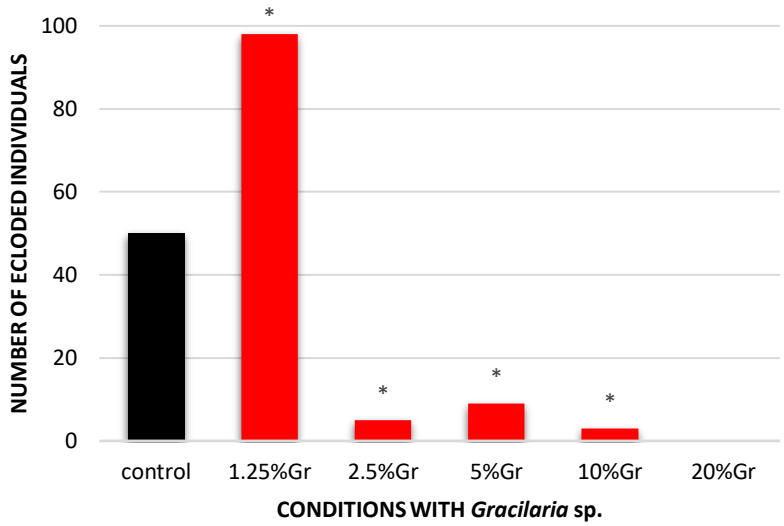

Figure 4.6 - Total number of ecloded individuals in the control and in the different conditions with *Gracilaria* sp. * significant when compared individually with the eclosions in control, χ^2 values(1) > 3.84, *p*s < 0.05.

Despite the significances obtained, it was possible to verify that only in 1.25%Gr the number of ecloded individuals was significantly higher than in the control. For 2.5%Gr, 5%Gr, and 10%Gr, ecloded individuals were significantly inferior than control's eclosions.

The result of Spearman's rank order correlation coefficient was -0.89, which defined a very strong downhill (negative) linear relationship, i.e., the higher the concentration of *Gracilaria* sp. the smaller the number of ecloded individuals.

Conditions with *Grateloupia turuturu*

The χ^2 goodness of fit tests performed for comparing the replicates for each condition with *Grateloupia turuturu* in relation to the total number of eclosions gave non-significant results for 1.25%G, 2.5%G, 10%G, and 20%G, χ^2 values(1) < 3.84, *p*s > 0.05. However, a statistically significant difference appeared between the replicates of 5%G, χ^2 value(1, N = 73) = 9.99, p < 0.002.

The replicates were summed for each condition with *Grateloupia* relatively to the total number of ecloded individuals to compare each of them with the control using χ^2 goodness of fit tests (Figure 4.7). Despite the 5%G significant difference between their replicates, they were summed and treated statistically, nevertheless, this concentration was discarded to be chosen for the SMART assay.

The total numbers of individuals ecloded in 1.25%G (62 individuals), 2.5%G (56 individuals), 5%G (73 individuals), 10%G (92 individuals), and 20%G (82 individuals) were superior relative to control's total eclosions (50 individuals) (Figure 4.7). However, the significance of these comparisons had only been proven to: control vs. 5%G, χ^2 value(1, N =

123) = 10.58, p = 0.001; control vs. 10%G, χ^2 value(1, N = 142) = 35.28, p = 2.86E-9; control vs. 20%G, χ^2 value(1, N = 132) = 20.48, p = 6.03E-6. Thus, 10%G obtained the highest significance, followed by 20%G and 5%G.

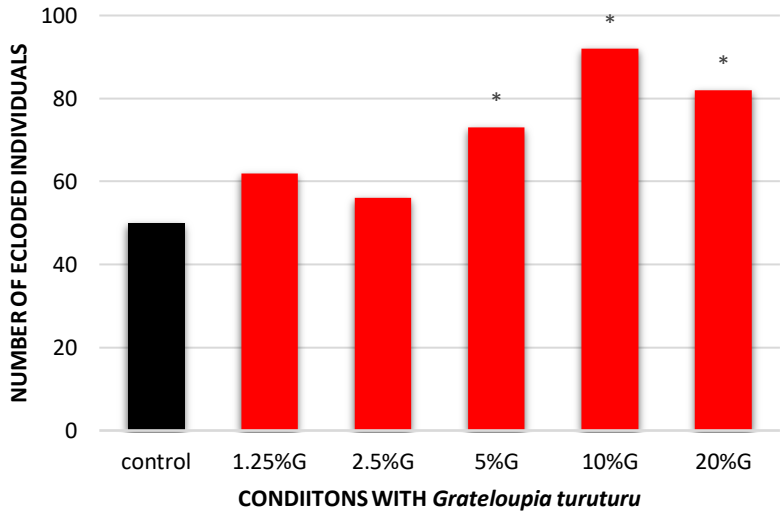

Figure 4.7 - Total number of ecloded individuals in the control and in the different conditions with *Grateloupia turuturu.*
* significant when compared individually with the eclosions in control, χ^2 values(1) > 3.84, ps < 0.05.

The Spearman's rank order correlation coefficient was 0.89, defining a very strong uphill (positive) linear relationship, i.e., the higher the concentration of *Grateloupia turuturu* the greater the number of ecloded fruit flies.

Conditions with *Porphyra umbilicalis*

Non-significant results were obtained for the χ^2 goodness of fit tests performed for comparing the replicates for each condition with *Porphyra umbilicalis* relative to the number of ecloded individuals, χ^2 values(1) < 3.84, ps > 0.05. Thus, the replicates were summed for each condition with *Porphyra* and comparisons with control using χ^2 tests were made.

As can be seen in Figure 4.8, the comparison of the number of eclosions of control vs. 1.25%P gave statistically significant results, χ^2 value(1, N = 77) = 10.58, p = 0.001. However, in 1.25%P only 27 fruit flies ecloded (in contrast to 50 in control), which demonstrated a statistically significance inferior to control. In 2.5%P the number of eclosions was 51, only 1 more fruit fly than in the control, which led to this small difference being considered as non-significant. For 5%P (97 eclosions), 10%P (68 eclosions), and 20%P (85 eclosions), the differences comparatively to the control were significant (statistically superior): control vs. 5%P, χ^2 value(1, N = 147) = 44.18, p = 3.00E-11; control vs. 10%P, χ^2 value(1, N = 118) = 6.48, p = 0.011; control vs. 20%P, χ^2 value(1, N = 135) = 24.50, p = 7.43E-7.

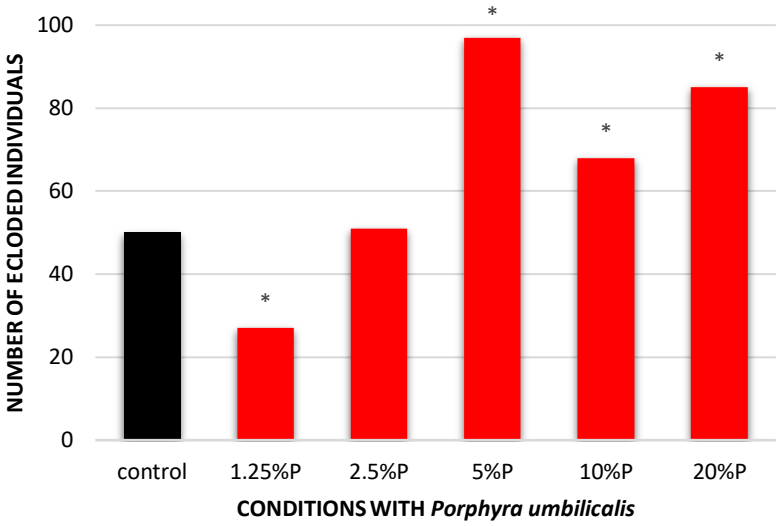

Figure 4.8 - Total number of ecloded individuals in the control and in the different conditions with *Porphyra umbilicalis*.
* significant when compared individually with the eclosions in control, χ^2 values(1) > 3.84, *p*s < 0.05.

The value of Spearman's rank order correlation coefficient was 0.77, a strong uphill linear relationship, i.e., the higher the concentration of *Porphyra umbilicalis* the greater the number of *Drosophila melanogaster* eclosions.

Conditions with *Ulva* sp.

For the χ^2 goodness of fit tests performed for comparing the replicates for each condition with *Ulva* sp., non-significant results were obtained regarding the number of ecloded individuals, χ^2 values(1) < 3.84, *p*s > 0.05. Thus, the replicates were summed for each condition with *Ulva* sp.

Under all conditions with *Ulva* sp. a greater number of eclosions than in control was observed. For 1.25%U (55 fruit flies) and for 20%U (59 fruit flies), despite the numbers of eclosions being higher than in control, the χ^2 tests failed to demonstrate significant differences. For the χ^2 tests performed to compare individually 2.5%U (107 fruit flies), 5%U (88 fruit flies), and 10%U (80 fruit flies) with the control, the results were found to be significant: control vs. 2.5%U, χ^2 value(1, *N* = 157) = 64.98, *p* = 7.57E-16; control vs. 5%U, χ^2 value(1, *N* = 138) = 28.88, *p* = 7.70E-8; control vs. 10%U, χ^2 value(1, *N* = 130) = 18.00, *p* = 2.21E-5. Therefore, these three conditions demonstrated to be statistically superior to the control (Figure 4.9).

A weak/moderate uphill linear relationship was reached in the Spearman's rank order correlation coefficient with 0.43, i.e., the degree of linear association between the concentrations of *Ulva* sp. and the numbers of individuals ecloded is not strong enough to state with safety that the higher the concentration of *Ulva* sp. the higher the numbers of eclosions.

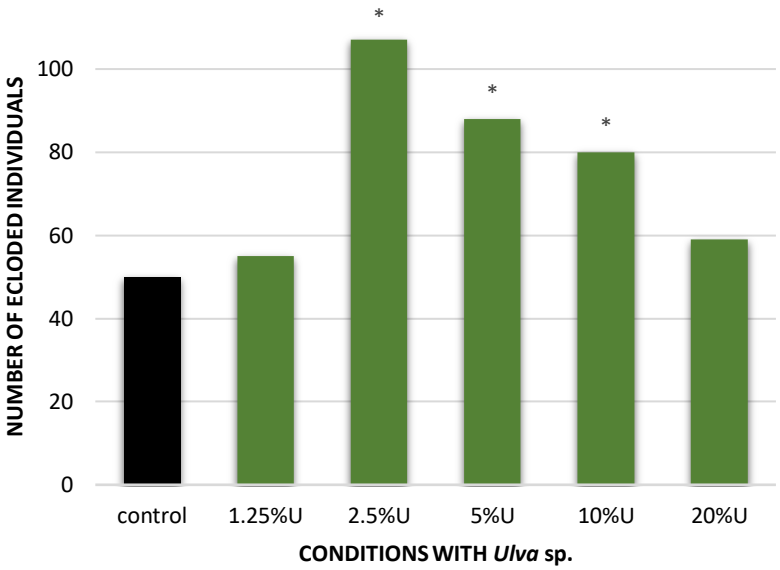

Figure 4.9 - Total number of ecloded individuals in the control and in the different conditions with *Ulva* sp. * significant when compared individually with the eclosions in control, χ^2 values(1) > 3.84, *p*s < 0.05.

Conditions with *Fucus vesiculosus*

For the χ^2 goodness of fit tests performed for comparing the replicates for each condition with *Fucus vesiculosus* in terms of total eclosions, non-significant results were obtained, χ^2 values(1) < 3.84, *p*s > 0.05. Then, the replicates were summed relatively to the total number of eclosions for each condition with *Fucus* and comparisons were made with control through χ^2 tests.

Taking into account Figure 4.10, the numbers of eclosions of 1.25%F (64 fruit flies), 2.5%F (78 fruit flies), and 5%F (67 fruit flies) gave statistically higher results than control: control vs. 1.25%F, χ^2 value(1, N = 114) = 3.92, p = 0.048; control vs. 2.5%F, χ^2 value(1, N = 128) = 15.68, p = 7.50E-5; control vs. 5%F, χ^2 value(1, N = 117) = 5.78, p = 0.016. Relative to 10%F (38 fruit flies), the number of eclosions was lower than control's (without significance) and the remaining concentrations of *Fucus*. In 20%F (61 individuals) a number close to 1.25%F and 5%F was obtained, although statistically non-significant in relation to control.

The result of Spearman's rank order correlation coefficient was -0.14, which defined a very weak downhill linear relationship, i.e., the degree of linear association between *Fucus vesiculosus* concentrations and the number of ecloded individuals was not proven.

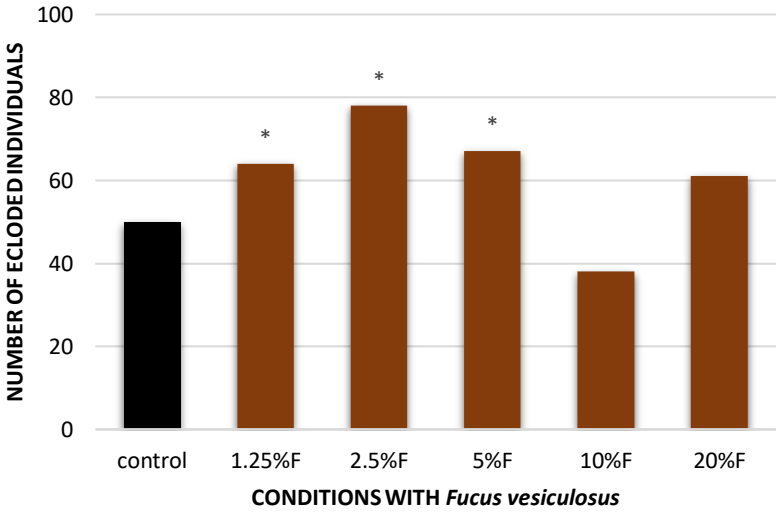

Figure 4.10 - Total number of ecloded individuals in the control and in the different conditions with *Fucus vesiculosus*.
* significant when compared individually with the eclosions in control, χ^2 values(1) > 3.84, *p*s < 0.05.

4.1.3. Sex Ratio

The control resulted in 25 males and 25 females, a perfect 1:1 sex ratio.

Conditions with *Gracilaria* sp.

For the χ^2 goodness of fit tests performed between males and females for each of the following: 1.25%Gr(1), 1.25%Gr(2), 2.5%Gr(2), 5%Gr(1), 5%Gr(2), 10%Gr(1), and 10%Gr(2), non-significant differences occurred, χ^2 values(1) < 3.84, *p*s > 0.05. An exception occurred in the test performed for 2.5%Gr(1), since 4 males ecloded in this concentration but not a single female, χ^2 value(1, $N = 4$) = 4, $p = 0.046$. Relative to 20%Gr(1) and 20%Gr(2) there were no eclosions, as previously mentioned.

In this way, the males of each two replicates for the same condition were summed, happening the same to females (Figure 4.11). Despite the significant differences between males and females of 2.5%Gr(1), they were added with the males and females (respectively) of 2.5%Gr(2) to observe their behaviour in the next statistical treatment.

When comparing with χ^2 tests the males and females in each of the conditions (summed replicates), the maintenance of the previous statistical behaviour was observed in 1.25%Gr, 5%Gr, and 10%Gr with non-significant results, χ^2 values(1) < 3.84, *p*s > 0.05. For 2.5%Gr, there was a significant difference between males (5 individuals) and females (0 individuals), χ^2 value(1, $N = 5$) = 5, $p = 0.025$ (Figure 4.11).

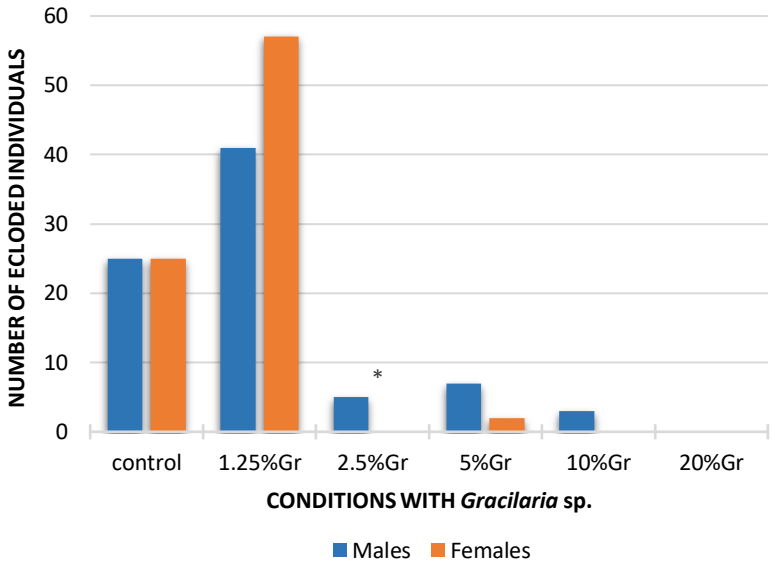

Figure 4.11 - Total number of ecloded males and females in the control and in the different conditions with *Gracilaria* sp.
* significant when comparing males and females of 2.5%Gr, χ^2 value(1, N = 5) > 3.84, p < 0.05.

Conditions with *Grateloupia turuturu*

The results of the χ^2 goodness of fit tests performed between males and females of the replicates with *Grateloupia turuturu* showed non-significant differences, χ^2 values(1) < 3.84, *p*s > 0.05. The males of the replicates of the same condition were summed, and the same happened for the females (Figure 4.12).

When comparing with χ^2 tests males and females in each of the conditions after summing replicates, non-significant results were observed for all comparisons, χ^2 values(1) < 3.84, *p*s > 0.05. Thus, the flies of the conditions with *Grateloupia* displayed a 1:1 sex ratio (Figure 4.12).

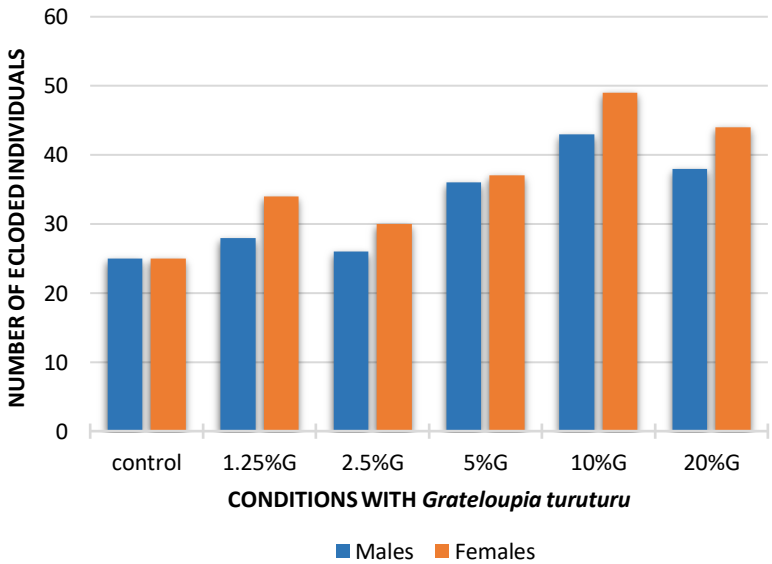

Figure 4.12 - Total number of ecloded males and females in the control and in the different conditions with *Grateloupia turuturu.*

Conditions with *Porphyra umbilicalis*

The results of the χ^2 goodness of fit tests performed between males and females of all *Porphyra umbilicalis* replicates showed non-significant differences, χ^2 values(1) < 3.84, *p*s > 0.05. In this way, the males of each two replicates (for the same condition) were summed, occurring the same for females (Figure 4.13).

In the comparison between males and females in each condition (summed replicates), non-significant results were observed for all comparisons, χ^2 values(1) < 3.84, *p*s > 0.05. Thus, a sex ratio of 1:1 was displayed for all conditions (Figure 4.13).

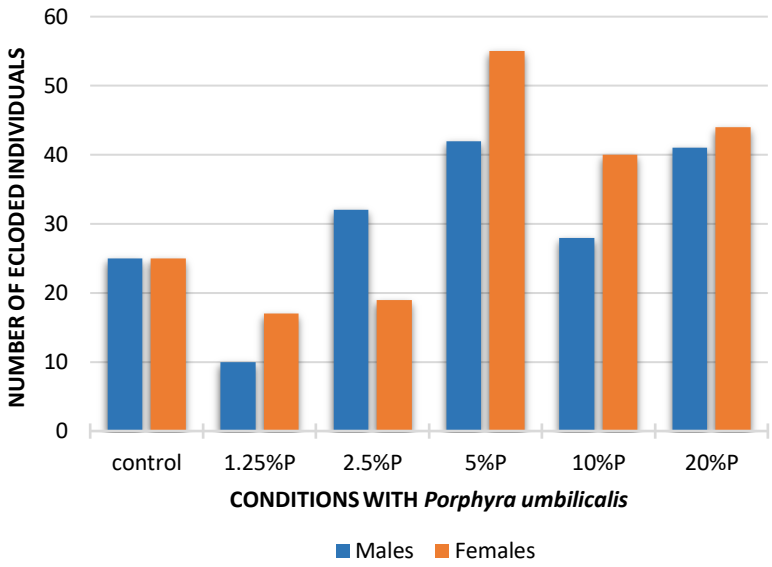

Figure 4.13 - Total number of ecloded males and females in the control and in the different conditions with *Porphyra umbilicalis*.

Conditions with *Ulva* sp.

The conditions with *Ulva* sp. did not present significant differences between the males and females of each replicate, as well as for the comparison between males and females already with the replicates summed for each condition, χ^2 values(1) < 3.84, *p*s > 0.05 (Figure 4.14). Thus, a sex ratio of 1:1 was maintained for all conditions with *Ulva* sp.

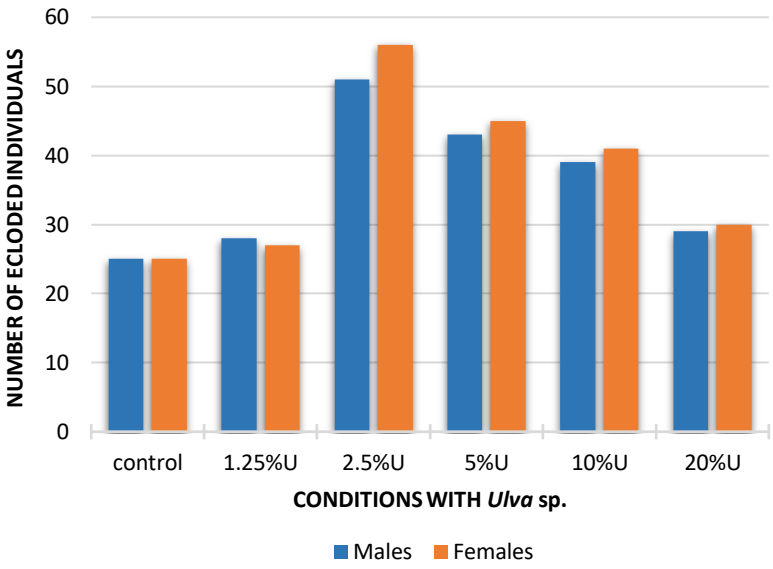

Figure 4.14 - Total number of ecloded males and females in the control and in the different conditions with *Ulva sp.*

Conditions with *Fucus vesiculosus*

For the χ^2 goodness of fit tests performed between males and females of each *Fucus vesiculosus* replicate, there were non-significant differences, χ^2 values(1) < 3.84, *p*s > 0.05, with only 20% F(1) as an exception. In 20%F(1), the eclosion of 6 males in contrast to 20 females resulted in a significant result for this comparison, χ^2 value(1, N = 26) = 7.54, p = 0.006.

Then, the males of each two replicates were summed, occurring the same for females (Figure 4.15). Despite the significant differences between males and females in 20%F(1), these were summed with the males and females (respectively) of 20%F(2) to observe their behaviour in the χ^2 test performed posteriorly.

When comparing with χ^2 goodness of fit tests the males and females in each condition (after summing), non-significant differences were observed for all comparisons, including in 20%F, thus displaying a 1:1 sex ratio for all conditions (Figure 4.15). The significant difference obtained for 20%F(1) was annulled when joining the fruit flies ecloded in 20%F(2) to the fruit flies ecloded in 20%F(1).

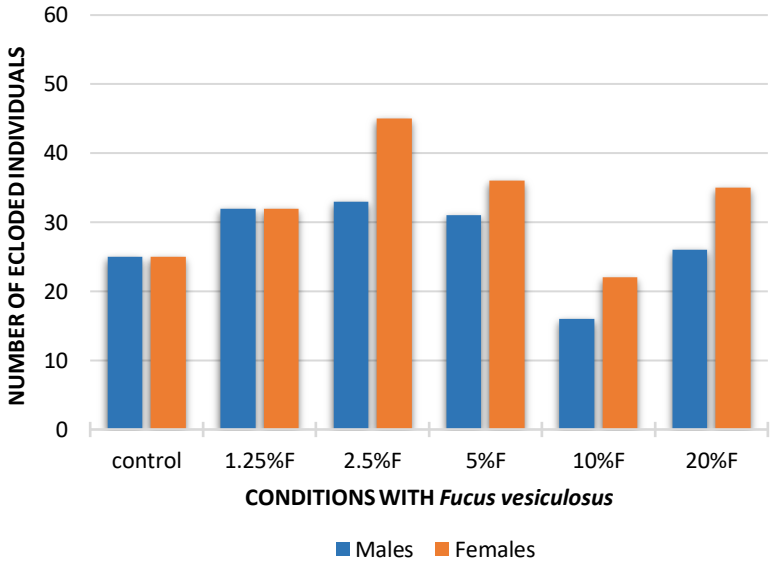

Figure 4.15 - Total number of ecloded males and females in the control and in the different conditions with *Fucus vesiculosus.*

4.2. Longevity Assay

As referred in the previous chapter, and analysing subchapter 4.1, the two seaweeds from initial toxicological screening that presented the strongest uphill linear relationships from Spearman's rank order correlation coefficient, *Grateloupia turuturu* and *Porphyra umbilicalis,* were selected to be followed in the longevity assay.

The information stated in the beginning of subchapter 4.1 relative to the abbreviations for *Grateloupia turuturu* and *Porphyra umbilicalis* is intended to be followed in this subchapter.

The comparison between males and females of the control relative to survivors per day demonstrated statistically non-significant results for Mantel-Cox test, χ^2 value(1) < 3.84, p > 0.05, as well as for Wang-Allison tests, ps > 0.05, i.e, mean, median, and maximum longevities were equal between males and females for the control.

4.2.1. Conditions with *Grateloupia turuturu*

Males

Comparing the replicates for each condition with *Grateloupia turuturu* relative to the behaviour of males' longevity, were obtained non-significant results when comparing mean longevities (Mantel-cox tests), χ^2 values(1) < 3.84, ps > 0.05, and non-significant results for median and maximum longevities (Wang-Allison tests), ps > 0.05. In this way, replicates were summed for each condition relative to the number of surviving males on each day. Kaplan-

Meier survival analysis was performed to obtain survivorship curves (Figure 4.16) and mean, median, and maximum longevities (Table 4.6) for each condition.

Taking into account Figure 4.16, a quite disparate behaviour was observed between males of the different conditions with *Grateloupia* and the control relative to their survivorship over time. However, the curve of 20%G has stood out almost all the time with more percent survivorship, as it is possible to prove with the information presented in Table 4.6.

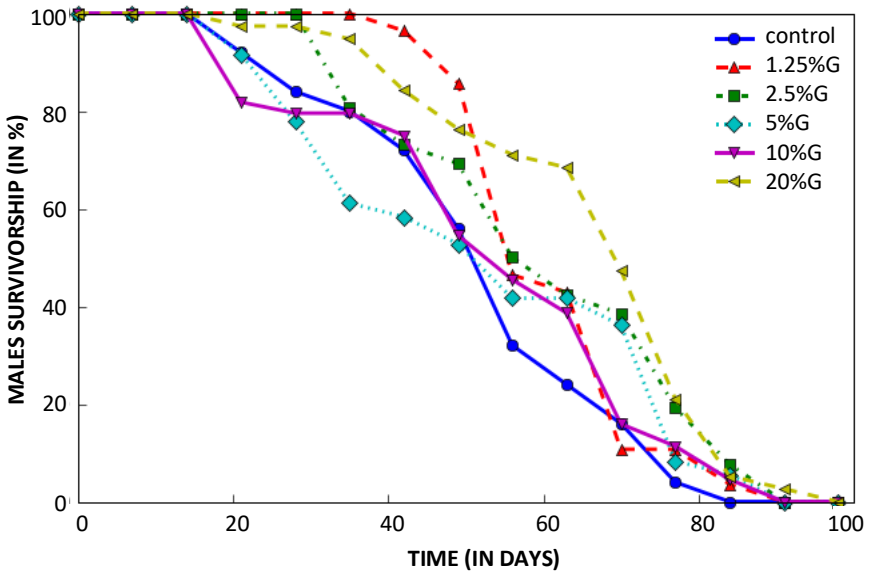

Figure 4.16 - Percentage of male survivors in the control and in the different conditions with *Grateloupia turuturu* throughout the days (survivorship curves).

For the mean longevity (Table 4.6), an increase of 27.09% relative to control was observed for the 20%G males (from 53.20 days to 67.61 days). For the remaining conditions there were also increases in relation to the control, although less remarkable.

Table 4.6 - Mean, median, and maximum longevities obtained for the males of the conditions with *Grateloupia turuturu* after performing Kaplan-Meier survival analysis. Mean longevity has SD and 95% CI associated, and median longevity has 95% CI. The control (males) is present as well for comparison purposes.

Grateloupia CONDITIONS (MALES)	*n*	Mean longevity (DAYS)	*SD*	95% CI	Median longevity (DAYS)	95% CI	Maximum longevity (DAYS)
control	25	53.20	3.45	[46.43, 59.97]	56	[49, 56]	77
1.25%G	28	62.75	2.16	[58.51, 66.99]	63	[56, 63]	84
2.5%G	26	61.65	3.67	[54.46, 68.85]	63	[56, 70]	84
5%G	36	54.25	3.77	[46.85, 61.65]	56	[35, 70]	84
10%G	44	55.05	3.15	[48.88, 61.21]	56	[49, 63]	84
20%G	38	67.61	2.78	[62.15, 73.06]	70	[70, 70]	84

Statistically higher results were proved for 20%G. Comparing control vs. 20%G relative to: mean longevity, the Mantel-Cox test showed a significant result, χ^2 value(1) = 10.50, p = 0.001; median longevity, the Wang-Allison test also showed a statistically significant result, p = 0.011. Comparing 1.25%G vs. 20%G the same was demonstrated: for mean longevity, the Mantel-Cox test gave a significant result, χ^2 value(1) = 3.88, p = 0.049; for median longevity, the Wang-Allison test also showed a significant result, p = 0.002. For 10%G vs. 20%G as well: for mean longevity, Mantel-Cox test showed a significant result, χ^2 value(1) = 6.39, p = 0.012; for median longevity a significant result performing Wang-Allison was displayed, p = 0.002.

For the remaining comparisons, there were non-significant differences between mean longevities, χ^2 values(1) < 3.84, ps > 0.05, and median longevities, ps > 0.05. Maximum longevities differences were found to be non-significant for all comparisons, ps > 0.05 (Wang-Allison tests).

Females

For the comparisons between the replicates for each condition with *Grateloupia turuturu* relative to the longevity of females, were obtained non-significant results when comparing mean longevities (Mantel-cox tests), χ^2 values(1) < 3.84, ps > 0.05, and non-significant results for median and maximum longevities (Wang-Allison tests), ps > 0.05. The replicates for each condition were summed in terms of surviving females per day. Kaplan-Meier survival analysis was performed to obtain survivorship curves (Figure 4.17) and mean, median, and maximum longevities for each condition (Table 4.7).

Taking into account Figure 4.17, 20%G stood out comparatively to the control curve and the curves of the other conditions. The fact that 20%G presented a higher percentage of survivors on most days relative to the other curves, led to the focus of this survivorship curve.

For the mean longevity of 20%G, there was an increase of 22.05% with respect to the mean longevity of the control (from 54.88 days to 66.98 days). For the remaining conditions, there were also increases in relation to the control, although less remarkable (Table 4.7).

Statistically superior results were demonstrated for 20%G. Comparing control vs. 20%G relative to: mean longevity, the Mantel-Cox test gave a significant result, χ^2 value(1) = 5.23, p = 0.022; median longevity, a significant result occurred using Wang-Allison test, p = 0.005. Comparing 1.25%G vs. 20%G: Mantel-Cox test (mean longevity) showed a significant result, χ^2 value(1) = 4.02, p = 0.045; Wang-Allison test (median longevity) also showed a significant result, p = 3.00E-4. For 2.5%G vs. 20%G, a significance occurred for the median longevity, p

= 0.008. For 10%G vs. 20%G, relative to: mean longevity, the Mantel-Cox test gave a significance, χ^2 value(1) = 6.29, p = 0.012; median longevity, the Wang-Allison test also showed a significant difference, p = 0.025. Also for a comparison without 20%G, 1.25%G vs. 10%G, significant higher results occurred relative to median longevity for 10%G (p = 0.045) and relative to maximum longevity for 1.25%G (p = 0.045) using Wang-Allison tests.

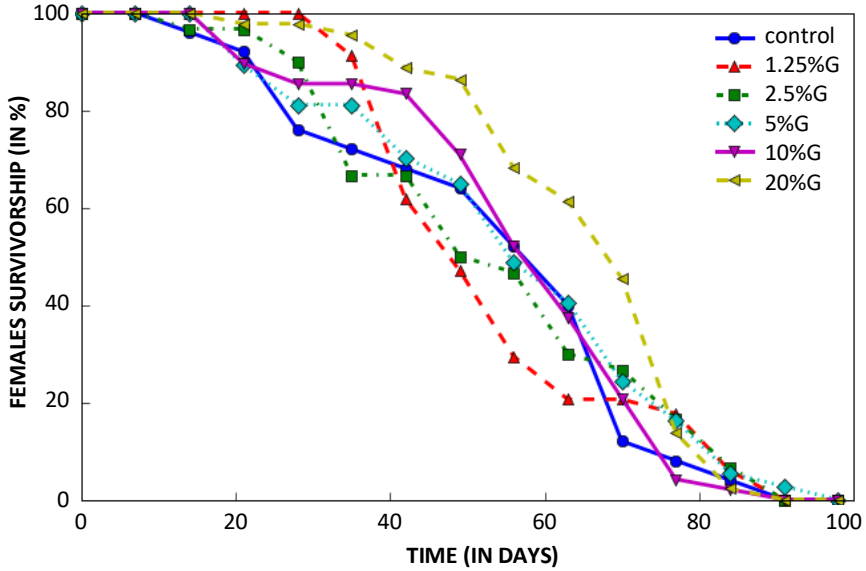

Figure 4.17 - Percentage of female survivors in the control and in the different conditions with *Grateloupia turuturu* throughout the days (survivorship curves).

Table 4.7 - Mean, median, and maximum longevities obtained for the females of the conditions with *Grateloupia turuturu* after performing Kaplan-Meier survival analysis. Mean longevity has *SD* and 95% CI associated, and median longevity has 95% CI. The control (females) is present as well for comparison purposes.

Grateloupia CONDITIONS (FEMALES)	*n*	Mean longevity (DAYS)	*SD*	95% CI	Median longevity (DAYS)	95% CI	Maximum longevity (DAYS)
control	25	54.88	4.15	[46.75, 63.01]	63	[42, 63]	77
1.25%G	34	55.59	2.90	[49.91, 61.26]	49	[42, 49]	84
2.5%G	30	55.53	3.83	[48.02, 63.05]	49	[35, 56]	84
5%G	37	57.70	3.50	[50.84, 64.57]	56	[49, 63]	84
10%G	48	58.19	2.59	[53.12, 63.25]	63	[56, 63]	77
20%G	44	66.98	2.25	[62.56, 71.40]	70	[63, 70]	84

For the remaining comparisons there were non-significant differences between mean longevities, χ^2 values(1) < 3.84, ps > 0.05, and also for median and maximum longevities (ps > 0.05).

Males vs. Females

When comparing the longevity of the fruit flies for both sexes, non-significant differences were found for all comparisons. For mean longevity, χ^2 values < 3.84, *p*s > 0.05, and for median and maximum longevities, *p*s > 0.05 (Table 4.8). I.e., the differences of longevity observed between the two sexes after ingestion of different concentrations of *Grateloupia turuturu* in the larval phase were due to chance.

Table 4.8 - Comparisons of the mean, median, and maximum longevities for males and females of the same condition with *Grateloupia turuturu*. Mean longevity was compared with Mantel-Cox test; median and maximum longevities were compared with Wang-Allison test.

COMPARISONS	MEAN LONGEVITY (DAYS)		MEDIAN LONGEVITY (DAYS)	MAXIMUM LONGEVITY (DAYS)
	χ^2 value	*p*	*p*	*p*
1.25%G males vs. 1.25%G females	2.09	0.149	0.179	0.974
2.5%G males vs. 2.5%G females	0.60	0.437	0.850	0.996
5%G males vs. 5%G females	0.10	0.758	0.629	1
10%G males vs. 10%G females	0.03	0.856	0.549	0.240
20%G males vs. 20%G females	0.48	0.487	0.914	0.673

4.2.2. Conditions with *Porphyra umbilicalis*

Males

The comparisons between the replicates for each condition with *Porphyra umbilicalis* regarding the longevity of males displayed non-significant results when comparing mean longevity (Mantel-cox tests), χ^2 values(1) < 3.84, *p*s > 0.05, and median and maximum longevities (Wang-Allison tests), *p*s > 0.05. In this way, the replicates were summed for each condition in terms of surviving males per day. Survivorship curves (Figure 4.18) and mean, median, and maximum longevities were obtained for each condition after Kaplan-Meier survival analysis (Table 4.9).

In Figure 4.18, the focus goes to the survivorship curves of 10%P and 20%P comparatively to the control and the remaining conditions with *Porphyra*. Following the behaviour of these two conditions, and using the longevity parameters (Table 4.9), it was possible to verify differences relative to mean and median longevities of these conditions compared to the control and the remaining conditions with *Porphyra*. Relative to maximum longevity, males of 10%P and 20%P presented a higher value than control, although similar to the other conditions.

Focusing on 10%P and 20%P, mean longevities increased 17.95% (62.75 days) and 14.15% (60.73 days), respectively, in relation to the control (53.20 days). Increases were also observed for the remaining conditions, although lower, and at 1.25%P mean longevity reached the same value as the control, but with a *SD* and 95% CI higher than those of control (Table 4.9).

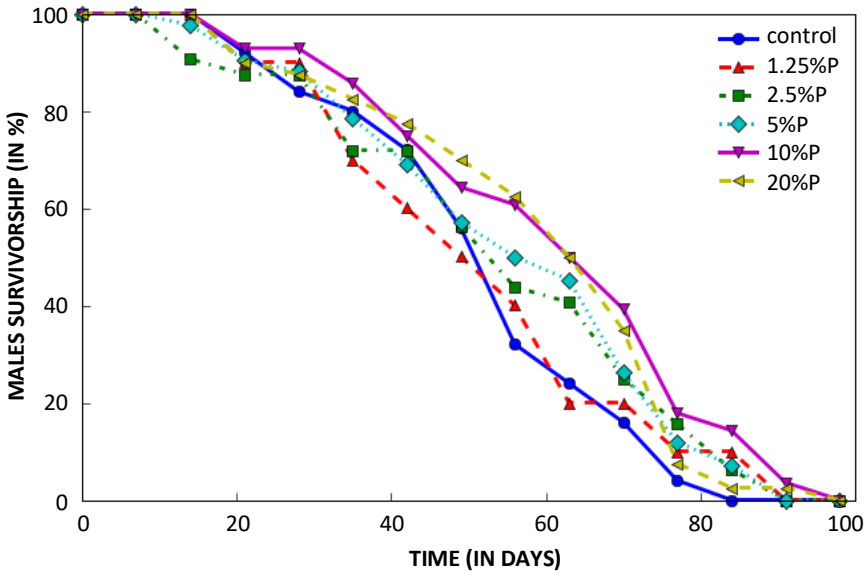

Figure 4.18 - Percentage of male survivors in the control and in the different conditions with *Porphyra umbilicalis* throughout the days (survivorship curves).

Table 4.9 - Mean, median, and maximum longevities obtained for the males of the conditions with *Porphyra umbilicalis* after performing Kaplan-Meier survival analysis. Mean longevity has *SD* and 95% CI associated, and median longevity has 95% CI. The control (males) is present as well for comparison purposes.

Porphyra CONDITIONS (MALES)	*n*	Mean longevity (DAYS)	*SD*	95% CI	Median longevity (DAYS)	95% CI	Maximum longevity (DAYS)
control	25	53.20	3.45	[46.43, 59.97]	56	[49, 56]	77
1.25%P	10	53.20	6.35	[40.75, 65.65]	56	[35, 56]	91
2.5%P	32	55.78	3.97	[48.00, 63.56]	56	[49, 63]	84
5%P	42	57.50	3.24	[51.16, 63.84]	56	[49, 63]	84
10%P	28	62.75	3.98	[54.96, 70.54]	63	[49, 70]	91
20%P	40	60.73	3.14	[54.56, 66.89]	63	[56, 63]	84

Statistically superior results were proved for 10%P when comparing control vs. 10%P relative to: mean longevity, the Mantel-Cox test displayed a significant result, χ^2 value(1) = 4.69, p = 0.030; median longevity, the Wang-Allison test showed, as well, a significant result, p = 0.039. Although, when comparing the control vs. 20%P, median longevity displayed a significant superior result for 20%P (Wang-Allison test), p = 0.045.

For the remaining comparisons, there were non-significant differences between mean longevities, χ^2 values(1) < 3.84, *p*s > 0.05, neither median and maximum longevities, *p*s > 0.05.

Females

The longevity comparisons made between the females of the replicates for each condition with *Porphyra umbilicalis* led to non-significant results when comparing mean longevities (Mantel-cox tests), χ^2 values(1) < 3.84, *p*s > 0.05, and median and maximum longevities (Wang-Allison tests), *p*s > 0.05. Thus, survivorship curves (Figure 4.19) and mean, median, and maximum longevities were obtained for each condition (Table 4.10) after the sum of the surviving females per day between the replicates for each condition and Kaplan-Meier analysis.

In Figure 4.19, the survivorship percentage per day of 10%P stood out relative to control and the other conditions with *Porphyra umbilicalis*. Over the course of days, the percentage of live individuals in this condition decreased more slowly than in the control and in the remaining conditions, which positively influenced mean, median, and maximum longevities (Table 4.10).

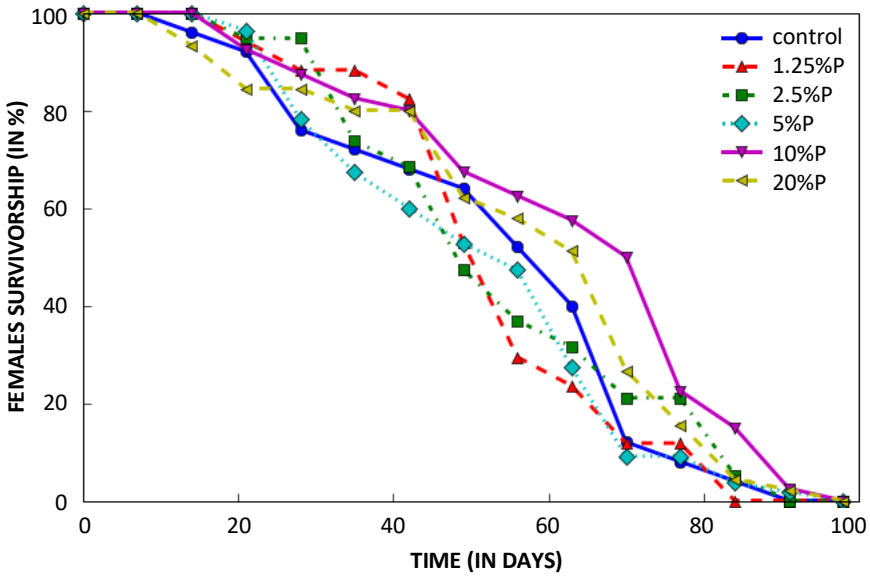

Figure 4.19 - Percentage of female survivors in the control and in the different conditions with *Porphyra umbilicalis* throughout the days (survivorship curves).

For the females of 10%P, mean longevity increased 17.35% (64.40 days) in relation to the control (54.88 days). For 2.5%P and 20%P mean longevity increased as well, although lower than for 10%P, 1.37% and 7.43% respectively (Table 4.10). Some longevity parameters of the females from the conditions with *Porphyra* displayed inferior results to those of the control. At 1.25%P the mean longevity of females decreased, as well as in 5%P, being both percentages negative (-0.22% and -4.16% respectively). The median longevities of 1.25%P, 2.5%P, and 5%P were lower than control's median longevity (Table 4.10).

Table 4.10 - Mean, median, and maximum longevities obtained for the females of the conditions with *Porphyra umbilicalis* after performing Kaplan-Meier survival analysis. Mean longevity has *SD* and 95% CI associated, and median longevity has 95% CI. The control (females) is present as well for comparison purposes.

Porphyra CONDITIONS (FEMALES)	*n*	Mean longevity (DAYS)	*SD*	95% CI	Median longevity (DAYS)	95% CI	Maximum longevity (DAYS)
control	25	54.88	4.15	[46.75, 63.01]	63	[42, 63]	77
1.25%P	17	54.76	3.92	[47.09, 62.44]	56	[49, 56]	84
2.5%P	19	55.63	4.53	[46.76, 64.50]	49	[42, 63]	84
5%P	55	52.69	2.64	[47.51, 57.87]	56	[42, 56]	77
10%P	40	64.40	3.49	[57.55, 71.25]	70	[56, 70]	91
20%P	45	58.96	3.33	[52.42, 65.49]	70	[49, 63]	84

Statistically superior results were proved for 10%P. Comparing control vs. 10%P relative to: mean longevity, the Mantel-Cox test showed a significant result, χ^2 value(1) = 5.46, p = 0.019; median longevity, the Wang-Allison test displayed a significant result as well, p = 0.002. Comparing 1.25%P vs. 10%P relative to: mean longevity, the Mantel-Cox test gave a significant result, χ^2 value(1) = 5.32, p = 0.021; median longevity, with Wang-Allison giving a significant p, p = 0.019. Comparing 5%P vs. 10%P, regarding to: mean longevity, the Mantel-Cox test showed a significant result, χ^2 value(1) = 9.02, p = 0.003; median longevity, Wang-Allison test displayed a p = 0.003, a significant result. And comparing 10%P vs. 20%P regarding to median longevity, the Wang-Allison showed its significance, p = 0.031.

The comparison of females of 5%P vs. 20%P showed a significant superior result for the median longevity of 20%P, p = 0.016. For the remaining comparisons, there were non-significant differences between mean longevities, χ^2 values(1) < 3.84, ps > 0.05, and median longevities, p > 0.05. Maximum longevities differences were found to be non-significant for all comparisons, p > 0.05, using Wang-Allison tests.

Males vs. Females

When comparing the longevity of the fruit flies for both sexes, non-significant differences were found for all of the comparisons: for mean longevities, χ^2 values < 3.84, ps > 0.05, and for median and maximum longevities, ps > 0.05 (Table 4.11). I.e., the differences in longevity observed between the two sexes after intake of different concentrations of *Porphyra umbilicalis* in the larval phase were due to chance.

Table 4.11 - Comparisons of the mean, median, and maximum longevities for males and females of the same condition with *Porphyra umbilicalis*. Mean longevity was compared with Mantel-Cox test; median and maximum longevities were compared with Wang-Allison test.

COMPARISONS	MEAN LONGEVITY (DAYS)		MEDIAN LONGEVITY (DAYS)	MAXIMUM LONGEVITY (DAYS)
	χ^2 value	*p*	*p*	*p*
1.25%P males vs. 1.25%P females	0.01	0.912	0.632	0.234
2.5%P males vs. 2.5%P females	0.02	0.891	0.658	0.999
5%P males vs. 5%P females	1.66	0.197	0.825	0.586
10%P males vs. 10%P females	0.18	0.673	0.515	1
20%P males vs. 20%P females	0.002	0.970	0.466	0.977

4.3. SMART

As referred in the previous chapter, and analysing initial toxicological screening results, the two lowest concentrations from *Grateloupia turuturu* and *Porphyra umbilicalis* with a survival significantly higher than the control's survival and with a DT75 inferior than the control's DT75 were chosen for SMART: 5%P, 10%P, 10%G, and 20%G.

For this subchapter (and the referent in Chapter 5), when using 5%P, 10%P, 10%G, and 20%G, it is intended to refer to the respective seaweed concentration, but also the instant medium and PBS. When using 5%P+SN, 10%P+SN, 10%G+SN, and 20%G+SN, it refers to the concentrations of the respective seaweeds, the instant medium, and the PBS with SN. In order to indicate the replicates of each of the previous conditions and also of NC and PC, the numbers 1, 2, or 3 are used after the abbreviations.

4.3.1. Survival

The χ^2 tests performed to compare the three replicates for each of the 10 conditions in terms of survival demonstrated to be non-significant for most conditions, χ^2 values(2) < 5.99, *p*s > 0.05. However, for 20%G and for PC the results showed significant differences between replicates, χ^2 values(2) > 5.99, *p*s < 0.05. The three replicates were summed for each condition, and χ^2 tests were performed to compare survivals between conditions.

The conditions 10%P (711 eclosions), 10%G (849 eclosions), and 20%G (693 eclosions), showed numbers of eclosions statistically superior to those of NC (643 eclosions), χ^2 values(1) > 3.84, *p*s < 0.05. For the conditions with seaweed plus SN, 5%P+SN (606 eclosions), 10%P+SN (659 eclosions), 10%G+SN (654 eclosions), and 20%G+SN (645 eclosions),

statistically higher numbers of eclosions were verified relative to PC (521 eclosions), χ^2 values(1) > 3.84, *p*s < 0.05 (Figure 4.20).

Only the condition with 5%P (compared to NC) obtained a non-significant result, χ^2 value(1, *N* = 1,285) = 0.003, *p* = 0.969. This significance was due to the eclosion of 642 individuals in 5%P, 1 individual less than in NC (Figure 4.20).

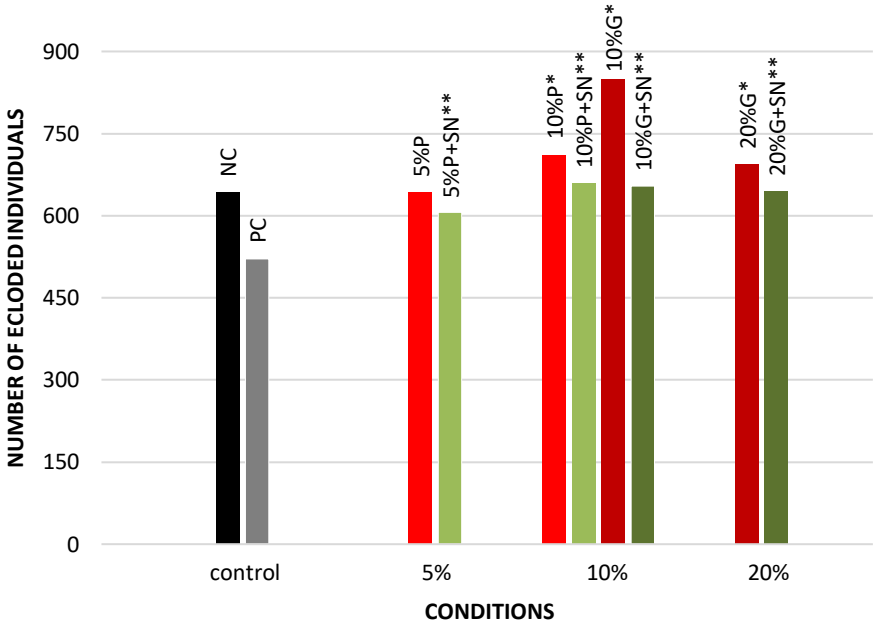

Figure 4.20 - Number of ecloded fruit flies in the 10 different conditions used in SMART.
* significant when the comparison was between NC, χ^2 values(1) > 3.84, *p*s < 0.05; ** significant when the comparison was between PC, χ^2 values(1) > 3.84, *p*s < 0.05.

A statistically superior result was obtained for NC when comparing NC vs. PC, χ^2 value(1, *N* = 1,164) = 12.79, *p* = 3.49E-4.

For the comparison of 10%G vs. 20%G a statistical difference was also observed, χ^2 value(1, *N* = 1,542) = 18.57, *p* = 1.63E-5, demonstrating a statistically superior difference of 10%G in relation to 20%G. For 5%P vs. 10%P, 5%P+SN vs. 10%P+SN, and 10%G+SN vs. 20%G+SN, non-significant differences were found, χ^2 values(1) < 3.84, *p*s > 0.05.

In the comparisons of the same condition with and without SN, significant results were obtained for: 10%G vs. 10%G+SN, χ^2 value(1, *N* = 1,503) = 43.45, *p* = 4.34E-11, being the number of ecloded individuals of 10%G statistically higher than the individuals of the condition with SN; 20%G vs. 20%G+SN with the number of eclosions of 20%G being statistically superior in relation to 20%G+SN, χ^2 value(1, *N* = 1,338) = 6.69, *p* = 0.010. For 5%P vs. 5%P+SN and 10%P vs. 10%P+SN, non-significant differences were found, χ^2 values(1) < 3.84, *p*s > 0.05.

4.3.2. Sex Ratio

For the χ^2 goodness of fit tests performed to verify if there were differences between males and females of each replicate in each condition, non-significant results were obtained for all, χ^2 values(1) < 3.84, *p*s > 0.05. Thus, replicates were summed for each condition in terms of numbers of ecloded males and numbers of ecloded females. The χ^2 tests performed between the numbers of males and numbers of females in each condition (after summed), resulted in non-significant differences for conditions without SN (Figure 4.21) and conditions with SN (Figure 4.22), χ^2 values(1) < 3.84, *p*s > 0.05.

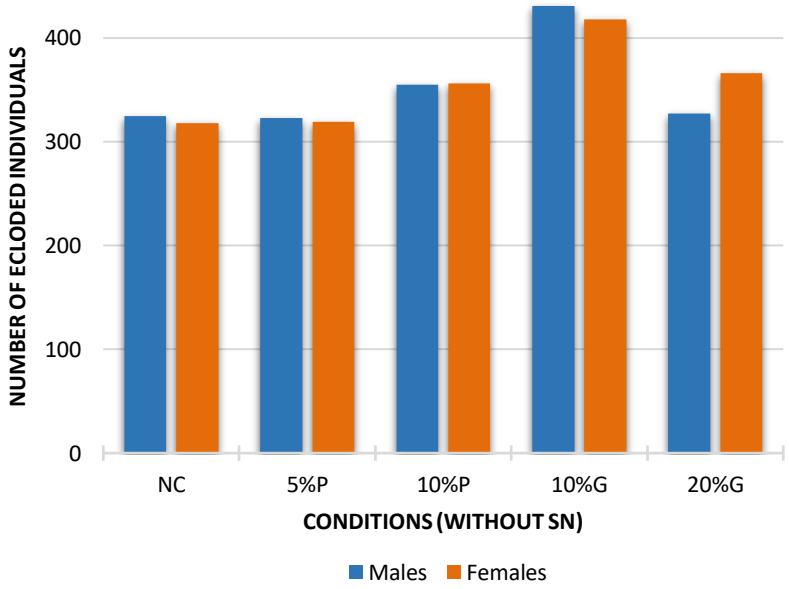

Figure 4.21 - Number of ecloded males and females (fruit flies) in the conditions without SN used in SMART.

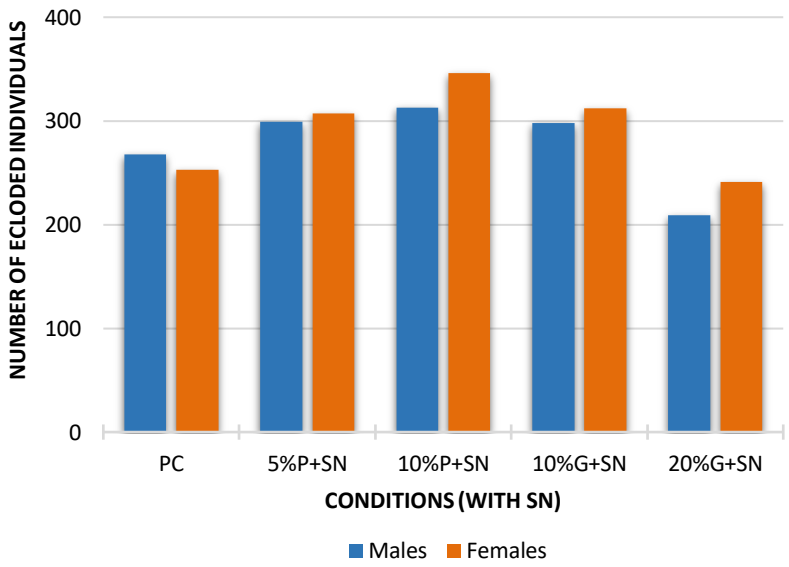

Figure 4.22 - Number of ecloded males and females (fruit flies) in the conditions with SN used in SMART.

4.3.3. Spots per 400 eyes

The χ^2 goodness of fit tests performed between the three replicates of each condition relative to spots per 100 eyes gave non-significant results for all conditions**,** χ^2 values(2) < 5.99, *p*s > 0.05. The replicates were summed in terms of total spots for each condition, resulting in spots per 400 eyes for each one (Figure 4.23).

It is visible in Figure 4.23 that the parameter spots per 400 eyes resulted in decreases in 5%P (42 spots), 10%P (36 spots), 10%G (38 spots), and 20%G (33 spots) relative to NC (60 spots). The multiple-decision procedure results (after χ^2 test for proportions) for NC vs. 10%P, NC vs. 10%G, and NC vs. 20%G, showed that the number of spots per 400 eyes decreased by half in 10%P, 10%G, and 20%G in relation to NC. Relative to NC vs. 5%P, the multiple-decision procedure resulted in inconclusive (Table 4.12).

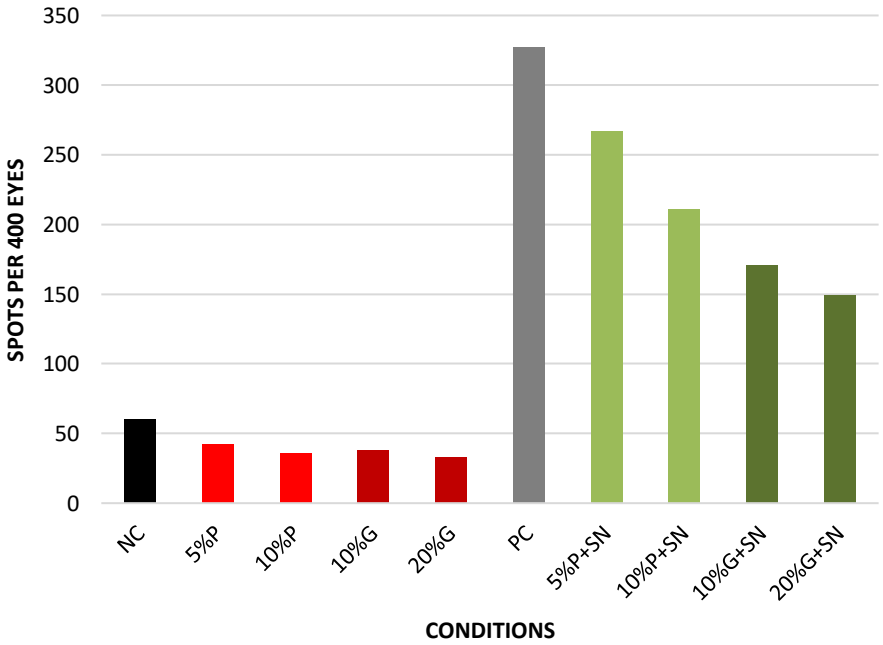

Figure 4.23 - Spots per 400 eyes in the different SMART conditions.

Taking into account Figure 4.23 and Table 4.12, when comparing 5%P vs. 10%P, 10%G vs. 20%G, and also 10%P vs. 10%G, the statistical testing showed negative results, meaning that the differences observed between these conditions where due to chance.

The spots verified in the flies of 5%P+SN (267 spots), 10%P+SN (211 spots), 10%G+SN (171 spots), and 20%G+SN (149 spots) decreased relative to PC's spots (327 spots). The multiple-decision results for PC vs. 10%G+SN and PC vs. 20%G+SN showed the reduction of

the spots by half in 10%G+SN and 20%G+SN in relation to the PC. Relative to PC vs. 5%P+SN and PC vs. 10%P+SN, the results were weak positive (Figure 4.23; Table 4.12).

Table 4.12 - Multiple-decision procedure results for the comparisons of the spots per 400 eyes between the different SMART conditions.

	NC								
5%P	i	5%P							
10%P	+	-	10%P						
10%G	+		-	10%G					
20%G	+			-	20%G				
PC	+					PC			
5%P+SN		+				w^+	5%P+SN		
10%P+SN			+			w^+	w^+	10%P+SN	
10%G+SN				+		+		w^+	10%G+SN
20%G+SN					+	+			-

diagnostic	m = 0.5
diagnostic	m = 5

+ (positive result); w^+ (weak positive result); - (negative result); i (inconclusive result).

Taking into account Figure 4.23 and Table 4.12, in the comparison of 5%P+SN vs. 10%P+SN and 10%P+SN vs. 10%G+SN the multiple-decision showed weak positive results, and in 10%G+SN vs. 20%G+SN a negative result occurred.

For SN conditions the spots were much higher than the spots obtained for the fruit flies of the conditions without SN (Figure 4.23). Comparing each of the conditions without SN with their equivalents with SN, positive results were obtained for all comparisons, with a m = 5, i.e., the number of spots per 400 eyes in the fruit flies of the conditions with SN were five times higher than the spots in the fruit flies of the conditions without SN (Table 4.12).

For the inconclusive result shown in Table 4.12, one-tailed Mann-Whitney *U* test demonstrated that the difference between the spots per 400 eyes was non-significant, $z > -1.645$, $p > 0.05$, i.e., the difference of spots for NC vs. 5%P was only due to chance.

4.3.4. IP

For all conditions with seaweed plus SN, inhibitory results of the number of spots in relation to the PC occurred, with increasing IPs the higher the seaweed concentration used, i.e., 5%P+SN displayed a 21.72% inhibition of spots but 10%P+SN displayed a higher IP (38.12%). The same happened for the conditions with 10%G+SN (49.89%) and 20%G+SN (56.33%). Comparing the same concentration for the two seaweeds, a higher IP was displayed in 10%G+SN comparatively to 10%P+SN (Figure 4.24).

Concerning the conditions with seaweed but without SN, there were also inhibitory results of the number of spots but in relation to the NC. Inhibition was also enhanced with the increasing concentration of seaweed used. In 5%P, a IP of 29.93% was obtained, but in 10%P the IP increased to 40.07%, happening the same with 10%G (36.60%) and 20%G (46.93%). Comparing the same concentration for the different seaweeds, a result opposite to that obtained for SN conditions was observed, the IP of 10%P was higher than the 10%G IP, although less pronounced (difference of 3.47%) (Figure 4.24).

A higher IP was observed at 5%P relative to 5%P+SN and at 10%P relative to 10%P+SN. However, in relation to 10%G, the IP obtained was lower than the 10%G+SN IP, and the same occurred in 20%G compared to 20%G+SN (Figure 4.24).

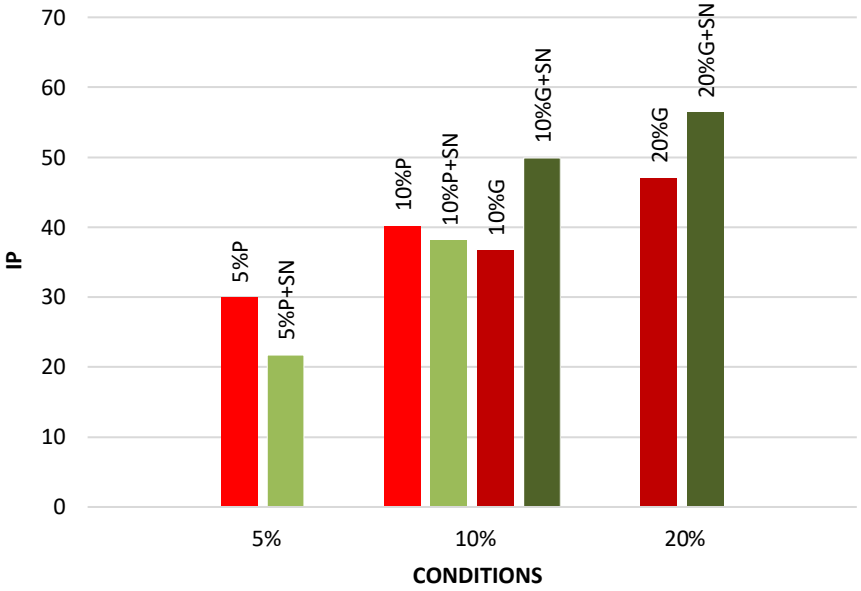

Figure 4.24 - IPs of the conditions with seaweed and the conditions with seaweed plus SN.

4.3.5. Average Clone Size

For the conditions without SN, in the NC it was obtained the highest value with 3.4 average clone size and in 10%G the lowest, with 2.2. For the conditions with SN, the highest value was reached in 5%P+SN, with 2.7 average clone size, and the lowest in 10%P+SN, with 2.3. After performing the χ^2 goodness of fit test to compare all values, it was found that the observed differences were non-significant, χ^2 value(9, N = 26.2) = 0.38, p = 0.999. The mean of the 10 average clone sizes was 2.6 with SD = 0.3.

4.3.6. Spots per 10^4 Cells

For NC, the f was 12.8, the highest f obtained for the conditions without SN. At 5%P the value of f dropped to 7.4, however the NC vs. 5%P statistical comparison (χ^2 test) proved the

non-significance of this decrease, χ^2 value(1, N = 20.2) = 2.28, p = 0.131. The statistical results for 10%P (f = 5.6), 10%G (f = 5.2), and 20%G (f = 5.6), showed values of f statistically lower than the one from NC, χ^2 values(1) > 3.84, ps < 0.05 (Figure 4.26).

Regarding the SN conditions, in PC the f was the highest of all conditions (with and without SN) with 53.1. In 5%P+SN, the f was equal to 45.1, being the difference in respect to PC non-significant, χ^2 value(1, N = 98.2) = 1.21, p = 0.272. The fs of 10%P+SN (f = 34.3), 10%G+SN (f = 24.6), and 20%G+SN (f = 22.4), were statistically lower than the PC's f, χ^2 values(1) > 3.84, ps < 0.05 (Figure 4.26).

The fs of the conditions with SN were statistically superior to the fs of the conditions without SN, χ^2 values(1) > 3.84, ps < 0.05.

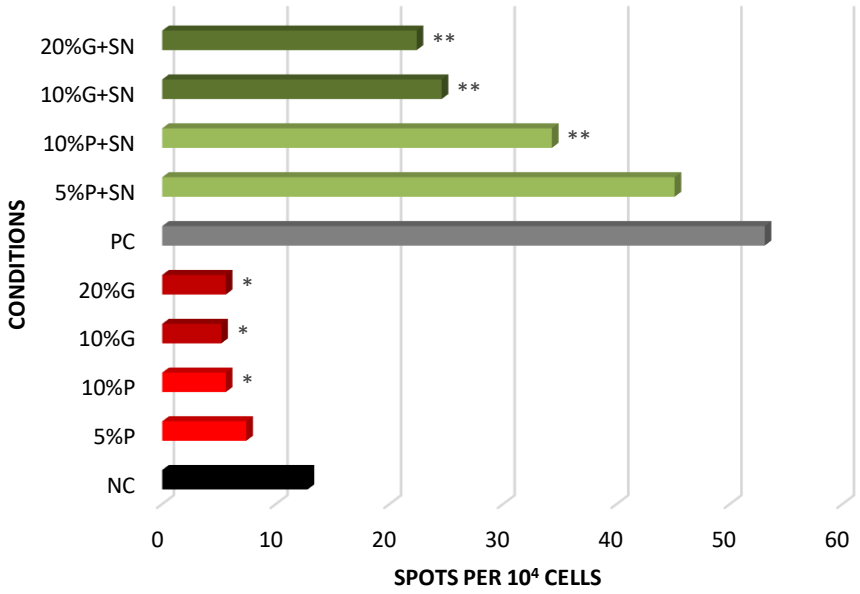

Figure 4.26 - Spots per 10^4 cells for the 10 SMART conditions.
* χ^2 values(1) > 3.84, ps < 0.05 in comparison with NC's f; ** χ^2 values(1) > 3.84, ps < 0.05 in comparison with PC's f.

Chapter 5 - Discussion

5.1. Development Time, Survival, and Sex Ratio

Organisms from fruit flies to humans regulate their nutrient intake to optimize a wide range of characteristics (M. A. Rodrigues et al., 2015). When fruit flies are subject to feeding in its larval phase with nutritionally rich foods, beneficial effects arise relative to parameters such as development time and survival (Güler et al., 2015; Neethu et al., 2013). During fruit flies development phase, larvae consume target amounts of macronutrients, including proteins, carbohydrates, and lipids, in addition to micronutrients, such as vitamins and minerals, to sustain their growth in the least time possible and to ensure their survival. Larvae do not survive on a diet that does not contain a source of protein, making it a limiting macronutrient for survival, and develop faster on diets containing sugars (Schwarz et al., 2014).

Considering the results for *Gracilaria* sp., the sex ratio of 1:1 was not maintained only for 2.5%Gr, which was due to the number of fruit flies being very low. Although, considering development time and survival, there may be some type of toxicity associated with the *Gracilaria* sp. that was only verified in the highest concentrations, from 2.5%Gr, being that in 1.25%Gr beneficial effects occurred.

One of the possible causes for this result may be related to the presence of metals, minerals, some organic compounds, or other, associated to the place of origin of the *Gracilaria* sp. (Torrão do Lameiro, Ria de Aveiro), which may have been concentrated by the seaweed and are in quantities superior to those tolerated by fruit flies with the intake of 2.5%Gr, 5%Gr, 10%Gr, and 20%Gr (Cheney, 2016; Holdt & Kraan, 2011). Several studies detected the presence of heavy metals such as arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), Cu, mercury (Hg), nickel (Ni), lead (Pb), and Zn in the sediments and fauna along the Ria, mainly derived from wastes from local industries (Martins, Dias, et al., 2013; Martins, Frontalini, et al., 2013). Moreover, in Coelho, Pereira, Duarte, and Pardal (2005), Hg was detected in specimens of *Gracilaria* collected at several sites along the Ria, although sampling sites did not contemplate Torrão do Lameiro. Hence, according to Martins, Dias, et al. (2013), the presence of Zn and Cu is very noticeable in Torrão do Lameiro in relation to the rest of the Ria, being that local fauna and flora function as bioaccumulators and biomagnifiers of these metals.

Instead of a contamination, there may be a antinutritional effect. There may be some constitutive compound in *Gracilaria* sp. which prevents the absorption of essential nutrients (an antinutrient) for the development of fruit flies (Cheney, 2016). One of the examples appears in relation to studies with *Gracilaria* spp. tested as supplements for fish, in which the toxicity

caused by the higher *Gracilaria* concentrations may be due to the presence of soluble non-starch polysaccharides (NSPs) in their constitution. NSPs bind minerals, exchange cations, and adsorb organic compounds, causing stress in the digestion of proteins, lipids, and others, leading to poor growth and less survival (Al-Asgah, Younis, Abdel-Warith, & Shamlol, 2016).

In relation to the other tested seaweeds, as significant differences between the two replicates of 5%G relative to survival were obtained, as well as significant differences between the numbers of males and females for 20%F(1), the ideal would be to perform more replicates or increase the number of fruit flies in each replicate to confirm these results.

Taking into account the results obtained for the five seaweeds, it is possible to infer that the nutrition of the fruit flies with *Gracilaria* sp. at 1.25%, *Grateloupia turuturu* at 5%, 10% and 20%, *Porphyra umbilicalis* at 5%, 10% and 20%, *Ulva* sp. at 2.5%, 5% and 10%, and *Fucus vesiculosus* at 1.25% and 5%, promoted beneficial/antitoxic effects in accelerating the numbers of eclosions, culminating in large numbers of total eclosions.

Despite the lack of studies involving development time, survival, or sex ratio in *Drosophila melanogaster* after seaweeds intake, a study was conducted in which a mixture of three seaweeds containing *Fucus vesiculosus*, *Laminaria digitata* (brown seaweed), and *Lithothamnium calcareum* (red seaweed) (from Algarve coast), was administered in different concentrations (from 14.3 g/L to 85.7 g/L) to *Drosophila melanogaster* (OK strain) during the larval development phase, which resulted in numbers of eclosions superior to the control, but non-significant, *p*s > 0.05 (Valente, 2014). The non-significant results contrast with the ones obtained for the present work for *Fucus* at 1.25, 2.5 and 5%, which may be influenced by the other seaweeds in the mixture or the concentrations tested.

In another model organism, the European seabass *Dicentrarchus labrax*, fish meal was supplemented with 5%, 10% and 15% of *Ulva lactuca* that was harvested from the Mediterranean coastal waters of Egypt. The 5% concentration produced significantly better growth and survival than control (Wassef, El-Sayed, & Sakr, 2013). As previously reported, 5%U in the results of the present study also demonstrated its nutritive potential.

Seaweeds are mainly constituted by H_2O, proteins, polysaccharides (such as sulphated polysaccharides), sugars and polyols, vitamins (such as A, C, and E), minerals (such as B, Ca, I, and Se), pigments (such as chlorophylls and carotenoids), lipids (such as ω-3 PUFAs), and phenols (such as polyphenols) (Holdt & Kraan, 2011; Lopes, 2014; Mendis & Kim, 2011). The chemical compositions of specimens from Atlantic European coast representative of the seaweeds used in the present work (from Table 1.1 to Table 1.5 in Chapter 1), demonstrate the

presence of essential proteins for the survival of fruit flies (from 6.2% in *Fucus* up to 29% in *Ulva*), carbohydrates (including sugars), minerals, and all the other nutritional compounds, creating a food that is able to increase the survival of fruit flies and allow them to develop faster, maintaining the sex ratio of 1:1.

However, it would be pertinent to perform the chemical characterization of the specimens of this work in order to have an accurate composition of them. It would be essential to carry out the chemical characterization of the *Gracilaria* sp. used in the present work so that the source of this toxicity could be uncovered. The collection of more samples of *Gracilaria* from the same site and other sites along the Ria de Aveiro would be pertinent, and also from more distant sites along the Portuguese coast, to perform the same toxicity test performed in the present work. It would also be essential to analyse the DNA of the *Gracilaria* sp. tested to confirm whether it is a *Gracilaria gracilis* or a *Gracilaria vermiculophylla*. *Ulva* also needs DNA analysis to confirm whether *Ulva lactuca* or *Ulva rigida* was tested.

Survival and sex ratio were calculated for the SMART conditions to confirm and give more statistical reliability to the initial toxicological screening results for the conditions with 5% and 10% *Porphyra umbilicalis* and 10% and 20% *Grateloupia turuturu*, in addition to test the effects of feeding fruit flies with each one of these seaweed concentrations plus SN.

Regarding survival, although differences between replicates of the same condition were non-significant for most conditions, differences were observed in 20%G and PC. Again, in order to draw conclusions about the disparity of these values for these two conditions, and although the numbers of fruit flies and the number of replicates were higher in this case than in the initial toxicological screening, larger numbers of replicates are required.

The 10%P, 10%G, and 20%G showed significantly higher numbers of eclosions than NC, confirming the results observed in the initial screening. For 5%P, the results were disparate with respect to the initial screening results, since a statistically higher significance was not proved relative to NC and less eclosions occurred in 5%P relative to 10%P. Moreover, 10%G was the condition that achieved the highest survival among all SMART conditions, and for initial screening was 5%P among *Porphyra* and *Grateloupia* conditions. In 10%G vs. 20%G, 10%G showed a significantly higher survival, which matches the survivals ratio between these two concentrations in the initial screening, although its significance was not tested in that case.

The differences between initial toxicological screening conditions and SMART conditions could be due to the different numbers of fruit flies and replicates used or even the

use of PBS in SMART and dH_2O in initial screening. Hence, further studies are needed to know the cause of these differences.

All the conditions with seaweed plus SN demonstrated their potential in inhibiting the toxic effects caused by SN. For conditions with *Porphyra*, in both 5%P and 10%P, the results indicate that a larger inhibition of SN activity may have occurred, since it was not possible to demonstrate significant differences when comparing 5%P vs. 5%P+SN and 10%P vs. 10%P+SN. However, the survivals were very identical between the conditions with *Porphyra* plus SN and conditions with *Grateloupia* plus SN.

For the sex ratio, the results remained unchanged with respect to the initial screening, being obtained sex ratios of 1:1 for all conditions without SN. Relatively to SN conditions, no change in the ratio of 1:1 was observed as well, which demonstrates that, at a concentration of 20 μM, SN equally affects males and females in relation to their ability to develop into adults.

In addition to the chemical composition of the seaweeds used capable of nourishing the fruit flies and promoting antitoxic effects in decreasing development time and increasing survival, joins here the ability to inhibit the toxic action of SN, at a 20 μM concentration, which is demonstrated relative to survival.

The most common toxic action of SN, and therefore the most studied, is genotoxicity (Donohoe et al., 2011; Donohoe et al., 2013). The SN linkage to DNA itself and ROS continuous production leads to alterations in DNA and RNA (Deepa et al., 2011; Gaivão et al., 1999), which could be the cause of the survivals decrease in the conditions with SN. Damage caused to proteins, carbohydrates, and/or lipids of fruit flies may also have affected their survival, however, this type of damage caused by SN is underexploited. SN clearly demonstrated toxicity relative to survival, which may be related to its ability to induce oxidative stress to *Drosophila melanogaster*'s macromolecules, being that seaweeds thus display an antioxidant protection, which could include antigenotoxic one as well.

Taking into account the chemical composition of *Porphyra umbilicalis* and *Grateloupia turuturu*, it is possible to combine antioxidant action (as well as antigenotoxic) of many of its components: phycobiliproteins, the complexes of pigment-protein present only in red seaweeds (between green and brown), demonstrated its antioxidant potential (Baweja et al., 2016); phycoerythrin (a phycobiliprotein) derived from a *Grateloupia sp.* reduced ROS induced by H_2O_2 in rat astrocytes (Jung et al., 2016); sulphated polysaccharides from a *Grateloupia* sp. demonstrated its potential in scavenging free radicals *in vitro* (Ye et al., 2015); I (iodine) may act as an antioxidant as well (Brown et al., 2014); fucoxanthin, β-carotene, and lutein found in

seaweeds exhibit powerful antioxidant properties (Baweja et al., 2016; Mendis & Kim, 2011; Škrovánková, 2011); a study with diabetic rats administrated with ω-3 PUFAs revealed decreased oxidative stress (Pan et al., 2012); vitamin C acts in trapping free radicals and also regenerates vitamin E, which inhibits the oxidation of low density lipoproteins (L. Pereira, 2011); vitamin B12, that in seaweeds is only present in some *Porphyra* spp. (including *Porphyra umbilicalis*) and *Ulva* spp., prevented DNA damage induced by paclitaxel in human blood lymphocytes (Alzoubi et al., 2014); a great number of studies have shown the ROS scavenging potential of phlorotannins (S.-K. Kim & Wijesekara, 2011).

In relation to crude extracts representative of seaweeds in its total: a *Grateloupia turuturu* displayed a great antioxidant activity *in vitro* (Liu & Pang, 2010); a *Grateloupia* sp. collected in Brittany coast (France) showed antioxidant potential *in vitro* as well (Zubia, Fabre, Kerjean, & Deslandes, 2009); a *Porphyra umbilicalis* collected in the Atlantic coast also displayed antioxidant potential *in vitro* (Cofrades et al., 2010).

According to Fernández-Bedmar et al. (2011), Mezzoug et al. (2006), and Panieri and Santoro (2016), the most common antioxidant mechanisms of dietary antioxidants such as those of *Porphyra umbilicalis* and *Grateloupia turuturu*, are scavenging and giving e^- or H^+ for ROS conversion in H_2O, and chelating metal ions (metal chelators) to avoid Fenton reaction.

5.2. Longevity Assay

The feeding quality of fruit flies larvae, besides influencing development time and survival, is also directly related to the longevity of adults (Galenza et al., 2016).

The oxidative damage in the major macromolecules is believed to be a major contributor to the functional decline associated with aging (Luisa & Giorgio, 2015; Pan et al., 2012; Peng, Chan, et al., 2011). As ROS correlates negatively with animal longevity, antioxidant mechanisms that can decrease ROS levels in cells by modulation of enzymes activity and direct antioxidant scavenging activity that delays the aging process (enhanced longevity) are required (Luisa & Giorgio, 2015). One of the ways to get a greater antioxidant protection is through supplementing the regular diet with dietary antioxidants (Galenza et al., 2016; May et al., 2015).

The mean longevities for control males and females are inserted in the interval referred by Gonzalez (2013), Ong et al. (2014), and Paaby and Schmidt (2009), 40 to 60 days for fruit flies subjected to conditions similar to those of the control of the present work, thus demonstrating the replicability of this assay. Second, it has been known that females have longer longevities than males across many species including humans, rats, fruit flies, and others

(Niveditha, Deepashree, Ramesh, & Shivanandappa, 2014; Tower & Arbeitman, 2009). Despite the superior longevity for the females of the control relative to males' longevity, it was not possible to verify significant differences. The numbers of males and females were very low, being that the use of more individuals would possibly lead to these differences becoming significant.

For both males and females of *Drosophila melanogaster*, it was verified that the ingestion of a medium with 20% of *Grateloupia turuturu* during the larval phase leads to the increase of mean and median longevities relative to control fruit flies and to others that ingested the inferior concentrations of seaweed tested. Although maximum longevity was not significantly affected for the fruit flies of 20%G, the differences obtained for mean and median longevities confirm the effect of 20%G on increasing longevity.

For the comparison 1.25%G vs. 10%G for the females, despite the significant differences for median longevity as well as for maximum longevity, there were non-significant differences relative to control for both conditions, which means that the significances obtained between the two conditions are not relevant.

The males and females exposed to *Grateloupia turuturu* did not show differences in longevity between the same conditions, as for control males and females, being necessary to increase as well the number of individuals to confirm this result.

For both males and females of *Drosophila melanogaster*, it was found that the ingestion of a medium with 10% of *Porphyra umbilicalis* during the larval phase leads to the increase of mean and median longevities relative to fruit flies fed with control medium and others that ingested the lower concentrations and the higher concentration of seaweed. Maximum longevity was not significantly affected for 10%P fruit flies. However, the data is sufficient to state that the ingestion of 10%P by fruit flies in the larval phase increases their longevity.

The highest concentration of *Porphyra* for males, 20%P, also demonstrated a higher significance of median longevity over control. However, as the median despises the outliners, and the mean longevity of 20%P displayed a non-significant result relative to control, it does not allow to infer a real alteration of the longevity for the fruit flies fed with 20%P. For the females, the significantly higher median longevity of 20%P in relation to 5%P is not relevant since there were non-significant differences to the control relative to median and mean longevities for 20%P.

The non-significant differences for the comparisons between males and females of the same condition of *Porphyra* is in agreement with the results for the control as well as for the

conditions with *Grateloupia*. Thus, in the same way, the increase of the number of individuals to confirm this result is necessary.

Comparing the obtained positive results, it is possible to verify that for fruit flies exposed to 20%G, the mean and median longevities were superior to those exposed to 10%P, in both males and females. In 20%G males (67.61 ± 2.78 days) the mean longevity was 7.75% higher than for 10%P males (62.75 ± 3.98 days), and the median longevity was also superior in 20%G at 70 days relative to 63 days. Regarding females, the differences were not as pronounced, but in 20%G (66.98 ± 2.25 days) more 4% of mean longevity appeared in relation to fruit flies of 10%P (64.40 ± 3.49 days), and median longevity was similar for both (70 days), although with a 95% CI indicating a greater median longevity to 20%G. Thus, *Grateloupia turuturu* presents results that demonstrate a greater longevity enhancing potential relative to *Porphyra umbilicalis*, that can be reflected by a greater antioxidant potential.

However, for *Porphyra umbilicalis* only a concentration of 10% was required to increase longevity, in contrast to the 20% of *Grateloupia*. For 20%P, the longevity enhancing potential as 10%P was not demonstrated, which may show that a 10%P concentration or a small concentration interval of this seaweed is required for longevity enhancing actions. It would be pertinent to test more concentrations of both seaweeds to clarify this point.

As stated in subchapter 5.1., the seaweeds *Grateloupia turuturu* and *Porphyra umbilicalis* have a very rich composition in bioactive compounds that display antioxidant potential. Ingestion of 20%G and 10%P by fruit flies during the larval phase promote increased longevity possibly by the action of antioxidant mechanisms triggered by seaweeds dietary antioxidants.

In spite the lack of studies that have tested the direct impact on *Drosophila melanogaster*'s longevity, or in any other organism, after ingestion of the seaweeds used in the present work, some studies have focused on compounds that are present in these seaweeds and that demonstrated the capacity to increase longevity.

The sulphated polysaccharides from *Porphyra* spp., porphyrans, when administrated to *Drosophila melanogaster* in adult life, significantly increased the mean longevity for males, from 53.62 to 58.23 days (8.60% increase; using 1% concentration), and for females, from 49.69 to 55.79 days (12.29% increase; using 0.2% concentration). Thus, according to the authors, the porphyrans showed their potential related to their free radical scavenger action in enhancing mean longevity (Zhao et al., 2007). The mean longevities are similar to those obtained for the present work, although the percentages are not as relevant as those obtained

for *Porphyra* and *Grateloupia* for both males and females in the present work. Zhao et al. (2008) has proven the results of the previous article.

In *Drosophila melanogaster* (Canton-S strain), longevity was enhanced when two carotenoids, fucoxanthin and β-carotene, where administered (individually) during adult life. Both resulted in increased median longevities. Furthermore, the effects were more pronounced in females (Ekaterina et al., 2015). For other pigment, lutein, a study with males of *Drosophila melanogaster* (Oregon-R-C strain) demonstrated that 0.1 mg of lutein/ml diet (intake in adult life) could prolong their mean longevity from 49.00 to 54.60 days (11.35%), as well as median longevity (ps < 0.05). This was consistent with a significant increase in antioxidant enzymes activity of the flies fed with lutein treated diet compared with those fed with basal diet (Zhang et al., 2013). For the flies that ingested lutein, taking into account the increase of mean longevity in relation to control, this did not increase as much as those observed for the present study.

In Driver and Georgeou (2013), when *Drosophila melanogaster* males (Canton-S strain) ingested during the adult life 20 µg/ml of vitamin E, mean longevity increased 16%, although there was no effect at higher or lower concentrations. The increase in mean longevity is similar to those that occurred in the present study, especially for conditions with *Porphyra*.

For the previous studies, although it was verified the use of strains of *Drosophila melanogaster* different from the one used in the present study (OK strain) and that the ingestion of the compounds was carried out during the adult life (in the present work it was in the larval phase), the porphyrans, fucoxanthin, β-carotene, lutein, and vitamin E, were able to enhance the longevity of fruit flies. In other researches, other compounds such as phycoerythrin and polyphenols also display longevity enhancing potential (Pan et al., 2012; Sonani et al., 2014).

According to Galenza et al. (2016), when feeding provides a low proteins:carbohydrates, the longevity of *Drosophila melanogaster* significantly extends. Analysing the proteins and carbohydrates percentage for specimens from Atlantic European coast representative of *Grateloupia* (Table 1.2 in Chapter 1) and *Porphyra* (Table 1.3 in Chapter 1), it can be observed that the amount of proteins is lower than the amount of carbohydrates for both seaweeds, thus resulting in a low ratio of proteins:carbohydrates that can lead to the increases in the longevities of fruit flies verified after ingestion of 20%G and 10%P. Hence, it is fundamental to perform chemical characterizations of both seaweeds used to confirm this outcome.

5.3. SMART

As SMART has proven to be a good tool for detecting a broad range of genetic alterations, many foods have already been tested in order to know their antigenotoxic potential. Thus, high quality of the feeding of fruit flies larvae with antioxidants and antigenotoxics is essential in all life history characteristics of fruit flies, including the protection of genome from alterations that lead to cancerous events (Mishra et al., 2016; Stamenković-Radak & Andjelković, 2016).

The genetic alterations detected in SMART are mainly caused by oxidative stress triggered by genotoxics. The role of oxidative stress as the first major cause of mutations occurring in genes regulating cell cycle, to facilitating tumor growth in multiple ways by causing DNA damage and genomic instability, and ultimately, reprogramming cancer cell metabolism, it is beginning to be unravelled (Gosslau, 2016; Schumacker, 2015).

When administrating 10%P, 10%G, and 20%G to fruit flies in the larval development phase, the spots per 400 eyes were reduced relative to NC, which demonstrated the reduction of the genotoxicity triggered by the spontaneous genotoxics of fruit flies. Hence, 5%P was not able to significantly reduce genotoxicity, which appears to demonstrate that the concentration is too low for the reduction of the spontaneous genotoxics of *Drosophila*. The non-significant differences between the same concentration for both seaweeds, 10%, demonstrates a reducing potential of *Drosophila* spontaneous genotoxics similar for *Porphyra* and *Grateloupia*. The greatest potential occurred for 20%G.

The significant reductions in the numbers of spots per 400 eyes for all conditions with SN (compared to PC) reflect the ability of seaweeds to reduce the genotoxic activity induced by SN and by the spontaneous genotoxics of fruit flies. However, for the conditions with *Porphyra* the reductions were not as significant as the reductions for *Grateloupia* conditions. The antigenotoxic action of 5% of *Porphyra* was more noticeable (was significant) when a higher genotoxicity was induced in fruit flies (conditions with SN), although, it was the one that developed lower antigenotoxic potential. Comparing the concentrations of 10% of both seaweeds, the induced genotoxicity of SN led to 10% of *Grateloupia* showing a greater antigenotoxic potential than 10% of *Porphyra*. However, the greatest potential continued to belong to the highest concentration tested, 20% of *Grateloupia*.

Regarding the IPs for SN conditions, corroborating the previous results for spots per 400 eyes, the highest potential was verified for the condition with 20% of *Grateloupia* with more than 56% inhibition of the genotoxic activities of SN and the spontaneous genotoxics, followed by 10% of *Grateloupia*, with about 50%. For conditions with *Porphyra*, the reduction was not

as expressive as for *Grateloupia*, with 5%P+SN showing the lowest potential. Comparing the conditions of 10%, *Grateloupia* showed more than 10% inhibition than *Porphyra*.

For the conditions without SN, 5% of *Porphyra* showed again the lowest inhibition, which according to the spots per 400 eyes results was a non-significant inhibition. The highest concentration of *Grateloupia*, 20%, displayed once more the largest inhibition (about 47% inhibition). The difference in IPs from the 10% seaweed conditions was very little pronounced compared to the SN concentrations, which shows that the genotoxicity induced only by the spontaneous genotoxics is not sufficient to trigger a significantly greater inhibition for *Grateloupia* as it was verified under conditions with SN.

The direct comparison between SN conditions and conditions without SN did not show concise results, which may have resulted only from the fact that in conditions without SN the number of spots was very low, which led, e.g., to an inhibition of about 30% in 5%P but did not show significant reduction of the spots in relation to NC, and in 5%P+SN the IP was lower but a significant reduction of the spots was confirmed relative to PC.

The average clone size of each of the conditions did not show significant differences between them. However, in Vogel and Nivard (1993) the opposite was shown for the average clone sizes triggered by several chemicals, since they presented very different values among themselves and between the controls used in the eye-spot test in *Drosophila melanogaster*, the same test performed in the present work. Currently, doubts still arise about this subject but the results for the present work demonstrate that both the genotoxic activity of 20 μM SN that induced a large number of spots and the antigenotoxic activity of the seaweeds that reduced the number of spots, do not change the size of the spots in *Drosophila melanogaster*.

For spots per 10^4 cells, SN conditions showed significantly higher fs than the fs of the conditions without SN, confirming the previous results. All the results for both conditions without SN and with SN were lower than the respective controls, although the significance was not verified for conditions with 5% of *Porphyra* (with and without SN), which proves, again, the lowest antigenotoxic potential for this seaweed concentration. Interestingly, for the conditions without SN was 10%G that obtained the smallest f and not 20%G, because the average clone size of 20%G was higher than the 10%G average clone size, culminating in this result, although with a difference that could be despised. However, for 20%G+SN the lowest f was obtained from SN conditions, which corroborates its protective action.

The protective actions of macromolecules, antioxidant and antigenotoxic, previously mentioned for the bioactive compounds of both *Porphyra umbilicalis* and *Grateloupia turuturu*,

can explain the results obtained for SMART. Antioxidants and antigenotoxics can reduce ROS, that prevents oxidative stress and genetic alterations which can, consequently, prevent cancer events (Mishra et al., 2016; Stamenković-Radak & Andjelković, 2016). There are some studies which focus these actions from some of these compounds in SMART for *Drosophila melanogaster*, although wing-spot tests are used instead of eye-spot tests.

B (boron) different concentrations (from 0.1 mg/ml to 40 mg/ml) were able to abolish 0.1 mM of EMS genotoxicity when exposure of *Drosophila melanogaster* in the larval development phase (Sarıkaya et al., 2016). Despite these results, conditions with only B (without EMS) gave negative results relative to their antigenotoxic potential against the *Drosophila* spontaneous genotoxics. It was possible to verify that the IPs in relation to the conditions with EMS were quite high, and that in the highest concentration of B tested the inhibition was more than 57%, a similar IP to 20%G+SN.

In Rizki et al. (2001) the antigenotoxic effects of sodium selenite (0.01, 0.05, and 0.1 mM), a Se compound, were demonstrated in the cotreatment with the genotoxic potassium dichromat in SMART. However, also for this study, sodium selenite did not demonstrate antigenotoxic potential when tested alone against the spontaneous genotoxicity of *Drosophila*. For the IPs, very high values were obtained in the administration of Se against the activity of potassium dichromat (and also spontaneous genotoxics), above 79% inhibition.

Dias et al. (2009) examined the effects of β-carotene (1, 2, and 4 mg/ml) on the genotoxicity of doxorubicin using SMART in *Drosophila melanogaster*. All β-carotene concentrations did not demonstrate antigenotoxicity against spontaneous genotoxics, however, when added to doxorubicin, β-carotene neutralised ROS, as shown by the IPs of more than 78%. Furthermore, there are many studies that have shown the correlation between a diet rich in carotenoids (such as β-carotene) and a diminishing risk of lung cancer in different organisms (Guiry, 2016; Holdt & Kraan, 2011; Mendis & Kim, 2011).

In other study, chlorophyll a (0.5, 1, and 2 µM), as well as vitamin C (25, 75, and 250 mM), demonstrated their antigenotoxicity against acrolein and also malondialdehyde (separately) in *Drosophila melanogaster* (SMART). The IPs obtained for both the inhibition of acrolein and malondialdehyde activities were of the order of magnitude of the above compounds, although vitamin C demonstrated a larger protective effect. However, no antigenotoxicity was demonstrated only against the spontaneous genotoxics (Esref Demir et al., 2013). In Graf et al. (1998), vitamin C inhibited the genotoxicity displayed by methyl urea together with sodium nitrite in SMART. Thus, chlorophyll a and vitamin C have significant

roles in cancer prevention by neutralizing free radicals before they can damage DNA and initiate tumor growth (Mendis & Kim, 2011; Škrovánková, 2011).

The exposure to the conditions in the previous articles began only in the three-day-old larvae and occurred until the eclosion of the adults, contrasting with the chronic treatment carried out in the present work (from egg to adult), which may have led to different outcomes. The IPs obtained were very high in the referred articles due, possible, to the fact that wing-spot tests were used instead of eye-spot tests, as well as the different strains used specifically for this type of SMART. No compound has demonstrated antigenotoxicity only against the spontaneous genotoxics, which contrasts with the results for 10%P, 10%G, and 20%G, which may indicate a greater sensitivity of both seaweeds in terms of antigenotoxic action relative to each of its compounds individually.

In spite of the absence of SMART studies in *Drosophila melanogaster* using seaweeds, there is a study already focused previously in which a mixture of three seaweeds was administered to *Drosophila melanogaster* (OK strain) during larval phase (Valente, 2014). The mixture (55.9 g/L) was tested with the eye-spot test, which reduced the percentage of eyes with spots from 8.14% to 1.66%, although it was not performed a statistical treatment to confirm this reduction. When adding 20 μM of SN to the mixture of seaweeds, the same genotoxic with the same concentration as the one used in the present work, the reduction occurred again but now from 70.68% to 18.02%, although again without statistical treatment to verify the significance of the results. A "significant conclusion" about these results is not possible, but many similarities in relation to the present work exist in the procedure and results obtained for this mixture that also contains a red seaweed.

As in all studies with model organisms, the ultimate goal of the present work is the possibility of extrapolating the results to human beings. Despite the differences that characterize both species, *Drosophila melanogaster* and humans have evolutionarily conserved genes, genetic pathways, and biochemical processes. A great amount of the analogous organ systems that control nutrient intake, digestion, absorption, storage, and metabolism in humans are present in fruit flies (Lemaitre & Miguel-Aliaga, 2013; Ong et al., 2014).

Taking into account the bioactive compounds present in *Grateloupia turuturu* and *Porphyra umbilicalis* and the antioxidant and antigenotoxic actions that they play in the protection of macromolecules, taking into account the results of the present work in decreasing development time, increasing survival, increasing longevity, and decreasing the genotoxicity

for fruit flies subjected to both seaweeds, it has been proven that *Grateloupia turuturu* and *Porphyra umbilicalis* have the potential to be considered as functional food.

Japanese eat between 10 to 25% of seaweeds relatively to their total food intake, being seaweeds biggest consumers (Collins et al., 2016; Peinado et al., 2014). In Europe, including in Portugal, the consumption of seaweeds is very low (Fleurence, 2016). Thus, food provides different nutrients for the Japanese, e.g., on average, the Japanese consume ≥ 12 mg of I (iodine) per day (*Porphyra* spp. are the major sources), a much greater amount than the quantities consumed in Europe like UK's 166 μg/day (Brown et al., 2014).

In this way, taking into account the consumption of seaweeds by the Japanese, is it possible to associate this consumption with the longevity achieved in Japan, which leads the WHO list as the country with the highest life expectancy (World Health Organization, 2016)? And cancer incidence? Studies have shown that Japanese females in Okinawa, Japan, have the longest life expectancy with lowest cancer rates than other people in the world (Pan et al., 2012). As cancer incidence is much lower among populations who consume a seaweed rich diet in comparison with those who consume a Western-style diet (Brown et al., 2014), epidemiological studies suggest that high mineral content in seaweeds must have accounted for the low prevalence of cancer in some Asian countries (Mendis & Kim, 2011).

However, more studies are needed with seaweeds in organisms closer to humans and even in humans, to give greater relevance to the results obtained in this study, and to attempt to associate the consumption of this functional food with the longevity enhancing and the diminishing of the incidence of different types of cancer. In this way, European countries with large coastal areas, such as Portugal, could explore more the harvest of wild seaweeds, as well as the development of seaweeds aquaculture, with the aim of socio-economic development.

Chapter 6 - Conclusions

Taking into account the specific objectives of this work and the results obtained, the main conclusions that can be withdrawn are:

- *Gracilaria* sp. at 2.5%, 5%, 10% and 20% demonstrated toxicity with late development times and low survivals in *Drosophila melanogaster*;
- *Gracilaria* sp. at 1.25%, *Grateloupia turuturu* at 5%, 10% and 20%, *Porphyra umbilicalis* at 5%, 10% and 20%, *Ulva* sp. at 2.5%, 5% and 10%, and *Fucus vesiculosus* at 1.25% and 5%, promoted beneficial/antitoxic effects in *Drosophila melanogaster* through the diminution of development times and increase of survivals;
- *Porphyra umbilicalis* at 5% and 10%, and *Grateloupia turuturu* at 10% and 20% demonstrated their antioxidant potential in inhibiting the toxic action of SN in the survival of *Drosophila melanogaster*;
- The antioxidant actions of 10% of *Porphyra umbilicalis* and 20% of *Grateloupia turuturu* were able to enhance the longevity of *Drosophila melanogaster*, in both males and females, with *Grateloupia* reaching the highest potential;
- In SMART, the antigenotoxic action was accentuated with the increased incorporation of *Porphyra umbilicalis* and *Grateloupia turuturu*, with and without SN, showing the potential for cancer prevention. *Grateloupia* conditions demonstrated higher antigenotoxic potential, with 20% of *Grateloupia* showing the highest.

Thus, the results obtained for *Grateloupia turuturu* and *Porphyra umbilicalis*, and the fact that they are constituted by a large number of bioactive compounds with antioxidant and antigenotoxic capacity, demonstrates the potential of these seaweeds as functional food.

However, there is a need for a greater number of studies, in other organisms, culminating in human beings, in order to prove the longevity enhancing effects and the genome protective actions in cancer prevention demonstrated by these two seaweeds in *Drosophila melanogaster*.

The *Ulva* sp. and the *Fucus vesiculosus* used in the work should be subject to longevity assay and also SMART, in order to better understand the potential unveiled by them in the initial toxicological screening. The *Gracilaria* sp. should be explored in the contemplation of knowing the cause of the toxicity demonstrated.

In this way, it is intended to boost toxicology studies with wild seaweeds from the Portuguese coast, with the interest of proving that its use as human food can promote a higher quality and longer lasting life, and consequently exploring the socio-economic potential of Portugal's exclusive economic zone in the harvest and aquaculture of seaweeds.

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