UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

ECOTOXICOLOGICAL EFFECTS OF ENVIRONMENTAL STRESSORS IN THE MUSSEL *MYTILUS GALLOPROVINCIALIS*

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RESUMO

EFEITOS ECOTOXICOLÓGICOS DE STRESSORES AMBIENTAIS NO MEXILHÃO *MYTILUS GALLOPROVINCIALIS*

A espécie *Mytilus galloprovincialis* (mexilhão do mediterrâneo) alimenta-se através da filtração da água e por esse motivo, entre outros, tem sido muito utilizado como espécie-sentinela em estudos de monitorização da contaminação ambiental e seus efeitos no meio marinho.

Os objetivos deste estudo foram: (i) determinar parâmetros físico-químicos na água e de biomarcadores ambientais em *M. galloprovincialis* utilizados em estudos de monitorização da qualidade ambiental e de avaliação dos efeitos da poluição e outras pressões ambientais em populações de organismos selvagens; (ii) avaliar a toxicidade de microplásticos (MPs) e da deltametrina (DELT), isoladamente e em mistura, para *M. galloprovincialis*.

Para atingir o primeiro objetivo foram recolhidos mexilhões e amostras de água em quatro locais da zona costeira do Norte de Portugal: Vila Praia de Âncora, Carreço, Cabo do Mundo e São Félix da Marinha. Os parâmetros morfométricos e o estado de saúde dos mexilhões foram determinados utilizando o índice de condição e biomarcadores indicativos de neurotoxicidade, produção energética, stress oxidativo, dano oxidativo lipídico e dano oxidativo proteico. Concluiu-se que os mexilhões de certos locais se encontravam num estado de saúde mais precário do que os provenientes de outros.

Para atingir o segundo objetivo, foram recolhidos 38 mexilhões na zona costeira da cidade do Porto. Inicialmente, em 9 indivíduos foram determinados os biomarcadores anteriormente referidos. Os restantes foram aclimatizados durante 7 dias a condições laboratoriais e posteriormente foram determinados os biomarcadores em 9 indivíduos. Com os restantes 20, foi efetuado um bioensaio agudo. Os tratamentos do bioensaio foram os seguintes: controlo (água do mar artificial); 0,3 mg/L de MPs; 1 mg/L de DELT; 0,3 mg/L de MPs + 1 mg/L de DELT. Após 96h de exposição, foi determinada a taxa de filtração, os mesmos biomarcadores e as concentrações reais de MPs na água ao longo do bioensaio. Não se observaram diferenças significativas ($p > 0.05$) entre tratamentos em nenhum dos biomarcadores, o que permitiu concluir que nas concentrações testadas os MPs e a DELT, individualmente e em mistura, não induziram efeitos tóxicos em *M. galloprovincialis*.

Palavras chave: Mexilhão (*Mytilus galloprovincialis*), Monitorização ambiental, Biomarcadores, Microplásticos, Deltametrina

ABSTRACT

ECOTOXICOLOGICAL EFFECTS OF ENVIRONMENTAL STRESSORS IN THE MUSSEL *MYTILLUS GALLOPROVINCIALIS*

Mytilus galloprovincialis (Mediterranean mussel) feeds through water filtration and for this reason among others it has been widely used as sentinel species in monitorization studies of environmental contamination and its effects in the marine environment.

The objectives of the present study were:(i) to determine water physicochemical parameters and environmental biomarkers in *M. galloprovincialis* widely used in monitoring studies to assess environmental quality, and the effects of pollution and other environmental stressors in populations of wild organisms; (ii) to assess the toxicity of microplastics (MPs) and deltamethrin (DELT) individually and in mixture to *M. galloprovincialis*.

In order to reach the first objective, mussels were collected in four different sampling sites of the northern Portuguese coastal shore: Vila Praia de Âncora, Carreço, Cabo do Mundo and São Félix da Marinha. In the laboratory, morphometric parameters were determined, and the health *status* of mussels collected in different sampling sites was compared using the condition index and neurotoxicity, energy production, oxidative stress, lipid oxidative damage and protein oxidative damage biomarkers. The results indicated that mussels collected in certain sampling sites showed a more precarious health *status* than mussels collected from other sampling sites.

To address the second objective, 38 mussels were collected in a sampling site on the shore near the Porto city and transported to the laboratory. Biomarkers were determined in 9 of the mussels. The other organisms were acclimatized to laboratory conditions for 7 days. After this period the biomarkers were determined in 9 specimens. With the other 20 mussels, a short-term toxicity bioassay was performed. The bioassay treatments were: control (artificial seawater); 0.3 mg of MPs; 1 mg/L of DELT; 0.3 mg/L of MPs + 1 mg/L of DELT. After 96h of exposure to test substances, the filtration rate and the previously mentioned biomarkers were determined in all the mussels. The actual concentration of MPs in the water of all treatments containing this test substance was determined in three different times. No significant differences ($p > 0.05$) among treatments in any biomarker were found. Thus, the concentrations of MPs and DELT tested (individually or in a mixture) did not induce toxic effects on *M. galloprovincialis.*

Keywords: Mediterranean mussel *(Mytilus galloprovincialis)*, Environmental monitoring, Biomarkers, Microplastics, Deltametrhrin.

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LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
APA	Agência Portuguesa do Ambiente (Portuguese Environment Agency)
CAT	Catalase
CI	Condition index
DELT	Deltamethrin
DNPH	2,4-dinitrophenylhydrazine
FAO	Food and Agriculture Organization of the United Nations
GST	Glutathione *S*-transferase enzymes
IDH	$NADPH^{+}$ -dependent isocitrate dehydrogenase enzyme
IPCS	International Programme on Chemical Safety
IUPAC	International Union of Pure and Applied Chemistry
LPO	Lipid peroxidation
MDA	Malondialdehydes
MPs	Microplastics
ODH	Octopine dehydrogenase enzyme
PA	Polyamide
PAH	Polycyclic aromatic hydrocarbons
PCB	Polychlorinated biphenyls
ROS	Reactive oxygen species
SEPNA	Serviço de Proteção da Natureza e do Ambiente (National Republican Guard's Nature and Environment Protection Service)
TBA	2-thiobarbituric acid
TBT	Tributyltin

I. Report on the activities developed during the curricular internship

This report concerns the curricular traineeship from the Integrated Master's Degree in Veterinary Medicine.

The trainee concluded traineeships. The first traineeship (curricular) took place in the Institute of Biomedical Sciences of Abel Salazar of the University of Porto (ICBAS-UP), Department of Population Studies, Laboratory of Ecotoxicology and Ecology. The second one took place in the Zeehondencentrum Pieterburen, in The Netherlands, included in the Erasmus programme.

The curricular traineeship at ICBAS-UP, under the guidance of Professor Doctor Lúcia Guilhermino, had a total duration of approximately 1530 hours, starting on the 16th September 2018 and finished on the 30th May 2019. It was performed under a flexible schedule that implied approximately 8 to 9 hours a day, 5 days per week, with time of the day variability, depending on the different protocols and work phases. In this internship, work was done in several places, like the algae-growing room, autoclave room, general workroom and the room with optical equipment and the field-work sites. Inside the laboratory facilities, several concepts were fundamental, such as forethought and organization, asepsis associated with the material maintenance and sterile conditions, ethical principles and procedures during experimental conditions with animals, hypothesis testing and experimental planning, detail-oriented work, care and safety when handling harmful substances and laboratory equipment, data analyses and results' interpretation, among other issues. During the fieldwork, organization and forethought were also required, as well as basic sampling notions.

The second traineeship (extracurricular), made at the Zeehondencentrum Pieterburen begun on the 9th of June 2019 and finished the 8th August of the same year. The work relied on seal behaviour, nutrition and quarantine-condition maintenance, necropsies and science communication. Common seal (*Phoca vitulina*) was the species present at the time of the traineeship, for during summertime was their calving period. At the time, the majority of the seals was still a pup. They were mostly admitted into the centre due to their mothers' abandonment. The adult seals present at the beginning and at the end of the internship had suffered from lungworms and/or viruses. There was also a grey seal (*Halichoerus grypus*) with an entangled fishnet in the front flipper. She had a

dislocated bone, severe cuts in muscles and tendons, and deep wounds throughout her body. She would not be able to express normal behaviour in nature and was euthanized.

II. Literature review

1. Introduction

The term ecotoxicology, introduced in 1969 by Truhaut, was initially used to describe methods to detect and determine chemicals present in samples obtained from animals or plants. Later on, a definition by Walker et al. (2012) was accepted as “the study of harmful effects of chemicals upon ecosystems”, which included the effects of those chemicals on an individual level up to a population level and even higher (Walker et al. 2012)

Species of the genus *Mytilus* have been used as a source of human food in the northwest of the Iberian Peninsula since the fifth-century b.C., where marine resources were valued, being both a food source and a construction material (using their shells) (Vázquez Varela and Rodríguez López 1999).

Mussels have often been used as bioindicators (Lima et al. 2008), a term that combines any reactions (abiotic or biotic) to ecological changes (Parmar et al. 2016). It is used to detect modifications in the environment (being natural or anthropogenic) and evaluate the responses of living organisms to environmental stress, being this assessment classified qualitatively (Holt and Miller 2010). It should have a good indicator ability (responding in proportion to the disturbance), be abundant and common (not rare or ubiquitous), well-studied and economically and commercially valuable (Holt and Miller 2010).

Mytilus sp. have been studied and used as a sentinel group as well as an effective bioindicator regarding the study of oxidative damage and the production of reactive oxygen species (ROS) related to water pollution, in spite the fact that the physiology and biochemistry of mussels can be influenced by factors like seasonality (Vlahogianni et al. 2007). Since mussels feed in their habitat’s water, they can efficiently accumulate biotoxins, such as dinophysis toxins (okadaic acid) or saxitoxin (Alexander et al. 2008). *Mytilus galloprovincialis* meet the bioindicator requirements by having a good tolerance to different environmental conditions, by having a broad distribution and restricted mobility and by allying a low metabolism with filter-feeding (allowing bioaccumulation to occur) (Lima et al. 2007; Parmar et al. 2016).

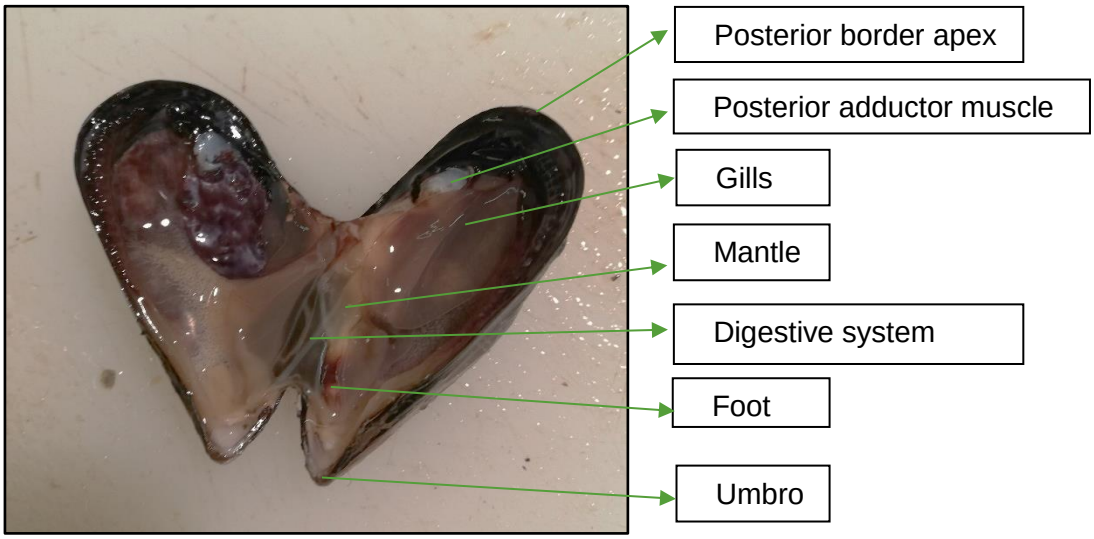
The importance of the marine debris problem has been increasingly recognized over the last 20 years, although monitorization has been done for at least 46 years (Wong et al. 1974). Microplastics are a ubiquitous group of oceans’ contaminants, whose impacts on human and fauna health, flora and

ecosystems have been studied as early as 1972 (Carpenter and Smith Jr. 1972) when it was questioned if they could be a surface on which bacteria or polychlorinated biphenyls (PCBs) could be adsorbed and therefore, ease their incorporation in marine organisms.

Microplastics, due to their uneven, irregular surface can be an easy substrate for adhesion and form of biofilms (and perpetuate bacteria with antibiotic or metal resistance genes) and have an active role in the transportation of chemical contaminants, drugs (such as antibiotics), pesticides, among others (Laganà et al. 2019). They can cause oxidative stress, cytotoxicity, the disruption of the energy homeostasis and the immune function; they can cause neurotoxicity and increase the likelihood of neurodegenerative diseases and finally, they can act as vectors of microorganisms as well as several chemical substances (Prata et al. 2019)

2. *Mytilus galloprovincialis*

Species of the genus *Mytilus* have historically been used as a human food source (Vázquez Varela and Rodríguez López 1999). The country that upholds the highest production of *Mytilus spp*. is China. Most southern European countries produce it too, as well as Russia, Ukraine, Morocco, Algeria, Tunisia and South Africa. In 2016, the global production of mussels was reported to be around 105 331 tones (FAO 2018).



Picture 1: The anatomy of *Mytilus galloprovincialis* with the designation of anatomic details according to Vernon et al. (2018) and Illesca et al. (2018). Photography: M.J. Gonçalves, April, the 22nd 2019.

Mytilus galloprovincialis is a bivalve species with symmetric valves and a black-violet outer shell belonging to the phylum Mollusca. Although adults commonly measure about 5 centimetres to 8 centimetres, they can grow up to 15 centimetres (Global invasive species database 2018).

The lifecycle begins with the fertilization of an egg, that withstands gametogenesis, after which, larvae are formed (Global invasive species database 2018). Afterwards, a juvenile is developed eventually becoming a sexually mature adult (during its first to the second year, according to the National Department of Agriculture of South Africa (National Department of Agriculture of South Africa 2012). As an adult, it undergoes a process of gonochoristic reproduction thorough simultaneous spawning (Global invasive species database 2018). This kind of reproduction is defined by the separation of the sexes and lasts the entire life of the individual (Maybank and Fostier 2008).

M. galloprovincialis tends to inhabit rocks (Observatório Marinho de Esposende) on which they attach through byssal threads produced by a mobile foot (National Department of Agriculture of South Africa 2012). *Mytilus sp*. tend to have a superior survivorship rate in habitats where predation is low, which lean towards being at the higher tidal elevations (Seed and Suchanek 1992). Consequently, the growth rate will be lower since it can only feed when underwater filtering particles in suspension as small as 2-3 µm (*e.g.* phytoplankton, bacteria or small pieces of organic detritus) (Seed and Suchanek 1992).

This species prefers waters clear from sediments, which means it flourishes in territories where an upwelling with plenteous nutrients is present (Global invasive species database 2018). It has been shown that *Mytilus* sp. have higher growth and absorption rates in water temperatures between 10 ºC and 20ºC, as well as the fastest ingestion (National Department of Agriculture of South Africa 2012). Although they can tolerate a wide range of habits or ecological conditions, there are extreme modifications in the environment on which mussels cannot survive. These can differ each season and, if combined, may lead to mass mortality. These factors are, as an example, excessive silt accumulation, high temperatures or even storms (Seed and Suchanek 1992).

Predators can also cause mortality. *M.galloprovincialis* predators are flounders, birds (Tyler 2019) crabs, starfish or sea snails (*Nucella lapillus*), amongst others (Seed and Suchanek 1992). Parasites and pathogens may also cause mortality: *Mytilus* sp. tend to shelter a large number of parasites (Seed and Suchanek 1992). However, the scientific community believes they do not cause

significant mortality, even though infected mussels may sometimes reveal some symptoms of a disease (Bayne and Bennet-Clark 1976). There is a parasite that settles in the gut of *M. galloprovincialis* named *Mytilicola intestinalis* that can inhabit this organ from one individual up to “several dozens” (Bayne and Bennet-Clark 1976). It causes little or no harm to *M. galloprovincialis*. Nonetheless, if present, it can decrease the body condition and eventually lead to death, depending on the degree of infestation (Bayne and Bennet-Clark 1976). Other parasites include *Ancistrum mytili* (that is present in the gills), parasites from the protistan class Ascetosporea, and parasites of the Mikrocytida, Paradinida and Paramyxida orders (Ward et al. 2019)

Before ingestion, in these animals, particles are captured, retained and sorted (Wright et al. 2013). Bivalves can select food through intracellular digestion by directing particles to the digestive system, or through longer retention of some particles, in order to make them have deeper extracellular digestion (MacDonald and Brillant 2000). These sorting areas are composed of edges and folds that are ciliated. While the heavy particles are prone to falling into the sorting tracts, the lighter ones will tend to be in suspension (MacDonald and Brillant 2000). According to Wright et al. (2013), “*Mytilus edulis* can ingest microplastic particles from 2 to 16 µm” (Wright et al. 2013). After ingestion, microplastics can be expelled with the faecal matter (Wright et al. 2013).

2. Microplastics

Generally, the classification of plastics into two groups has been accepted. This classification divides them into microplastics (with less than 5 mm) and macroplastics (with more than 5 mm) (Moore 2008). Nevertheless, some authors and groups, such as the Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP 2015) go further on and consider there to be four different groups: the macroplastics (from 2.5 cm up to 100 cm, that can be seen by the naked eye); the mesoplastics (from 0.1 cm to 2.5 cm, that can be observed with an optical microscope or by the naked eye); the microplastics (whose dimensions vary from 1 µm to 0.1 cm, that can no longer be observed with a naked eye, but rather an optical microscope) and, finally; the nanoplastics (which are smaller than 1 µm and can only be visualized through an electron microscope) (GESAMP 2015; Andrady 2017).

Moreover, some authors (Ter Halle et al. 2016) consider that classifying microplastics solely by their size can lead to reasoning errors because while the measuring method may vary with the experiment or protocols, microplastics weighing cannot be disputable. Another way to classify the microplastics is to divide them into primary and secondary microplastics (GESAMP 2015). The primary ones are those purposely produced with a specific size. They include microbeads on cosmetics, virgin resin pellets, industrial blasting surface cleaners. The secondary microplastics are the result of the degradation or the breakdown of larger plastics, such as plastic bags, textiles, tyres (GESAMP 2015).

According to Andrady (Andrady 2017), degradation is "the complete degradation or mineralization where the plastic is digested into small molecules, typically CO_2 and H_2O". This mineralization is significant when it comes to the marine environment because it eliminates the polymer from the area (Andrady 2017). The accelerated degradation of polymers in the environment is mainly caused by ultraviolet radiation exposure, due to its autocatalytic thermal oxidation effect, being much rapid on the sand (due to an existent higher temperature) than on the ocean's surface.

There are numerous microplastic polymers, such as polypropylene (PP) (specific density of 0.83-0.85 $g.cm^{-3}$), low-density polyethylene (LDPE) (with a specific density between 0.91 and 0.93 $g.cm^{-3}$), high-density polyethylene (HDPE) (with a specific density 0f 0.94 $g.cm^{-3}$)(GESAMP 2015), polyamide (PA) (specific density of 1.02-1.05 $g.cm^{-3}$), acrylic (specific density of 1.09-1.20 $g.cm^{-3}$) and polyvinyl chloride (PVC) (specific density of 1.16-1.58 $g.cm^{-3}$) (Hidalgo-Ruz et al. 2012) and thermoplastic polyester (PET) (with a specific density of 1.37 $g.cm^{-3}$) (GESAMP 2015).

Various factors can affect the bioavailability of microplastics, such as density, size, abundance or even colour. The density will determine the layer the microplastics occupy in the water column and, therefore, the organisms that may feed on them by mistake (Wright et al. 2013). Microplastics can have a specific gravity similar to the specific gravity of the algae on which some marine organisms feed, making microplastics likely to be ingested by them (Wright et al. 2013). For instance, feeders of the upper water column will be likely to ingest low-density plastics, since these tend to occupy, in the water column, the same territory (Wright et al. 2013). The tentacles in some animals, the size of the microplastics or the mouth size may limit the particles ingested. The colour can mislead the animal into thinking that plastic is prey. Finally, the abundance of microplastics can make their ingestion more likely (Wright et al. 2013).

3. Deltamethrin

Deltamethrin is a synthetic pyrethroid insecticide (Unger 1996). It is classified as a type II pyrethroid, due to the presence of the α-ciane group (Spinosa et al. 2006). It is used in crops, such as citrus, maize, soy and cotton (Unger 1996). When it comes to veterinary medicine, in farm animals is mostly used in a liquid formulation in a pour-on administration. For small animals, bath formulations exist for topical use (DGAV 2020).

It has been studied in recent years in order to determine if it might be used as an anticancer agent, by inducing apoptogenic signalling pathways (Kumar et al. 2015). Pyrethroids tend to be fastly degraded in plants and soil, due to its tight bind to it, being barely eluted with water (United Nations Environment Programme et al. 1990a). Synthetic pyrethroids are considered to be toxic for fish and aquatic arthropods (United Nations Environment Programme et al. 1990a). Bees should not be harmed if adequately used. (DGAV 2019a).

Even though communities should be aware that pyrethroid usage can be concerning when it comes to non-target organisms, if applied at low rates, pyrethroids can undergo into reduced residual environmental concentrations (Bradbury and Coats 1989).

Deltamethrin is known in human health as an endocrine disruptor (with a weak estrogenic activity), an IARC group 3 (Not classifiable as to its carcinogenicity to humans (International Agency for Research on Cancer 2019)) and a neurotoxicant (University of Hertfordshire and IUPAC 2019). It works through contact or ingestion and is because of its classification of being extremely dangerous for aquatic organisms which made the Portuguese Directorate-General of Food and Veterinary (DGAV) state that it should not be applied in adjoining lands of watercourses nor should it contaminate waters (DGAV 2019a).

Pyrethroids depolarize the nerve's membranes and impede impulse conduction, throughout an overly prolonged sodium current. The activity of these pyrethroids is associated with the binding in the GABA-receptor complex (Bradbury and Coats 1989). At the end of depolarization, the organism that was in contact with deltamethrin has a sodium tail current with a slow influx of sodium

(National Center for Biotechnology Information; 2019)[1]. Synthetic pyrethroids interact with sodium channels in animals, acting on the axons of the central and peripheral nervous system. In mammals, even a single dose can cause hyperexcitability, tremors, salivation and paralysis. “At near-lethal levels” it can produce axonal swelling and “myelin degeneration in the sciatic nerves”(United Nations Environment Programme et al. 1990a). Temperature can affect toxicity since nerve function can change along with sensitivity when cold temperatures are present (Bradbury and Coats 1989).

Deltamethrin may cause both neurotoxicity and cardiotoxicity in fish, due to the irregularities it can cause in the electrical activity, that alter the function of sodium channels (Haverinen and Vornanen 2016). In cats, due to their deficient glucuronoconjugation, are hypersensitive to pyrethroids (Beugnet and Franc 2012).

[1] Ellenhorn M, Barceloux D. 1988. Medical Toxicology - Diagnosis and Treatment of Human Poisoning.1st ed. New York: Elsevier Science Publishing Co., Inc.

4. Biomarkers

The definition of biomarker has been accepted to be a measurement of a change in biological response that can be the result of exposure to a given hazard (Van der Oost et al. 2003).

According to the International Program of Chemical Safety, biomarkers can be divided into three classes: biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility (International Program on Chemical Safety 1993)

The first mentioned detect and measure, throughout their metabolites or themselves, an exogenous substance or the interaction between a specific target and a xenobiotic agent. Biomarkers of effect help assessing the health *status* of an organism, by measuring tissue or fluid alterations. Lastly, the biomarkers of susceptibility evaluate the ability of an organism of responding to the peril of a specific substance (Van der Oost et al. 2003).

The health *status* of an individual can be measured by different health indicators (such as the presence or absence of certain diseases, the behaviour of the individual, mortality and several other indicators) (Depoorter et al. 2015)

4.1. Acetylcholinesterase

Acetylcholine is the neurotransmitter used in the neuromuscular somatic synapsis (Klein and Cunningham 2007). Acetylcholinesterase (AChE) is an enzyme that inactivates the acetylcholine into esters of choline molecules and acetic acid. This process occurs in the synaptic cleft (Klein and Cunningham 2007).

Vertebrates possess two types of cholinesterases: acetylcholinesterase and the butyrylcholinesterase The first one, acetylcholinesterase (AChE, EC 3.1.1.7), is fundamental for the nervous system and in vertebrates is also found on ganglia, blood, among other sites (Garcia et al. 2000). According to Moreira et al. (2001), the cholinesterase that is present in the haemolymph of *M. galloprovincialis* is acetylcholinesterase which is also identified and characterized in several aquatic invertebrates, like bivalves, that have high levels of this enzyme's activity in the haemolymph (Rickwood and Galloway 2004). When using acetylcholine as a substrate, a higher rate of hydrolysis is achieved, comparing to a very low rate of butyrylcholine. The second type, butyrylcholinesterase (BuChE, 3.1.1.8), is known as a non-specific

cholinesterase that in vertebrates takes more action in the heart, liver and pancreas, and the serum. However, it is less specialized (Garcia et al. 2000).

Assessing the activity of AChE is critical when it comes to evaluating an existent AChE inhibition. In case this inhibition does occur, neurotransmission can be disturbed, as a consequence of hyperstimulation of muscarinic and nicotinic receptors caused by an accumulation of acetylcholinesterase (Colovic et al. 2013).

The causes of the specific activity of AChE variation could be related to xenobiotics (Moes et al. 2014) present in marine pollution, *e.g*. organophosphorus and carbamates pesticides; metals (such as zinc, copper, mercury and cadmium) (Frasco et al. 2005); polycyclic aromatic hydrocarbons (PAH) (Moes et al. 2014) and tributyltin (TBT) (Sousa 2009).

Organophosphorus bind to the AChE irreversibly inactivating AChE (English and Webster 2012). Some derivates of petrol, such as oil, petrochemical contaminants or PAHs may inhibit acetylcholinesterase activity in mussels disregarding if in the field or in laboratory conditions (Moreira et al. 2004). The presence of iron, cadmium and lead have a negative correlation with AChE activity (Oliva et al. 2012). The presence of tributyltin (TBT) can increase this enzyme's activity. Tributyltin is known as a molluscicide and antifoulant agent, commonly found on fishing equipment such as boats, fishing nets, buoys (among others). It also has other functions in other industries. However, due to its high phototoxicity, it is not used in agriculture (United Nations Environment Programme et al. 1990b). In Portugal, TBT is present on the shore but also in port areas (Sousa 2009). Exposure to pyrethroids insecticides can be accessed by measuring acetylcholinesterase activity (Singh et al. 2018)

4.2. Octopine dehydrogenase

In bivalves, octopine dehydrogenase (ODH) is an important enzyme involved in the processes of cellular energy production (Oliveira, Barboza, et al. 2018) being a pyruvate oxidoreductase (Lima et al. 2007). It is a critical marker for the evaluation of the anaerobic response (Capaz et al. 2017), evaluating chronic hypoxia exposure (Murphy and Richmond 2016)[2].

[2] De Zwaan A, Putzer V. 1985. Metabolic adaptations of intertidal invertebrates to environmental hypoxia (a comparison of environmental anoxia to exercise anoxia). Symposia of the Society for Experimental Biology.39:33-62.

ODH requires NADH, NAD^+, amino acids and pyruvate (Lee et al. 2011). It oxidizes NADH obtained by anaerobic glycosylation, transforming it into NAD^+ by oxirreducting pyruvate (Lima et al. 2007). It produces energy (in the form of octopine) in case of functional anoxia, which happens when, despite the oxygen supply, tissues are in anaerobiosis, due to its excessive consumption (Carroll and Wells 1995; Lee et al. 2011). There is another kind of anaerobiosis called environmental anaerobiosis, that takes place when the whole organism is exposed to anoxic conditions caused by external, physical factors in the microhabitat, arginine, phosphate, glycogen and aspartate are the substances for the metabolism (Gäde 1983)

Studies have found that the accumulation of HCO_3^- in body fluids, in order to compensate hypercapnia induced by acid-base disturbance, can contribute to an hypercalcification of $CaCO_3^-$ structures, like crustacean carapaces as an example (Hu et al. 2011). Hypercapnia is defined as the increase in seawater partial pressure of carbon dioxide (PCO_2) (Hu et al. 2011). However, hypercapnia is common in upwelling regions in continental shelves and also systems that happen to be hypoxic seasonally. Therefore, it is considered to be an environmental stressor, expected to rise during this century, from “0,04 kPa up to 0,14 kPa” (Caldeira and Wickett 2003).

A study by Storey et al. (1979) found that in *Sepia officinalis* subjected to hypoxia, octopine was elevated, and throughout its recovery, this substance was promptly cleared from the blood (Storey et al. 1979). Octopine levels of *Sepia officinalis* in the mantle were higher in hypoxic animals rather than in normoxic (Storey et al. 1979).

4.3. $NADP^+$-dependent isocitrate dehydrogenase

Isocitrate is a molecule involved in the Citric Acid Cycle (that produces energy in the form of ATP) (Nelson et al. 2013). $NADP^+$-dependent isocitrate dehydrogenase (IDH) is, therefore, an excellent biomarker to evaluate the production of aerobic cellular energy as well as an essential enzyme for the antioxidant system (Oliveira, Guilhermino, Lírio, et al. 2018). It controls the segregation of isocitrate into the citric acid and the glyoxylate cycle, being the first responsible for producing ATP and the second responsible amino acids and nucleotides (Nelson et al. 2013).

With the aim of evaluating the antioxidant defences of a given organism or its aerobic metabolism, scientists frequently select IDH and so, in the presence of ammonia, nitrates and phosphates, IDH activity is likely to increase (Lima et al. 2007). When inhibition of IDH is present among side with an ODH that did not increase its activity, one can consider that hypoxia could have been present (Oliveira, Guilhermino, Lírio, et al. 2018). However, lower levels might indicate lower levels of NADPH regeneration (Lima et al. 2007). If almost full inhibition of IDH activity occurs, changes might have occurred in the aerobic pathway of energy production (Oliveira, Guilhermino, Lírio, et al. 2018).

Studies of Lima et al. (2007) have found that IDH activities were higher in more polluted sites and that IDH activity had a positive correlation with metals in water (e.g. cadmium and copper) and in sediment (e.g. lead and cadmium) (Oliva et al. 2012). It is believed that copper has this positive correlation on this enzyme's activity due to its activation by metal cations (Oliva et al. 2012). Heavy metals react with sulfhydryl groups, and it is this reaction that is generally accepted to be the main reason for their toxicity (Oliva et al. 2012).

4.4. Glutathione *S*-transferase

Glutathione is a tripeptide involved in detoxification of xenobiotics (such as pollutants, carcinogens or drugs), protecting the cell from the oxidative stress (Pompella et al. 2003). Nevertheless, it can eventually generate free radicals and ROS (Pompella et al. 2003), also known as "oxygen radicals" (National Cancer Institute 2019). Asides from the previously mentioned, oxidative stress can also lead to the loss of the mitochondrial membrane potential (Hu et al. 2015).

Glutathione *S*-transferase (GST) is involved in the conjugation of glutathione to several electrophilic substances, due to the thiol group of glutathione, managing substances made by oxidative stress, such as products of lipid oxidation or S-glutathionylated proteins (Laborde 2010). While controlling cell proliferation and apoptosis, GST carries out as a modulator of their signal transduction pathways. It is also associated with the biosynthesis and metabolites of leukotrienes, prostaglandins and steroids, substances involved in inflammation responses (Laborde 2010). GST also shows peroxidase activity (Sheehan et al. 2001).

GST activity can increase when the organism is exposed to a xenobiotic and environmental levels of nitrate and ammonia (Lima et al. 2007) however if the activity is decreased, it may be due to a GST saturation (Hu et al. 2015).

4.5. Catalase

Catalase (CAT) is an enzyme that degrades two hydrogen peroxide molecules into water and oxygen. This reaction is critical in the process of avoiding cellular damage, for it removes ROS (Chelikani et al. 2004), which are involved in carcinogenesis, neurodegeneration, diabetes and ageing (Ray et al. 2012).

CAT activity can increase when the organism has been in contact with contaminants or at higher temperatures, salinity or a broader light period. Therefore, when collecting samples in the summer, it is expected that CAT levels will be higher (Khessiba et al. 2005).

A study performed in the North-western coast of Portugal (Lima et al. 2007)[3], suggests that ROS stimulated by contaminants, at low levels, can have a toxic effect on cells' membranes, even though enzymes responsible for the antioxidant defences are acting. This means that, at long-term exposure to ROS, CAT will be depleted, inducing oxidative damage in molecules (*e.g*. DNA, lipids or proteins) (Vlahogianni et al. 2007). Hypoxia can also inhibit CAT activity (Hu et al. 2015).

4.6. Lipid Peroxidation

There is a group of substances called Reactive Oxygen Intermediates (ROI) (Nathan and Ding 2010) that can lead to enzyme inactivation, lipid peroxidation (LPO), DNA damage and eventually cell death (Hampel et al. 2016). The production of ROI (e.g. superoxide radicals, H_2O_2 and hydroxyl radicals) (Nathan and Ding 2010) can be either induced by environmental stress agents

[3] Gravato C, Oliveira M, Santos MA. 2005. Oxidative stress and genotoxic responses to resin acids in Mediterranean mussels. Ecotoxicology and Environmental Safety. 61(2):221–229. doi:10.1016/j.ecoenv.2004.12.017

(*e.g.* by xenobiotics) (Hampel et al. 2016) or can occur naturally in several biological processes, such as by active phagocytosis, electron transport chains (Giulio et al. 1989) or oxidative phosphorylation (Hampel et al. 2016).

It is important to state, in order not to cause any mistakes, that sometimes the terms ROI and ROS are used interchangeably. Briefly, ROI are the electron reduction products of oxygen that are grouped under the ROS denomination, in which ozone and oxygen are also included (Nathan and Ding 2010).

Lipid peroxidation requires a long exposure period to be observed and appears to be affected by seasonality and the organisms' condition. LPO occurs when oxidants like free radicals act upon double-bonded carbon-carbon lipids, particularly polyunsaturated fatty acids. From this process, lipid peroxyl radicals (molecules that had one hydrogen atom removed from a carbon and an oxygen inclusion) are obtained as well as hydroperoxides such as aldehydes, acetone and malondialdehydes (MDA) (Hampel et al. 2016).

MDA is found when unsaturated fats are ruptured, and when in reaction with 2-thiobarbituric acid (TBA), it produces a red fluorescent adduct that can be measured by spectrophotometry (Hampel et al. 2016). It is also known to react with cysteine, histidine, arginine and lysine (Weber et al. 2015), which may falsify the results.

Damage in cells' structures due to oxidative processes are thought to be mainly caused by lipid peroxidation, after which DNA damage occurs, precipitating cell death (Hampel et al. 2016).

4.7. Protein carbonyls

There are several physiopathological processes on which metal-catalysed oxidation has effects, such as intracellular protein turnover or aging. The definitive method to estimate this metal-catalysed oxidation is done by assaying the carbonyl content of proteins (Levine et al. 1990). Protein carbonyls presented in an irreversible form of protein modification are comparatively more stable than lipid peroxidation products. The first ones may degrade in hours and cleared in days, whereas lipid peroxidation products may be removed in a matter of minutes (Weber et al. 2015).

Protein carbonyls are also known markers for overall protein oxidation being promptly formed on oxidative conditions and do not result in a reaction of a specific oxidant (Weber et al. 2015). There can be a fragmentation of the

polypeptide chain (Stadtman 1993). Deamination occurs in the presence of oxygen in proteins' N-terminal of amino acids, and ammonia can be released. This deamination can create α-ketoacyl derivates that can be quantified by reaction with 2,4-dinitrophenylhydrazine (DNPH). Oxidation of the side chains of residues of some amino acids, such as lysine or arginine, can lead to the formation of carbonyl groups (Stadtman 1993). When it comes to protein oxidation, there can exist species that do not endure bonded to proteins but are released instead. These can, for example, be acetaldehyde, acetone or formaldehyde (Weber et al. 2015).

There are nucleic acids that contain carbonyl groups and some biological compounds (such as retinoids, haemoglobin or myoglobin) that absorb at 370 nm. These events may falsify the result (Weber et al. 2015). Moreover, haemoglobin has four globin chains, each containing a heme component with a protoporphyrin ring and ferrous ion (Fe^{2+}) (Farid and Lecat 2019 Jan 13). Redox cations, such as Fe^{2+} or Cu^{2+} can bound into proteins, and perform a Fenton reaction, on which oxygen peroxide or oxygen play a role. If oxygen is present, aggregation barely occurs or does not occur at all (Stadtman 1993). It is important to remember that the resolubilization may be incomplete, that acidic conditions may introduce carbonyl groups or eventually, the DNP may be captured in the pellet, falsifying the results (Weber et al. 2015).

4.8. Condition index

Condition Index (CI) is an essential parameter for the processing industry as well as an ecophysiological one (Orban et al. 2002). It provides hard information on the growth and physiological state of the organisms (Andral et al. 2004) and is used in studies addressing exploited populations or the physiological evolution after a given event that may arise into variations of energy reserves (Gabbot and Walker 1971).

After spawning, the tissue mass is reduced (Benali et al. 2015) and spawning usually happens during late autumn and spring (Okaniwa et al. 2010). Somatic weight, shell length, growth rates are higher in spring, due to an increase of nutrients, the availability related to phytoplankton boom (Benali et al. 2015).

Mussels are filter feeders and can accumulate various pollutants (Moreira et al. 2004). The determination of the condition index, along with biomarkers, can

help better evaluate the impact of pollutants better when accumulated (Van Der Oost et al. 1996).

III. Four sites in the Northern Coast of Portugal: Monitorization

This study took place in ICBAS-UP, on the Laboratory of Ecotoxicology and Ecology in the Department of Population studies from mid-September 2018 to mid-March 2019, during the first part of the traineeship. It was performed in order to follow up on the laboratory previously studied sites, continuing their monitorization work.

1. Objectives

The purpose of this study was to compare the health *status* of *M. galloprovincialis* specimens collected in four different sampling sites throughout the evaluation of several biomarkers.

2. Material and Methods

2.1. Sampling sites

During the first months in the laboratory, four sites in the Northern coast of Portugal were monitored: Vila Praia de Âncora, in Caminha; Carreço, in Viana do Castelo; Cabo do Mundo, in Matosinhos; São Félix da Marinha, in Vila Nova de Gaia.

Vila Praia de Âncora is located near the mouth of the Âncora river (a small river about 19 km long, that is born in Serra de Arga). The Portuguese Agency for the Environment (APA) reports the presence of installations with a particularly high risk of accidental water pollution in this river: a phytopharmaceutical unit (1 unit, classified as high (4/5) on the severity index); gas stations (2 units, classified as very low (1/5) on the severity index) and a wastewater treatment plant (1 unit classified as moderate (3/5) on the severity index (Agência Portuguesa do Ambiente 2015).In this report is also stated that Duna do Caldeirão, a site nearby the mussel's collecting site had some changes (due to erosion) and that these could close the current mouth and that could also lead to siltation or/and deterioration of water quality on that part of the river (the estuary) (Agência Portuguesa do Ambiente 2015). This same agency stated that short-term pollution (≤ 72 hours) was likely to happen as a result of malfunctions in the wastewater pumping station or from ships spills. Faecal contamination can also

occur from Âncora river, the local sewage network or the fishing harbour (Agência Portuguesa do Ambiente 2019). Studies in this site were made by Tim-Tim et al. (2009) and Agência Portuguesa do Ambiente (2019).

Carreço is located next to a promontory, next to diverse geological formations but also in the vicinity of farming sites with corn production (Câmara Municipal de Viana do Castelo 2019), and it has been systematically recognized as a beach with excellent water quality, and 2018 was no different (Sistema Nacional de Informação de Recursos Hídricos 2019). Lima et al. (2007) and Lima et al. (2008) previously monitored this site.

Cabo do Mundo is located next to an oil refinery in Leça de Palmeira and was included in previous monitorization studies (Leal et al. 1997; Moreira and Guilhermino 2005; Lima et al. 2007; Tim-Tim et al. 2009). More than that, it is also located next to a very active mercantile harbour (that has traffic through the sea, roads, railways) and in the vicinity of an airport. This site is long known to be chronically exposed to heavy metals (Leal et al. 1997) and petroleum-derived hydrocarbons, such as polycyclic aromatic hydrocarbons (PAH) (Moreira and Guilhermino 2005), or even sulfur, nitrogen-oxygen compounds (Lima et al. 2007).

São Félix da Marinha is located in the south bank of the Douro hydrographic basin, in an extensive dune system (Associação Bandeira Azul da Europa 2019). It also had excellent water quality in 2018 (Sistema Nacional de Informação de Recursos Hídricos), having been given this site the Blue Flag, for quality beaches (Associação Bandeira Azul da Europa 2019). It has had interventions since 20 years ago either in dune walkways and dune regenerations (Águas de Gaia, oral communication, 7th November 2019). This site was previously studied by Moreira and Guilhermino (2005).

2.2. Sample collection

The mussels were collected in the four different sampling sites in three different days at low tide. The first ones were gathered in October 9th 2018, in the beach of São Félix da Marinha in Vila Nova de Gaia, around 10 a.m. On this site, 25 mussels were collected. The second day happened in October, 26th 2018 took place in Cabo do Mundo in Matosinhos, a place next to an oil refinery, around 11 a.m. On this site, 21 mussels were collected. On the third day, November, the 14th 2018, two sites were sampled: one in Vila Praia de Âncora and the other in

Carreço beach, both in Viana do Castelo's district. The collection took place around noon, where 20 mussels were collected and in the second, 22 mussels.

The collection of the mussels was done by two different people, which led to different sample numbers. Since the mussels could no longer be released, it was decided to use them. The exception was São Félix da Marinha, where the 25 mussels were collected with the purpose of using 5 to practice the procedures.

In each beach, mussels were collected and put in previously filled with water iceboxes. The buckets were preferred for the collection to ease the collecting on the rocks. The mussels were chosen based on their size (chosen by what appeared to the be the overall monitorization samples' standard size and the site's standard size, for homogenous samples) and the closed ones were preferred. This homogenous sampling was necessary to achieve a better comparison of the results.

Arriving at the laboratory, the following biomarkers were analysed from samples from the mussels: AChE in the haemolymph, ODH and IDH in the adductor muscle, and GST, LPO, CAT and oxidized proteins on both the digestive system and the gills.

2.3. Abiotic parameters determination

During the collection of the animals at each sampling site, three different water samples were collected along a perpendicular transept in relation to the waterline, for laboratory analyses of physic-chemical parameters. This transept comprehended a first sample collected in the first poodles on the rocks, in the intertidal zone, the second one 3 metres further in the same line and the last one the closest possible to the shoreline, keeping the collectors safe.

Water bottles destined for human use were used for the collection in previously washed with distilled water. In the same spots, the local water conductivity, salinity, temperature were measured with a multi-parameter probe (Hydrolab DS5X- Hach) and dissolved oxygen levels were also measured with a portable meter (LDO HQ 10- Hach Environmental).

On the same day, after arriving at the laboratory, the water in the bottles was analysed regarding its physic-chemical parameters, which were the concentration of nitrates, nitrites, ammonia, phosphates, iron, phenol and silica. The water hardness was also determined. All these parameters were determined using

commercial photometer kits (Photometer 7100, Palintest, Kingsway, England) and corresponding standard protocols provided with the analyses kits.

2.4. Biomarkers determination

Afterwards, when arriving at the laboratory, the mussels were measured with vernier callipers (for their length, in centimetres) and the total body mass weighed (in grams) in a scale. The length was defined as the anteroposterior diameter, from the umbo to the posterior border apex (see Picture 1).

These measurements were used to calculate de condition index, that, in this case, was chosen based on the length of the shell, according to the formula from Bodoy et al. (1986) on which condition index equals the weight of the soft tissues in grams divided for the length of the shell (in millimetres), multiplied by 10^4 factor.

The summary of the process can be observed in the flowchart (see Figure 1).

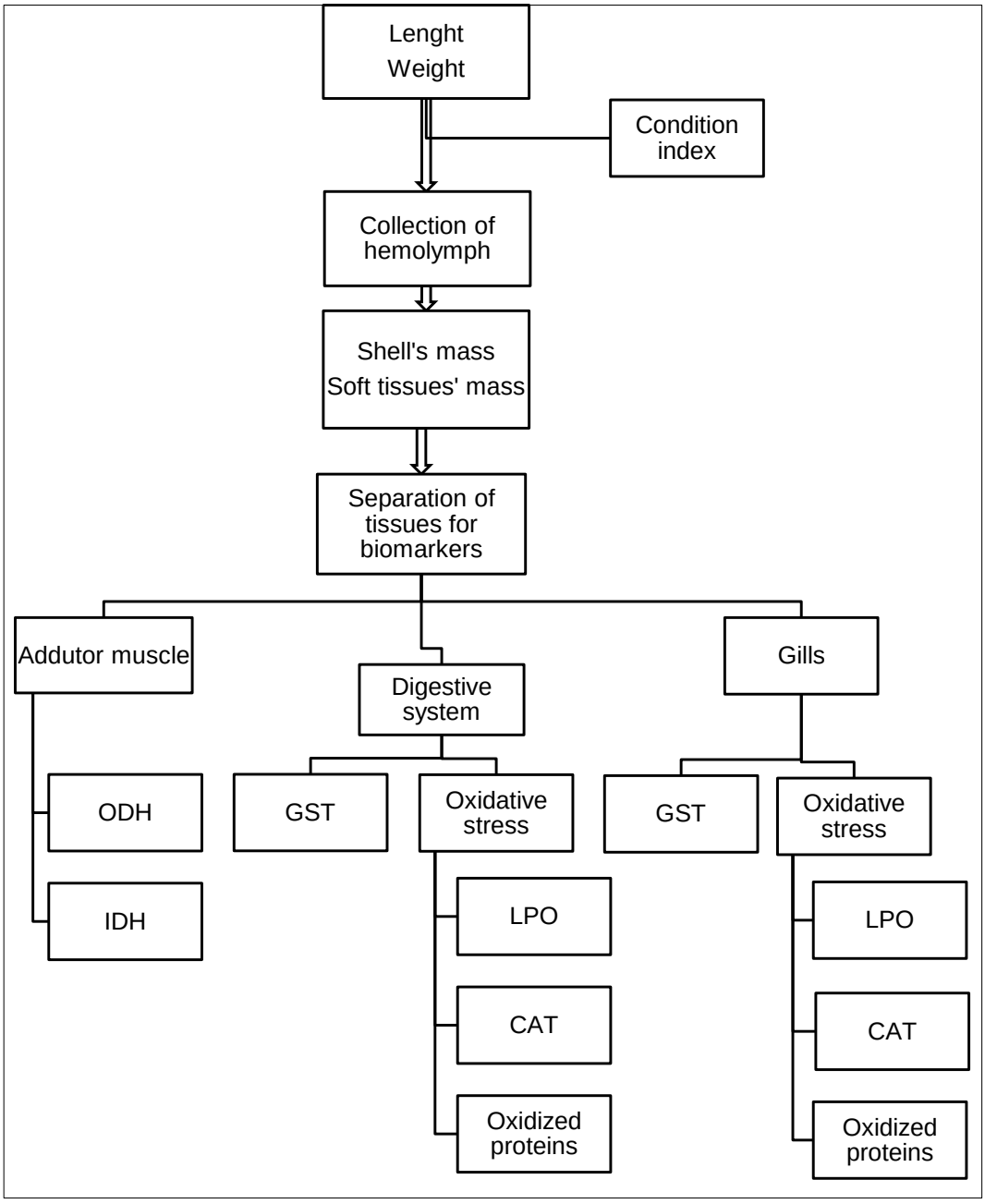
Promptly, the shells were slightly opened just as little as to expel the water from the bodies (with the purpose of not to mix the fluids from the mantle with the haemolymph) and the hemolymph was collected from the posterior adductor muscle with the help of a 2 ml syringe and put in a 2-millilitre microtube, being one microtube per mussel, to analyse the enzyme acetylcholinesterase. Then, a buffer solution (phosphate buffer at 0.1 M and a pH of 7.2) was added to the microtube, diluting the haemolymph sample in a 1:3 ice-cold buffer. The acetylcholinesterase was determined in the posterior adductor muscle, vital for the shell's opening and closing movements, where the neuromuscular function is critical (Oliveira, Barboza, et al. 2018).

At this moment, the mussels were sacrificed, and the shell's mass and the soft tissues weighted. Next, the tissues were separated into different microtubes of 2 ml according to the different enzymes that were to be analyzed, where a specific buffer (previously prepared) was present. All the chemicals used during the elaboration of this dissertation were purchased from Sigma-Aldrich (USA) or Merck (Germany).

The mussel's foot was collected for acetylcholinesterase's analyses; the adductor muscles were sorted into two different microtubes, one for octopine dehydrogenase (with a Tris 20 mM, 1mM Na_2EDTA, 1 mM DDT with a 7.5 pH buffer) and the other for isocitrate dehydrogenase (Tris 50 mM solution with a 7.8

pH buffer). The digestive system was sorted into two different microtubes, one for GST (phosphate buffer of 0.1 M with a 6.5 pH) and the other for oxidative stress enzymes (phosphate buffer of 0.1 M and 7.4 pH). This last separation process was repeated for the gills. The repetition of the biomarkers on both the gills and the digestive system was based on the state of the art proceedings. Then the enzymes were put in a freezer at the temperature of -80 degrees Celsius.

Homogenization was done with a Ystral homogenizer (Ballrechten-



Dottingen, Germany) during 3 seconds after unfreezing the frozen samples. It was done putting the microtube in a small goblet with ice, in order not heat the samples.

The acetylcholinesterase samples were centrifuged (Eppendorf, 5810R centrifuge, Germany) at 3300 G for 10 minutes at 4ºC, a protocol that can be observed in Table 1.

After centrifugation and collection of the supernatant, two microtubes were used: one to the measurement of the initial protein and the other one to evaluate the enzyme itself. In the other enzymes, the same two microtube method was used.

For the ODH and IDH assessment, the samples were centrifuged at 3300 G for 3 minutes at 4ºC.

For the GST enzyme, the centrifuge was set at 9000G for 30 minutes at 4ºC. Then, from the stress oxidation microtube, 250 µl of the homogenate was transferred to a microtube containing 4 µl of BHT at 4% to analyze LPO. After homogenizing again, 40 µl of it was separated to determine the initial protein.

With the remaining homogenate, destined for the stress oxidation enzymes, the microtubes were put in the centrifuge at 10.000 G for 15 minutes at 4ºC and then divided into three aliquots: one for initial protein (40 µl), one for CAT (150 µl), one for oxidized proteins (300 µl). After all these processes, the centrifugated samples were frozen again.

Biomarkers	Buffer	Centrifugation		
		Force (G)	Time (min)	Temperature (ºC)
AChE	Phosphate buffer at 0.1 M and a pH of 7.2	3,300	10	4

Figure 1: Flowchart of the process made in order to obtain samples for the study of the biomarkers.

IDH	Tris 50 mM solution with a 7.8 pH buffer	3,300	3	4
GST	Phosphate buffer of 0.1 M with a 6.5 pH	9,000	30	4
LPO	Phosphate buffer of 0.1 M and 7.4 pH	No centrifugation		
Oxidative stress	Phosphate buffer of 0.1 M and 7.4 pH	10,000	15	4

enzymes

In order to determine the initial and final protein content of the sample, the Bradford method (Bradford 1976) adapted by Frasco and Guilhermino (2002)

Table 1: Different biomarkers and their buffers and centrifugation processes.

was chosen, having been used ɣ-globulin as standard protein, according to the second protocol.

Acetylcholinesterase activity was determined at 412 nm using a protocol adapted by Guilhermino et al. (1996) based on the Ellman et al. (1961), for the activity of IDH, the Ellis and Goldberg (1971) protocol revised by Lima et al. (2007) was used, at 340 nm and ODH activity was determined using a protocol adapted by Lima at al (2007) according to Livingstone et al. (1990), also at 340 nm. Catalase activity was determined at 240 nm, according to Aebi (1984); GST activity was determined at 340 nm in consonance with Habig et al. (1974)adapted by Frasco and Guilhermino (2002); LPO levels were measured at 535 nm according to Bird and Draper (1984) and Ohkawa et al. (1979), where thiobarbituric acid reactive substances (TBARS) were quantified, and carbonyl content in oxidatively modified proteins was measured at 370 nm, according to Levine et al. (1990).

For CAT analyses, it was used a spectrophotometer (JASCO® V-630 UV/Vis spectrophotometer, USA), with a quartz cuvette. For all the others, photometer (BIO-TEK®, Powerwave 340, USA) was used to determine the activities and levels, in the case of the LPO.

All enzymatic activities were expressed in nanomoles of substrate hydrolysed per minute per milligram of protein (nmol/min/mg protein) except for CAT, protein carbonyl and LPO. CAT was expressed in µmol/min/mg protein, protein carbonyl quantification in nmol/mg protein and LPO levels were expressed in nmol TBARS/mg protein.

2.5. Statistical analyses

Results were analyzed with the statistic package SPSS version 25 (IBM, USA).

First, for each biomarker data set, the normality of the data distribution was tested with the Kolmogorov-Smirnov test. Then, the homogeneity of variances

was tested using the Levene test. If normality of distribution and/or homogeneity of variances were not achieved, data transformations were made (Zar 2010).

When normal distribution and homogeneity of variances were achieved, a one-way Analysis of Variance (ANOVA), followed by a multi-comparisons Tukey test was performed to compare means of the biomarker in mussels from different sampling sites.

When ANOVA assumptions could not be achieved, the nonparametric Kruskal-Wallis test was performed, followed by a Tukey-type non-parametric multi-comparison test (Zar 2010).

The significance level was lower than 0.05.

3. Results and discussion

3.1. Water analysis.

The results of water temperature and dissolved oxygen are shown in Fig.1. Significant differences of water temperature ($F_{3, 8}$ = 74.564, p<0.001) and dissolved oxygen ($F_{3, 8}$ = 11.237, p=0.003) among sites were found. In the other water parameters, no significant (p >0.05) differences among sites were found (Table 2). The n values below 12 (e.g. in ammonia, 11; in iron, 10; in phenol, 11) were inferior to the photometer limit of detection, and therefore, were taken out.

Table 2- Mean and standard error of the mean of several water parameters measured or determined in the water of distinct sampling sites, and results of the statistical analyses (ANOVA or Kruskal-Wallis , p ≤ 0.05). n = 3 per sampling site.

Parameters	N	Vila Praia de Âncora	Carreço	Cabo do Mundo	São Félix da Marinha	Statistical Analysis
Nitrates (mg/l)	12	2.767±0.37	1.980±0.21	4.267±2.32	2.933±0,33	H_3=2.939, p=0.401
Nitrites (mg/l)	12	0.06±0.00	0.043±0.01	0.570±0.52	0.047±0.01	H_3= 3.622, p=0.305
Ammonia (mg/l)	11	0.815±0.03	0.803±0.17	0.44±0.27	1.587±0.40	$F_{3, 7}$=3.127, p=0.097
Phosphates (mg/l)	12	0.160 ± 0.03	0.147±0.02	0.863±0.72	0.32±0.04	$F_{3, 8}$=1.370, p= 0.320
Iron (mg/l)	10	0.025±0.03	0.050±0.05	0,350±0.35	0.067±0.02	H_3=1.105, p=0.776
Phenol (mg/l)	11	0.087±0.03	0.107±0.03	0.077±0.04	0.06±0.04	$F_{3, 7}$=0.264, p=0.849
Silica (mg/l)	12	0.520±0.21	0.440±0.08	3.693±3.40	0.897±0.25	$F_{3, 8}$=0.547, p=0.664
Hardness (mg/l)	12	356.667±51.75	330.0±26.46	340.0±35.12	356.667±14.53	$F_{3,8}$=0.144, p=0.931
Conductivity (µS/m)	12	45.700±4.40	49.733±0.29	36.383±14.72	51.033±0.33	H_3=5.275, p=0.153
Salinity (‰)	12	29.400±3.15	32.2±0.21	23.267±9.78	33.50±0.12	H_3=6.651, p=0.084

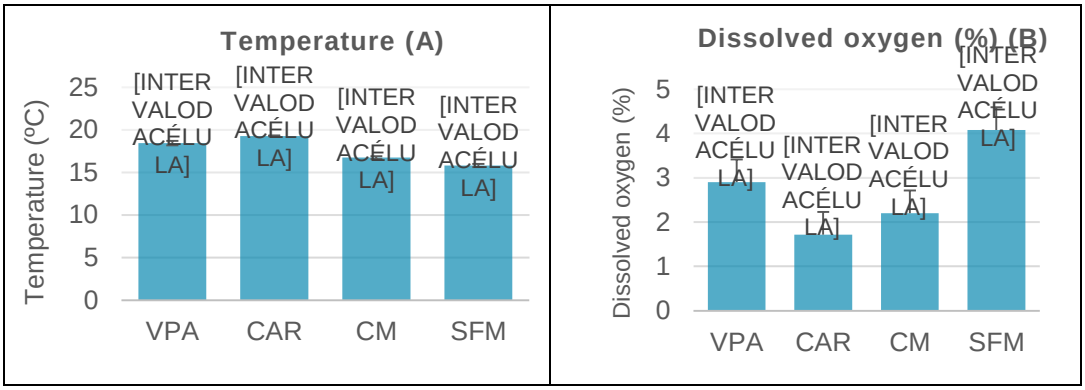


Figure 1 – Mean and standard error of the mean of water temperature (A) and dissolved oxygen (B) measured three times along a transept perpendicular to the water line at Vila Praia de Âncora (VPA), Carreço (CAR), Cabo do Mundo (CM) and São Félix da Marinha (SFM). Different letters above the bars indicate significant differences among sites (ANOVA and Tukey test, $p \leq 0.05$).

Understanding dissolved oxygen percentage is of the utmost importance when evaluating the fitness of an aerobic aquatic organism and its habitat (Kutty 1987). In water, the sources of dissolved oxygen are the atmosphere and photosynthesis from plants and algae. According to several gas laws (such as Henry's law and Dalton's Law), the quantity of dissolved gases in water is dependent on its proportion in the air and its solubility. When it comes to oxygen absorbance by the water, most of it comes from the water's agitation. With the increase of the temperature, dissolved oxygen promptly reduces (Kutty 1987).

Vila Praia de Âncora temperature was higher than São Félix da Marinha and Cabo do Mundo and lower that Carreço, but no significant differences were found in oxygen dissolution when comparing with the other sites.

Carreço presented a robust correlation with one of the lowest dissolved oxygen levels and the highest temperature, in agreement with Kutty (1987) conclusions.

Cabo do Mundo had one of the lowest dissolved oxygen levels, despite the fact of the site's temperature was the second-lowest. Given that the other analysed water parameters showed no significant differences (*e.g.* salinity) and taking in the location, it is possible that the air physicochemical characteristics might be related, but just like all the other sites, water currents could also be involved.

São Félix da Marinha presents a correlation with the lowest water temperature and higher dissolved oxygen, like Kutty (1987) suggests.

3.2. Biomarkers with no significant differences

There were found no significant ($p > 0.05$) differences in the protein carbonyls and CAT on the digestive system and LPO in the gills throughout all the sites (Table 3). Since these results showed no significant differences, they were put in a table, to easily understand them. The different sample numbers (n) were due to errors in dilutions when adjusting the protein content.

Table 3: Mean and standard error of the mean of several biomarkers determined in the digestive system (DS) and gills (G) determined in *M. galloprovincialis* of distinct sampling sites, and results of the statistical analyses (ANOVA or Kruskal-Wallis , $p \leq 0.05$).

Parameters	N	Vila Praia de Âncora	Carreço	Cabo do Mundo	São Félix da Marinha	Statistical results
Protein carbonyls (DS) (nmol.mg $prot^{-1}$)	87	11.689±2.29	12.178±1.83	15.461±3.48	14.901±2.44	H_3= 1.137, p=0.768
CAT (DS) (umol. min^{-1} mg $prot^{-1}$)	88	14.780±1.53	11.045±0.90	14.210±2.87	9.698±1.13	$F_{3, 84}$=1.19, p=0.319
LPO (G) (nmol.mg $prot^{-1}$)	85	2.185±0.19	1.945±0.09	2.159±0.14	2.548±0.43	H_3= 2.137, p=0.545

3.3. Morphometric parameters

The results of the morphometric measurements length, total weight, shell weight, soft tissues weight and condition index are shown in Fig.2. Significant differences of length (H_{3}= 8.132, p=0.043); total weight ($F_{3, 84}$= 6.9, p<0.001), shell weight ($F_{3, 83}$= 4.942, p=0.003), soft tissues weight ($F_{3, 84}$=11.252, p<0.001), condition index ($F_{3, 85}$=10.643, p<0.001) among sites were found.

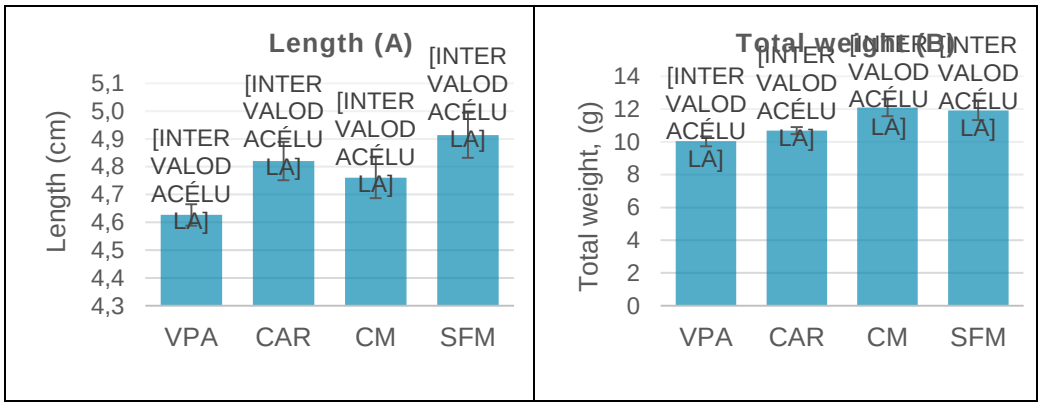
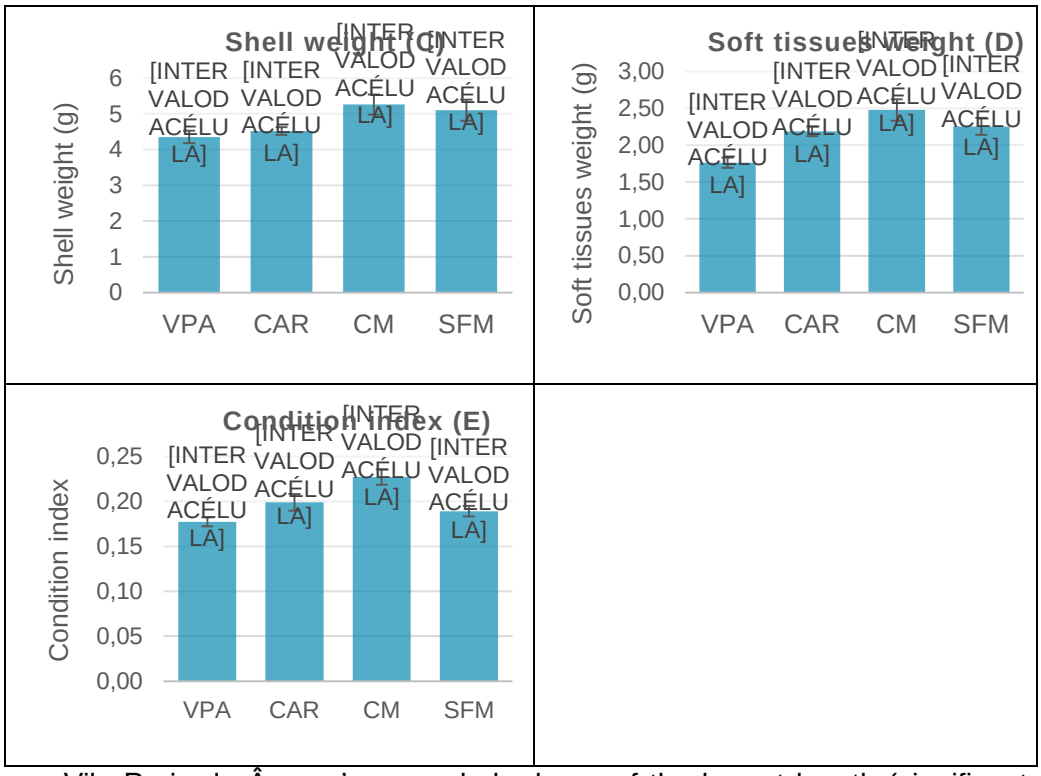


Figure 2: Mean and standard error of morphometric measurements (length (A), total weight (B), shell weight (C), soft tissues weight (D), Condition Index (E)) *in M. galloprovincialis* from Vila Praia de Âncora (VPA), Carreço (CAR), Cabo do Mundo (CM) and São Félix da Marinha (SFM). Different letters above the bars indicated significant differences among sites (ANOVA and Tukey test, p ≤ 0.05).



Vila Praia de Âncora's mussels had one of the lowest length (significant differences from São Félix da Marinha), one of the lowest total weight (significant differences from Cabo do Mundo and São Félix da Marinha), one of the lowest shell's weight, the lowest soft tissues' weight and one of the lowest condition index (with its only significant differences from Cabo do Mundo).

According to Alunno-Bruscia et al. (2001), the shell's weight is influenced by population density levels and food. These morphometric parameters seem to indicate that mussels collected in this site were the youngest mussels collected, but more parameters should be evaluated in order to reach that conclusion, like ring counting (Millstein and O'Clair 2001).

Carreço showed one of the lowest shell weight, one of the highest levels of soft tissues' weight and the condition index, with significant differences from Cabo do Mundo, this one having a higher value. From these results only, one cannot establish an accurate conclusion.

Cabo do Mundo only showed significant differences in the total weight from Vila Praia de Âncora, one of the highest shell weight, significant differences of soft tissues weight from Vila Praia de Âncora and the highest condition index.

Shell's composition is related to water chemistry and used to monitor heavy metal pollution (Bertine and Goldberg 1972; Fang and Shen 1984). Various factors can influence mussel's metal accumulation, such as physiological condition, sex, body size, reproductive status, salinity, organic matter and tidal height (Mubiana et al. 2006). In estuaries, it has been shown that salinity can

create different metal contents in overlaying waters (Phillips 1976). However, this stratification might not exist in well-mixed waters (Mubiana et al. 2006).

Also, it should not be forgotten the fact that hypercapnia (common in upwelling regions or systems that can be hypoxic seasonally) may lead to the hypercalcification of calcium carbonate structures (like shells) (Hu et al. 2011), which can result in higher shell's weight. Moreover, Cabo do Mundo's water analyses showed one of the lowest levels of dissolved oxygen.

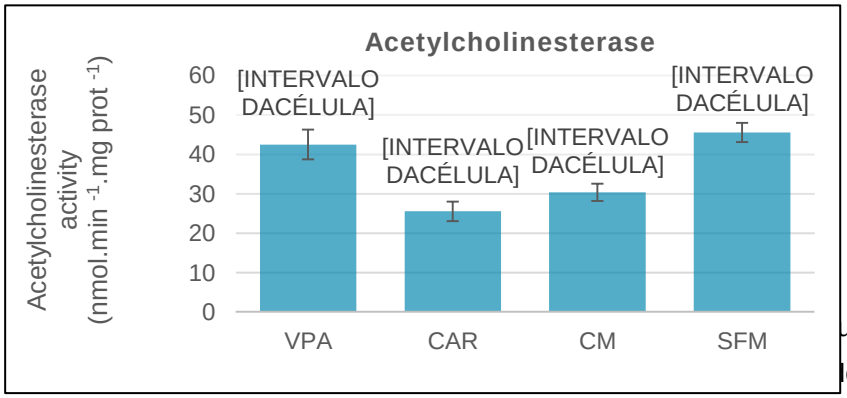
São Félix da Marinha's mussels had significant differences from Vila Praia de Âncora on the length, total weight, soft tissues weight, and condition index also had significant differences with Cabo do Mundo.

High population densities could elongate mussels' shells, increasing their length (Alunno-Bruscia et al. 2001)[4]. Density levels and food can influence the shell's weight, according to Alunno-Bruscia et al. (2001), that states that mussels located in a high-density site, but low food levels have a lower condition index (this one, however, calculated with the fresh dry mass per shell mass ratio). It is crucial to keep in mind that high-shell growth usually happens during spring and summer (Okaniwa et al. 2010). However, these muscles were collected on October, the 9th and the next ones (in Cabo do Mundo) were collected on October, the 20th and there were found no significant differences among them.

Growth can be predicted by associating the age and the length of an individual (Millstein and O'Clair 2001). Nonetheless, mussels were always collected in a similar group density in all places.

3.4. Acetylcholinesterase

The results of the acetylcholinesterase's activity are shown in Fig. 3. Significant differences of acetylcholinesterase (AChE: $F_{3, 87}$= 10,412, p <0.001)



ussel Mytilus Edulis. lom. 48(3):561–584. doi:10.1017/S0025315400019159.

among sites were found.

Figure 3: Mean and standard error of acetylcholinesterase's activity determined in *M. galloprovincialis* from Vila Praia de Âncora (VPA), Carreço (CAR), Cabo do Mundo (CM) and São Félix da Marinha (SFM). Different letters above the bars indicate significant differences among sites (ANOVA and Tukey test, p < 0.01).

According to Tim-Tim et al. (2009), acetylcholinesterase activity can be influenced by abiotic factors such as salinity and conductivity.

Both Cabo do Mundo and Carreço showed the lowest activity and both Vila Praia de Âncora and São Félix da Marinha showed the highest activity. In the bibliography found (*vide* II – Literature Review), references mainly addressed the decrease of AChE, from which was concluded that there is a basal level of this enzyme, depending on the different biological parameters. It was only found a study in a master thesis (Sousa 2009) stating that TBT could indeed increase these enzymes' activity. Nonetheless, Vila Praia de Âncora and São Félix da Marinha had both higher levels of acetylcholinesterase, but it is believed that these values could be physiological.

Carreço is located next to farming sites of corn production. A study of Frasco et al. (2005) concluded that the use of phosphate buffer (such as the one used in this monitorization) might inhibit acetylcholinesterase's activity when metal presence is involved, but no metal quantification was done. However, in corn production, some organophosphorus are allowed, such as chlorpyrifos, to act upon corn pests, such as *Forficula auricularia, Gryllotalpa gryllotalpa* and *Agrotis sp*. (WA Temple and NA Smith 2019; DGAV 2019b). Frasco et al. (2005) found that Organophosphorus can vary AChE (Frasco et al. 2005) and its compounds are irreversible acetylcholinesterase inhibitors (English and Webster 2012). Carbamates, quaternary and tertiary ammonium groups are reversible inhibitors of this enzyme (English and Webster 2012). Tests for these substances were not performed in this monitorization, so there are no proves that Carreço was contaminated at the time, although it may be likely. Therefore, by evaluating AChE activity alone, one cannot produce definite conclusions, other than the site might have been polluted.

As previously mentioned, Cabo do Mundo had lower levels of AChE. This is a known site of petrochemical contamination, and this factor can lower acetylcholinesterase activity (Moreira et al. 2004).

1

3.5. Octopine dehydrogenase and NADPH^{+}-dependent isocitrate dehydrogenase

The results of the ODH and IDH activity are shown in Fig. 7. Significant differences of ODH ($F_{3,\ 84}$= 7.070 p<0.001) and IDH ($F_{3,\ 84}$=6.822, p<0.001) among sites were found.

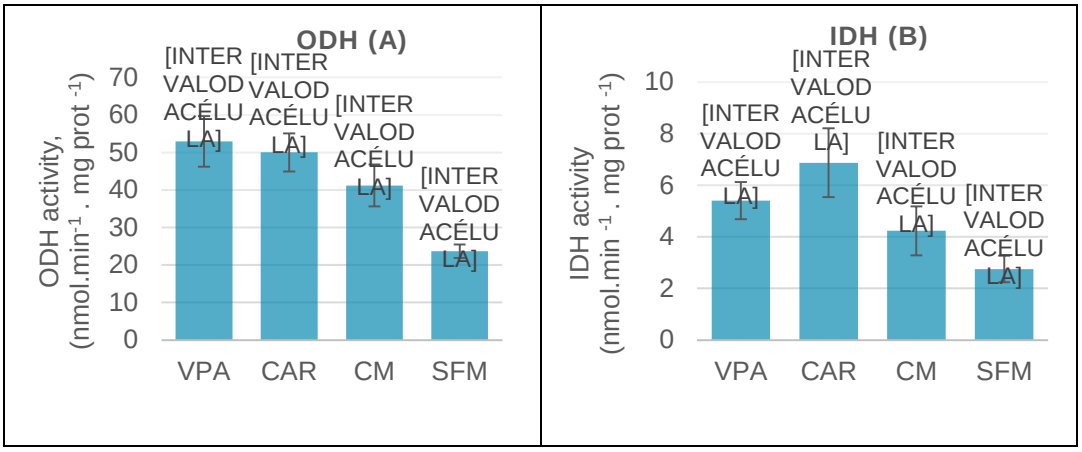


Figure 4: Mean and standard error of ODH (A) and IDH (B) activities determined in *M. galloprovincialis* from Vila Praia de Âncora (VPA), Carreço (CAR), Cabo do Mundo (CM) and São Félix da Marinha (SFM). Different letters above the bars indicated significant differences among sites (ANOVA and Tukey test, p ≤ 0.05).

ODH and IDHs samples were grouped, having the studied sites the same relative position concerning activity levels.

Vila Praia de Âncora showed similar results to Carreço, although dissolved oxygen showed to have no significant differences from all the rest of the groups.

Carreço had the lowest score on oxygen dissolution but still paired in a group with Cabo do Mundo, which correlates with one of the highest ODH levels. According to Murphy and Richmond (2016), ODH activity is an indicator of chronic exposure to anoxia. However, IDH is at its highest (although in the same group) in the analysis, which can mean that something other than dissolved oxygen was involved. Higher IDH levels can indicate the presence of ammonia, nitrates or phosphates (Lima et al. 2007). Notwithstanding water analyses showed no significant differences on these parameters.

Higher IDH can also occur in the presence of cadmium, copper and lead (Oliva et al. 2012). However, metal quantification was not done in this study. High levels of both IDH and ODH, according to Lima et al. (2007) can happen when mussels are exposed to stressful conditions. Organisms diminish their cellular

respiration, and therefore, the anaerobic metabolism is activated. It is believed that this situation was present in both Vila Praia de Âncora and Carreço.

In Cabo Do Mundo, values were lower compared to Vila Praia de Âncora and Carreço but higher than São Félix da Marinha. However, no significant differences between sites were found on both ODH and IDH.

A study by Oliveira, Lírio, et al. (2018) found that when IDH levels were low, and ODH levels were maintained, hypoxia was likely to be present. This situation is similar to the low IDH levels found and ODH levels with no significant differences from the other sites. Also, Lima et al. (2007) found that that lower levels of IDH can also indicate lower NADPH regeneration (related to the citric acid cycle).

São Félix da Marinha had one of the lowest ODH and IDH results. However, the dissolved oxygen level was one of the highest, which correlates with the low ODH levels (ODH is a biomarker used for anoxia conditions, according to Murphy and Richmond (2016)). It is believed that IDH and ODH are physiological, given that the oxygen dissolution was high, and their results paired.

3.6. Oxidative stress enzymes in the digestive gland

The results of the GST and LPO activity are shown in Fig.5.Significant differences of GST (H_3 = 22.410, p<0.001) and LPO (H_3 = 55.104, p<0.001) among sites were found.

There were only found significant differences in the GST and LPO in all the analysis performed in the digestive gland.

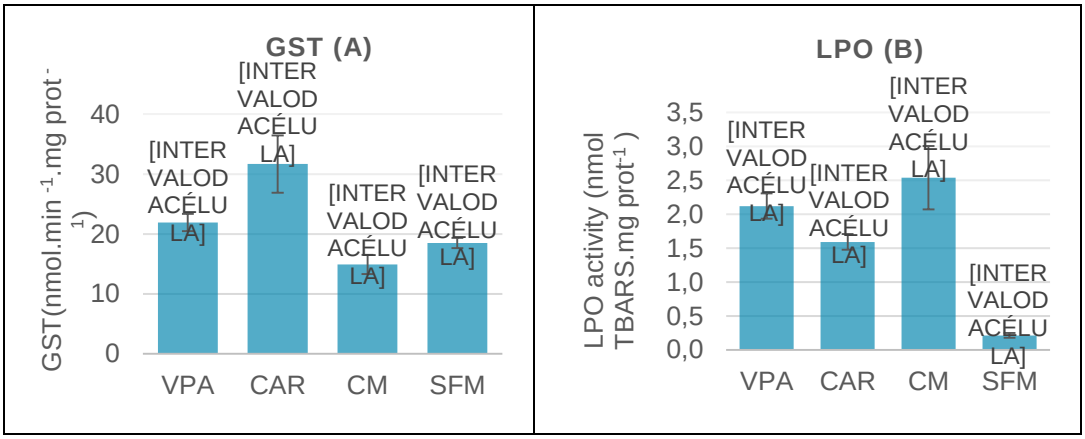


Figure 5: Mean and standard error of GST (A) and LPO (B) activities determined in the digestive system of *M. galloprovincialis* from Vila Praia de Âncora (VPA), Carreço (CAR), Cabo do Mundo (CM) and São Félix da Marinha

(SFM). Different letters above the bars indicate significant differences among sites (ANOVA and Tukey test, p≤ 0.05).

Vila Praia de Âncora's LPO levels only had significant differences having only significant differences with São Félix da Marinha and GST levels with Cabo do Mundo. According to Hampel et al. (2016), LPO requires a long exposure period to be observed, and it can happen when some degree of pollution is present. This way, one can infer that Vila Praia de Âncora might have been a place where some degree of pollution was present for a while, although water samples did not show any significant difference in their parameters. It appears, however, that GST might be physiological.

Carreço showed the highest GST activity, although significant differences existed only with Cabo do Mundo and São Félix da Marinha. GST is known to be related to the inflammatory response (Laborde 2010). GST activity, when high, can indicate the presence of xenobiotics, nitrates and ammonia (Lima et al. 2007). These last two were tested (Table 2), and there were found no significant differences between them. Being this site located next to farmland, different kinds of xenobiotics, that might go through the groundwater until they reach the beach, are expected to be present. Unfortunately, however, xenobiotics testing was not done. Carreço also had one of the highest ODH and IDH levels and lowest oxygen levels, which could be related to a higher LPO due to chronic exposure to ROS.

Cabo do Mundo had the lowest GST level, but without having significative differences from São Félix da Marinha. GST, as stated by Laborde (2010), manages products of LPO. However, this result does not mean this enzyme was not active. According to Hu et al. (2015), if GST has a decreased activity, it may indicate GST's saturation. Being Cabo do Mundo located where it is, the saturation hypothesis that seems very likely and, therefore, it corroborates the supposition that this site might be chronically polluted.

São Félix da Marinha had the lowest LPO level. Interestingly enough, it also showed the highest oxygen level, as well as the lowest ODH and IDH levels.

It appears that, even though oxygenation was the highest found in this monitorization, it did not portrait any particular differences in the producing ROIs, at least at the time monitorization was done since LPO takes a long exposure period to be observed, as reported by Hampel et al. (2016).

3.7. Oxidative stress enzymes in gills

The results of the GST, oxidized proteins and CAT activities are shown in Fig. 6. Significant differences of GST ($F_{3, 81}$=10.506, p<0.001), oxidized proteins ($F_{3, 83}$= 8.34, p<0.001) CAT ($F_{3,84}$= 3.284, p=0.025) among sites were found.

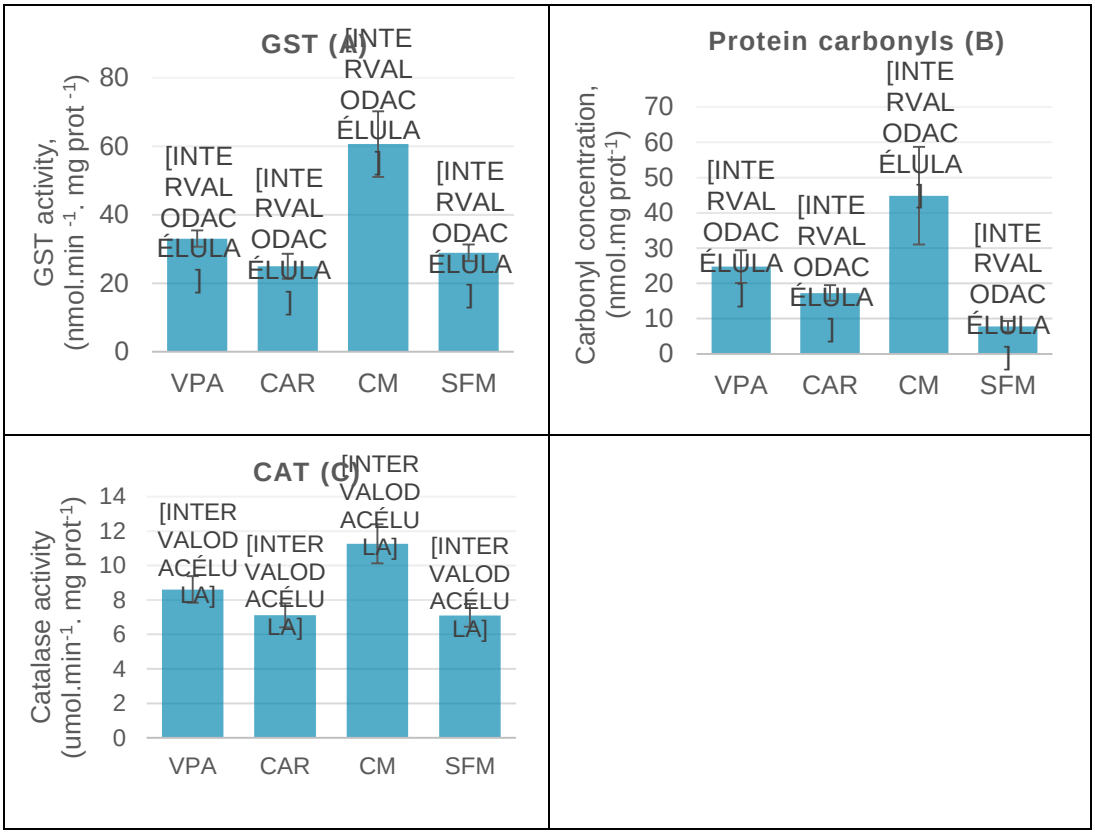


Figure 6: Mean and standard error of GST (A), protein carbonyls (b) and CAT (C) activities determined in the gill of *M. galloprovincialis* from Vila Praia de Âncora (VPA), Carreço (CAR), Cabo do Mundo (CM) and São Félix da Marinha (SFM). Different letters above the bars indicate significant differences among sites (ANOVA and Tukey test, p ≤ 0.05).

In Vila Praia de Âncora, CAT revealed no significant differences from all the other sites, just like dissolved oxygen in the water sample. Protein carbonyls were significantly different from São Félix da Marinha (being the levels higher) and similar to all the others, and GST had significant differences between Carreço, and Cabo do Mundo, being its value higher than Carreço, but lower

than Cabo do Mundo. Comparing the results from the digestive system, one could observe that either in the digestive system and in the gill, Vila Praia de Âncora and São Félix da Marinha showed no significant differences. With this information, it could be concluded to an extent, that this site might have some degree of xenobiotics (Hampel et al. 2016) or even oxidation by some metals (Levine et al. 1990).

Carreço had one of the lowest GST levels, significant differences in protein carbonyls with São Félix da Marinha (in this last one, levels were lower) and significant differences with Cabo do Mundo (which was higher). According to Lima et al. (2007), a higher GST activity is likely to compensate for lower levels of CAT in the gill, since GST also presents peroxidase activity. This situation is possible because CAT can be lower if long term exposure to ROS occurs (Vlahogianni et al. 2007). This supposition can be corroborated with the low GST levels (that can also happen when this enzyme is saturated (Hu et al. 2015)) and one of the higher protein carbonyls, that might indicate the presence of metal (Levine et al. 1990). However, given that this analysis was made in the gill, one cannot discard the hypothesis that hemocyanin might have contaminated and falsified the result (Weber et al. 2015). Nonetheless, all the other enzymes do help on the conclusion that there might have been some presence of metal and /or hypoxia.

Cabo do Mundo revealed the highest values in all the parameters, even though with some sites it presented no significant differences. LPO showed no significant differences in this site. This means all sites had similar levels of cells' lipid oxidation (Hampel et al. 2016). CAT removes ROS (Chelikani et al. 2004), and in Cabo do Mundo, CAT on gill had the highest result, although with no significant differences from Vila Praia de Âncora. CAT activity can be high in the presence of contaminants since it removes ROS (Khessiba et al. 2005) and this site known to be contaminated with PAHs (Moreira and Guilhermino 2005). PAHs are molecules that can bind to lipophilic sites because they are hydrophobic. This property makes PAHs be very quickly taken by marine organisms. If the target of the PAH is a crucial molecular in a cellular process, these molecules can induce a toxic response and eventually, if severe, the stability of the organism may be critically compromised (Meador et al. 1995). Hydrocarbons and its metabolic products can produce protein oxidation, lipid peroxidation and DNA damage that may lead to cellular damage throughout the increase of ROS (Lima et al. 2007). Unfortunately, heavy metals or PAHs analysis were not performed, but it is

believed that these higher results might be related to them, due to the vicinity of the oil refinery.

Protein carbonyls indicate aging, intercellular protein turnover and metal-catalyzed oxidation (Levine et al. 1990). The length might indicate age as well, (Millstein and O'Clair 2001) and Cabo do Mundo's length showed no significant difference from all the other mussels in other sites, so ageing is not believed to be a determinant factor or a reasonable explanation for these values.

Metal quantification was not done. However, qualitatively, Cabo do Mundo showed higher values for protein carbonyls, despite the fact that it only differed significantly from São Félix da Marinha. These results are consistent with the morphometric parameters analyses (where all the different weighted parameters showed to be systematically higher) and GST (that were the highest ones). GST protects cells against oxidative stress, and so, one can surmise mussels were in severe oxidative peril. It is interesting, however, to notice that the majority of the sites (except for Carreço) presented higher GST levels in the gills rather than in the digestive system. This fact is also consistent with Lima et al. (2007), where the same site was studied, also with *M. galloprovincialis,* and GST levels were at its highest. This study states that the digestive gland might have lower results because this organ may produce toxic intermediates, and these can inactivate the GST, leading to lower levels in the analysis of the digestive system. On the other hand, the gills, being highly exposed to the contaminants present in the environment, can reveal higher activities of this enzyme, as a result of higher rates of detoxification (Lima et al. 2007)[5].

São Félix da Marinha revealed significant differences in CAT with Cabo do Mundo only (this one having higher levels) and the lowest protein carbonyls result. Furthermore, GST was significantly different from Cabo do Mundo. After going through the water analysis, it was noted São Félix da Marinha exhibited the highest level the dissolved oxygen, which could justify the lower, nonetheless present oxidative stress enzymes.

[5] Cheung C, Zheng G, Li A, Richardson B, Lam P. 2001. Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, *Perna viridis*. Aquatic Toxicology. 52(3–4):189–203. doi:10.1016/S0166-445X(00)00145-4.

IV. Bioassay: Microplastics and Deltamethrin

1. Objectives

Microplastics have been studied for several reasons these last few decades, some of which were their availability to work as a conveyer of several chemicals (such as endocrine disruptors)

The purposes of this bioassay were to determine if, indeed, microplastics could work as a conveyer of deltamethrin, increasing its availability to *Mytilus galloprovincialis*, and to determine if a given concentration of deltamethrin could have an impact on their general health and fitness.

2. Material and methods

2.1. Preparation of the bioassay

2.1.1. *Tetraselmis chuii* culture

Before starting the bioassay, a source of *Tetraselmis chuii* was needed to feed the mussels. Firstly, all-glass material was previously decontaminated with nitric acid at 20%, being rinsed afterwards with distilled water.

To do so, 4 litres of an F/2 Guillard (Guillard and Ryther 1962; Guillard 1975) mean were prepared in a glass beaker of 5 litres, being autoclaved (AJC, Uniclave 88, Portugal) at 121ºC during 35 minutes, according to Prata et al. (2018), along with all the glass and plastic materials, just as described in Baltazar et al. (2014). All the chemicals used during all bioassay were purchased from Sigma-Aldrich (USA) or Merck (Germany). The seawater used as the main mean was reconstituted water (30 ‰), using sea salt (PRODAC Ocean Fish, Prodac International, Italy).

After that, the inoculum was added, along with the vitamin complex. Both were added after the sterilization because the inoculum itself would be sterilised and the vitamins would degrade. Then, the culture medium was supplied with sterilized air, with a sterile filter (Millex®- GS, 0.22 µm- Merck (Germany) and shaken twice a day, to avoid aggregation of *Tetraselmis chuii*. The algae were grown until they reached a visually a green colour. Attempts of spectrophotometry line were made, but the results were not satisfying.

According to Mihaylova et al. (2003), the best excitation length would be 488 nm, and the highest intensity at 683 nm. The laboratory tests showed that in the spectrophotometer (Jenway, 6405 UV/Vis spectrophotometer, UK), the excitation length would be 487 nm and the highest intensity at 682 nm. Spectrophotometry of the algae was performed every day since the inoculation, in order to facilitate the counting. However, the results were not satisfying, for the spectrophotometer was only able to read the samples at a very high concentration.

It was decided afterwards that the concentration of algae would be determined by using a Neubauer camera.

From the glass jars, algae were decanted to four tubes just up to two fingers from the top. Next, the tubes were put in the centrifuge (Thermo SCIENTIFIC, Sorvall BIOS 16 Bioprocessing Centrifuge, Germany) for 8 minutes at 5000 rcp and room temperature. The supernatant was left out and still with the pallet on the bottom, more algae solution was added, and the process was repeated until there were no algae left in the glass jar.

After this, the algae were put into a Schott® jar with salted water (30 ‰), slightly shaken and then approximately 40 millilitres were put into small jars and then stored in the freezer (-4ºC) until needed.

With the intention of feeding the mussels, a concentration of algae of $15x10^6$ cells per litre of water was present in the tank. This concentration was based on Prego-Faraldo et al. (2017) work, were *Tetraselmis suecia* (in a concentration of $12x10^6$ cells per litre) was used along with *Isochrysis galbana* of $3x10^6$ cells per litre. After some research, this article was found to be the most similar to the aim of this project and, therefore, it was adapted to the bioassay.

2.1.2. Microplastics

For this bioassay, red fluorescent plastic microspheres were used, purchased from Cospheric-Innovation in Microtechnology (USA). It is relevant to state, from the start, that the type of microplastics present was unknown since the fabricant did not provide that information, nor did it give a specific answer when contacted and therefore, their composition (*e.g.* polyamide or polyethylene) was unknown. Nonetheless, according to the supplier, the particle had a mean diameter of approximately 2 µm (from 1 to 5 µm), where 1 mg of the plastic microspheres were equivalent to 1.84 $x10^8$ particles with a 1.3 g/cm^3 density.

Their excitation wavelength was 575 nm, and their emission wavelengths were 607 nm.

To accurately establish conclusions after the bioassay, a reference line, connecting both fluorescence and known concentrations, was designed. This would allow later to distinguish nominal concentration from the measured concentration of microplastics. To do that, solutions containing microplastics and saltwater (30 ‰) were made. The concentrations were 0.078 mg/l, 0.154 mg/l, 0.313 mg/l, 0.625 mg/l, 1.25 mg/l and 2.5 mg/l. Then, flasks with different concentrations were put in a PGC1400 Bronson Climate (Bronson Incubator Services B.V., The Netherlands), being exposed at 16 hours of light and 8 hours of dark per day, at 16 ± 1ºC. The fluorescence of each flask (with the specific microplastics' concentration) was determined with the spectrofluorometer (JASCO, FP-6200, Netherlands) using the 575 and 607 nm for excitation and emission wavelengths, respectively. It was read at 0h, 3h, 6h 12h and 24h.

These results would also be used to calculate the decay of microplastics and, therefore, settle the best time for treatments' renewal.

In order to establish a more accurate line, a transformation of the values in the form of a logarithmical expression in base ten was done, and the following formula was used to calculate the concentration present.

The line was defined as $y= -2.068 + 0.944\ x$ (n=18, r= 0.988, p<0.001) from which the obtained formula was the following:

$$\log (\text{Microplastic Concentration}) = -2.068 + 0.944\ [\log (\text{Fluorescence})]$$

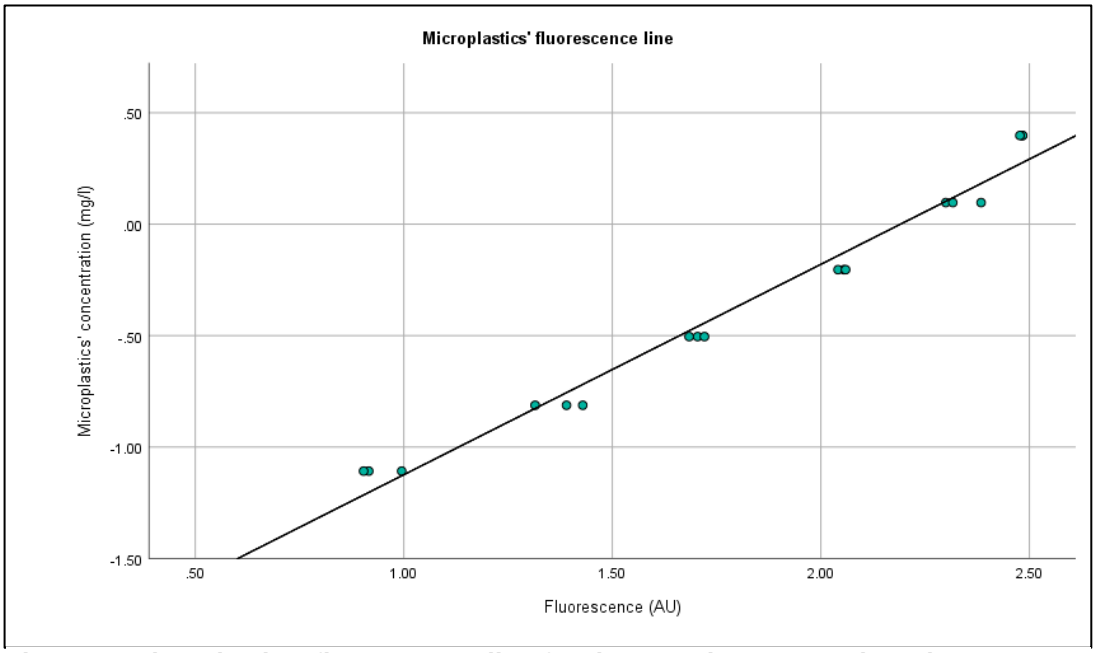


Figure 7: Microplastics' fluorescence line for the tested concentrations (0.078, 0.154, 0.313, 0.625, 1.25 and 2.5 mg/).

The concentration was measured in milligrams per litre of microplastics and the fluorescence in arbitrary units, given by the spectrofluorometer.

After analyzing the decay (Table 4), 24 hours was the chosen time to change the medium and the treatments.

The nominal concentration was 0.8 milligrams of microplastics per litre, but after analyzing the treatments' microplastics concentration, it was found that the measured concentration used was approximately 0.3 mg/l (0.297 ± 0.11). It was a consistent error, and therefore, all results were discussed according to this measured concentration.

Table 4: Microplastics' decay (%) after 3 and 24 hours.

Concentration (mg/l)	3 hours	24 hours
0.078	56.6	59.6
0.154	36.4	77.9
0.313	51.2	74.6
0.625	85.6	98.0
1.25	81.2	89.7
2.5	62.5	99.7

2.1.3. Deltamethrin

As University of Hertfordshire and IUPAC (2019) pointed out, deltamethrin solubility in water (at 20 ºC) is 0,0002 mg/L (classified by IUPAC as low), whereas in acetone is 450.000 mg/L and in methanol is 8.150 mg/L.

Given that deltamethrin had low solubility in water, it was decided to use acetone to increase deltamethrin availability. Acetone concentration (90 µg/L) was selected based on dissolution tests performed previously in the laboratory.

2.2. Bioassay

On April 29th, 2019, in Homem do Leme's beach, thirty-eight *M. galloprovincialis* were collected. Even though this site was not studied in monitorization (*vide* III- Four sites in the Northern Coast of Portugal: Monitorization) it was chosen because it was previously monitored by the laboratory, being used as a reference for one of the least polluted beaches in central Porto.

According to the same method previously explained, mussels were chosen based on the dimensions of the previously studied mussels (in the monitorization (*vide* III-Four sites in the Northern Coast of Portugal: Monitorization), to make a more accurate comparison.

The mussels were collected and posteriorly analysed in three different moments: at the arrival, after the acclimatization and after the bioassay.

On the same day, 29th April, nine mussels were sacrificed, and their organs divided into different microtubes with different buffers, just like it was done during the monitorization (*vide* III-Four sites in the Northern Coast of Portugal: Monitorization). Water analysis was performed in the same way it was done in the monitorization, in order to measure the concentration of the chemical substances present in it, using the Palin test protocol.

The other 29 mussels were put in an aquarium with seawater. On the day after, the water was changed to fresh seawater and reconstituted (30‰) in a proportion of 2:1, in a way that the mussels could adapt. This reconstituted water was made with filtered water and sea salt (PRODAC Ocean Fish, Prodac International, Italy). The day after, the same process was made with one part of the collected seawater and two parts of reconstituted water. The day after, all the water that was used was the reconstituted water (30‰). While acclimatizing, in a PGC1400 Bronson Climate incubator (Bronson Incubator Services B.V., The Netherlands) the mussels were fed on a concentration of $15x10^6$ cells per litre of water of *Tetraselmis chuii*. They were exposed to light, sixteen hours, and to the dark, eight hours per day, at 16 ± 1 ºC.

After acclimatization, that took 6 days, nine more mussels were sacrificed, with the purpose of controlling if there had been changes in the biomarkers.

The mussels were individually put in their different treatments (each with 250 mL of solution), according to the purpose of the study, for 96 hours.

They were:

- Four controls with saltwater (salinity of 30 ‰)
- Four flasks with saltwater and acetone (90 µg/L)
- Four flasks with deltamethrin (1 mg/L)
- Four flasks with microplastics (0.3 mg/L)
- Four flasks with microplastics and deltamethrin (0.3 mg/L and 1 mg/L respectively)

The treatments were changed after every 24 hours, in order to perform a semi-static test, according to the microplastics' decay (Table 4). Both 0h (the

fresh solution) and 24h (the old solution) treatments were read in the spectrofluorometer to measure the microplastics' concentration.

2.3 Filtration test

After the 96 hours, the 20 mussels were put individually in jars, with 250 ml of reconstituted seawater and an airway system. Twenty-four hours later, filtration rate testing was done.

The filtration rate is a biological biomarker that signals a bivalve's capacity to intake food, which reveals its state of fitness (Oliveira, Barboza, et al. 2018). The aim with for this test was to calculate mussels' algae filtration rate by measuring the number of algae present in the flasks at the beginning of the filtration test and by the end of it (Coughlan 1969). *M. galloprovincialis* were put in flasks with a concentration of 34.96 ± 1.40 cells per millilitre. After an hour, the mean of the flask was collected, and mussels sacrificed. The final concentration was calculated with a Neubauer camera.

To calculate the filtration rate, the following formula was used:

FR= [(V/nt) x In (Ci/Cf)], where *FR* is the filtration rate*, V* is the volume of water used during the test (ml), *n* is the number of mussels present in the jar (in this case one), *t* is the time (expressed in hours), *Ci* is the concentration of microalgae at the beginning of the test (expressed in number of cells/mL) and *Cf* is the concentration of microalgae after one hour.

2.4 Enzyme measurements

At the end of the bioassay, the enzymes were measured following the same steps as in the monitorization (*vide* III-Four sites in the Northern Coast of Portugal: Monitorization).

Acetylcholinesterase was analyzed in the haemolymph, collected with a syringe from the posterior adductor muscle and diluted in phosphate buffer (1:3) according to Moreira and Guilhermino (2005) and in the foot. ODH and IDH were analyzed in the posterior adductor mussel and finally; GST, LPO and CAT on both gills and digestive system.

3. Results of the bioassay and discussion

3.1. Bioassay's results

According to the protocol, mussels were weighted and measured. Their length was 5.029 ± 0.04 cm (n=38). Shell weight (4.715 ± 0.15 g (n=38)) was not relevant for this acute test, so it was only measured to agree with the protocol.

No significant differences were found in the water analysis between Praia do Homem do Leme and the sites monitored in part (*vide* III-Four sites in the Northern Coast of Portugal: Monitorization). This means that biomarkers evaluation (based on monitorization) in the bioassay from the *M. galloprovincialis* collected could be evaluated without fearing interference from the environment.

Table 5: Mean and standard error of several water parameters measured or determined in the water of Praia do Homem do Leme, and results of the statistical analyses (ANOVA or Kruskal-Wallis , p ≤ 0.05).

	Analyse in Praia do Homem do Leme		Comparative analysis with the sites studied in the monitorization
Parameters	N	Mean± standard error	Analyse with the previously studied sites
Nitrates (mg/l)	3	1.487±0.29	H_4=7.588, p=0.108
Nitrites (mg/l)	3	0.037±0.02	H_4=6.619, p=0.157
Ammonia (mg/l) *	1	0.020	H_4=8.128, p=0.087
Phosphates (mg/l)	3	0.250±0.2	H_4=7.595, p=0.108
Iron (mg/l)	3	0.050±0.05	H_4=1.155, p=0.886
Phenol (mg/l)	3	0.090±0.02	H_4=1.659, p=0.798
Silica (mg/l)	3	0.613±0.13	H_4=2.994, p=0.559
Hardness (mg/l)	3	333.3±35.19	H_4=1.114, p=0.892

(*) there were 3 samples, but two were inferior to the spectrophotometer's limit of detection, and therefore, were taken out. No significant differences were found when comparing to the tests performed in the monitorization

The results of total weight and IDH activity are shown in Fig.8. Significant differences of total weight ($F_{2, 19}$= 6.666, p=0.006) and IDH ($F_{2, 19}$=4.34, p=0.028) among the different times were found.

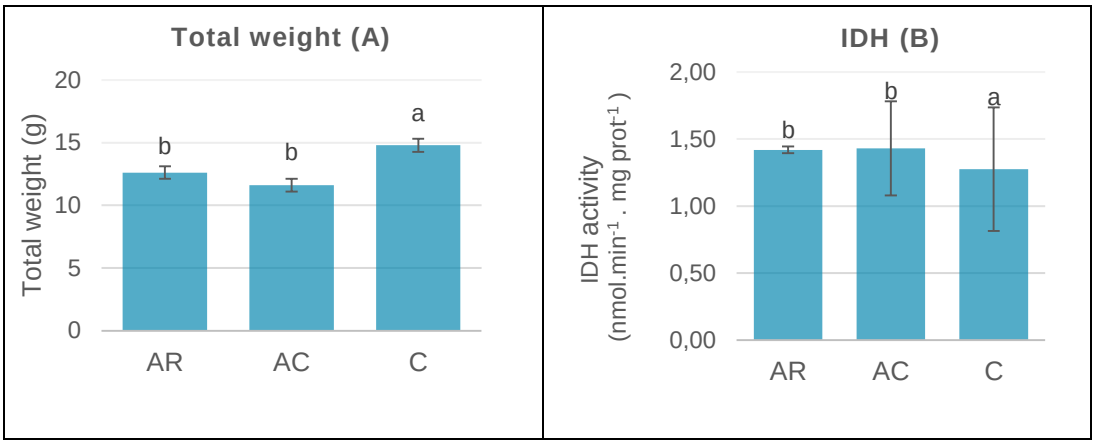


Figure 8: Mean and standard error of total weight (A) and IDH (B) determined in *M. galloprovincialis* at the time of arrival (AR), after acclimatization (AC) and control group after the bioassay (C). Different letters above the bars indicate significant differences among the different times. (ANOVA or Kruskal-Wallis , $p \leq 0.05$).

There were found significant differences during the time of arrival, after acclimatization and the control group. These were at total weight and IDH. Regarding the total weight, where the control group had significant differences from the mussels at the time of arrival and after acclimatization. It is believed that after the bioassay, *M. galloprovincialis* would have filtered more algae due to better acclimatization and depuration. The filtration test results (Fig. 11, explanation ahead) indicates that the control group had gotten a higher filtration result.

IDH showed significant differences in the control groups versus the mussels at arrival and acclimatization. It could have been due to inappropriate oxygenation, but as one can assess from Table 7, oxygenation during the bioassay showed no significant differences, and therefore one may conclude that this difference might be due to the depuration and acclimatization since IDH is an enzyme related to oxygenation.

There were no significant differences found when comparing the different parameters on fresh mean versus the treatment that was previously in contact with the mussel for 24 hours before (table 6).

Parameters	N	Time of arrival	After the acclimatization	Control group	Statistical analysis
Soft Tissues Weight (g)	22	2.446±0.35	1.752±0.05	1.90±0.20	$F_{2, 19}$= 2.275, p=0.13
Condition Index	22	0.176±0.02	0.153±0.005	0.132±0.02	$F_{2, 19}$= 0.291, p=0.751
AChE (H) (nmol.mg prot $^{-1}$)	22	47.10±9.46	51.354±7.79	47.141±14.39	$F_{2, 19}$= 0.086, p=0.934
AChE (F) (nmol.mg prot $^{-1}$)	22	8.260±0.60	7.458±0.48	8.215±1.27	$F_{2, 19}$= 0.509, p=609
ODH (nmol.mg prot $^{-1}$)	22	43.865±5.21	44.434±4.53	35.521±5.54	$F_{2, 19}$= 0.613, p= 0.552
GST (DS) (nmol.mg prot $^{-1}$)	22	25.705±1.60	26.734±2.22	18.213±1.90	$F_{2, 19}$= 3.485, p= 0051
LPO (DS) (nmol TBARS.mg prot $^{-1}$)	22	1.255±0.12	1.646±0.44	2.503±0.79	$F_{2, 19}$= 1.492, p=0.25
CAT (DS) (umol.mg prot $^{-1}$)	22	15.542±2.21	15.853±1.34	9.808±1.00	$F_{2, 19}$= 2.20, p=0.138
GST (G) (nmol.mg prot $^{-1}$)	22	32.086±3.22	30.107±3.143	28.753±1.42	$F_{2, 19}$= 0.228, p= 0.798
LPO (G) (nmol TBARS.mg prot $^{-1}$)	22	1.657±0.12	1.783±0.15	1.794±0.06	$F_{2, 19}$= 0.043, p=0.958.
CAT (G) (umol.mg prot $^{-1}$)	22	8.463±1.25	7.225±0.92	6.612±1.41	$F_{2, 19}$= 0.571, p=0.575

Table 6: Mean and standard error of morphometric parameters and biomarkers (in the haemolymph (H), foot (F), digestive system (DS) and gills (G)) with no significant differences (determined in *M. galloprovincialis* collected in Praia do Homem do Leme) at three different times and results of the statistical analyses (ANOVA or Kruskal-Wallis , $p \leq 0.05$).

Table 7: Mean and standard error of morphometric parameters and biomarkers (in the haemolymph (H), foot (F), digestive system (DS) and gills (G)) with no significant differences after the bioassay in the different treatments. Results of the statistical analyses (ANOVA or Kruskal-Wallis , $p \leq 0.05$).

Parameters	N	Control	Acetone	Deltamethrin	Microplastic	Mixture	Statistical Analysis
Soft Tissues Weight (g)	20	1.90±0.196	1.475±0.111	1.35±0.065	3.825±2.129	1.475±0.048	H_4= 7.855, p=0.097
Condition Index	20	0.132±0.02	0.126±0.006	0.109±0.007	0.296±0.158	0.109±0.005	H_4= 7.616, p=0.107
AChE (H) (nmol.mg prot $^{-1}$)	20	47.141±14.386	54.441±13.298	65.541±18.061	73.225±8.974	76.299±4.761	$F_{4, 15}$= 0.941, p=0.467
AChE (F) (nmol.mg prot $^{-1}$)	20	8.215±1.267	7.908±0.819	7.114±0.906	6.773±1.141	6.414±0.975	$F_{4, 15}$= 0.538, p=0.710
ODH (nmol.mg prot $^{-1}$)	20	35.521±5.542	43.530±4.811	32.945±6.134	45.658±7.351	30.175±8.130	$F_{4, 15}$=1.071, p=0.405
IDH (nmol.mg prot $^{-1}$)	20	7.363±0.662	8.252±1.460	6.884±0.696	6.486±1.664	5.505±2.753	$F_{4, 15}$= 0.388, p=0.814
GST (DS) (nmol.mg prot $^{-1}$)	20	18.213±1.896	24.253±1.744	21.332±1.753	36.685±12.109	21.674±3.805	$F_{4, 15}$=1.433, p=0.271
LPO (DS) (nmol TBARS.mg prot $^{-1}$)	20	2.503±0.790	2.251±0.509	1.098±0.195	1.257±0.195	1.665±0.280	$F_{4, 15}$=1.950, p=0.187
CAT (DS) (umol.mg prot $^{-1}$)	20	9.807±1.002	14.145±0.848	14.703±2.538	13.527±0.798	13.565±2.533	$F_{4, 15}$=1.223, p=0.343
GST (G) (nmol.mg prot $^{-1}$)	20	28.753±1.423	37.714±5.497	22.699±3.714	33.399±6.408	20.748±3.235	$F_{4, 15}$= 2.595, p=0.079
LPO (G) (nmol TBARS.mg prot $^{-1}$)	20	1.794±0.0570	1.987±0.465	1.986±0.132	2.339±0.452	2.442±0.284	$F_{4, 15}$=0.744, p=0.577
CAT (G) (umol.mg prot $^{-1}$)	19	6.612±1.407	9.399±2.643	10.294±1.322	8.606±1.0712	10.527±1.650	$F_{4, 14}$= 1.047, p=0.418
Treatment values variation							
pH	4	0.803±0.139	0.925±0.024	1.105±0.024	1.030±0.039	1.0175±0.050	$F_{4, 15}$= 2.746, p=0.068
O_2 (mg/l)	4	0.963±0.137	1.256±0.110	1.285±0.093	1.209±0.111	1.439±0.122	$F_{4, 15}$= 2.235, p=0.114
Temperature (ºC)	4	5.225±0.203	5.525±0.120	5.063±0.261	5.20±0.151	5.489±0.133	$F_{4, 15}$=1.199, p=0.352

1

The results of total weight and filtration test are shown in Fig.9.

Significant differences of total weight ($F_{4, 15}$= 3.466, p=0.034) and filtration test ($F_{4, 15}$=5.056, p= 0.009).

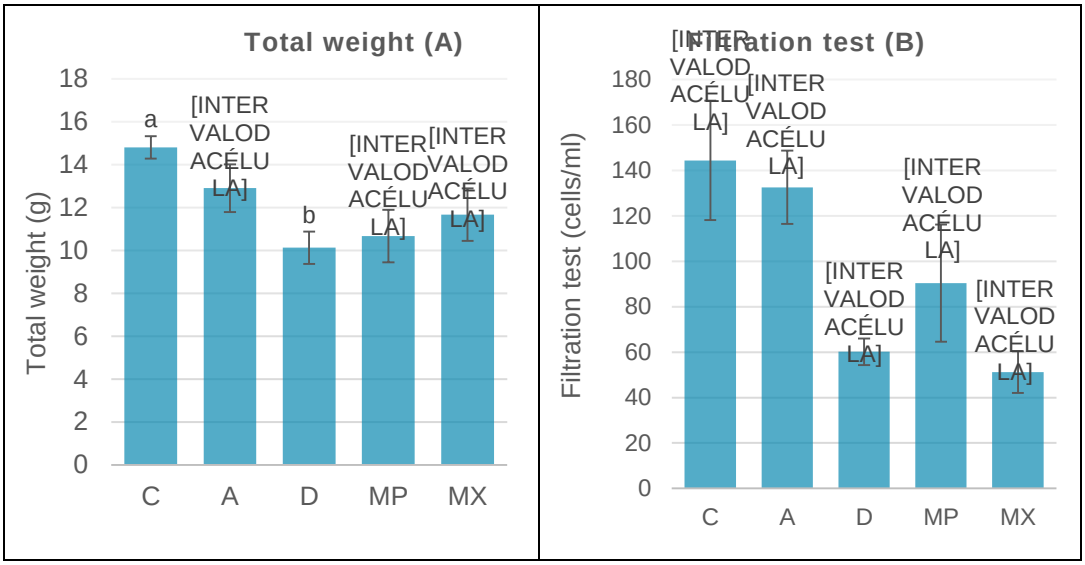


Figure 9: Mean and standard error of total weight (A) and filtration tests variation (B) determined in *M. galloprovincialis* exposed to different treatments (control group (C), acetone (A), deltamethrin (D), microplastics (MP) and mixture (MX)) for 96 hours. Different letters above the bars indicate significant differences among treatments (ANOVA or Kruskal-Wallis, p ≤ 0.05)

Douro river has the largest hydrographic basin in the Iberian Peninsula (Santos et al. 2010). It receives effluents from different industries, from agriculture runoffs to discharges from households that make up more than one million inhabitants.

This study (Santos et al. 2010) suggests that after being in contact with chronic pollution, eels appeared to have had a worsening in their health *status*. This situation might have also happened to the mussels present in this study, for this site is located near the Douro estuary, about 2 kilometres away. There were found no significant differences in the water analysis and the sites studied in the monitorization. These results indicate that the habitat where mussels were collected would not interfere with the results.

The filtration test showed significant differences between the control group and both deltamethrin and mixture, and between acetone and the mixture (Fig.9). The presence of deltamethrin showed to be constant in the treatments

with lower filtration rates. Nevertheless, these treatments also showed no significant differences with the microplastics treatment.

A study by Oliveira, Barboza, et al. (2018) found that *Corbicula fluminea*, a bivalve that inhabits freshwaters, when exposed to microplastics had its filtration rate decreased, expressing fitness reduction. However, this situation was not present in the essay's results because no significant differences were found between microplastic and control groups. For this reason, one can conclude that the presence of deltamethrin lowered the filtration rate.

Moreover, Horton et al. (2018) found that "polystyrene microplastics are unlikely to act as a significant sink, nor as a vector for the increased uptake of pesticides of aquatic organisms". In this study, they found that regardless of the chemical binding capacity, deltamethrin acute toxicity did not change in the presence of microplastics (Horton et al. 2018). However, since the microplastics' composition was unknown, this might or might be not verified in this essay, although it appears to be likely to have happened.

Another study, by Awoyemi et al. (2019) concluded that "pyrethroids are labile" and that there were differences in nominal concentration (1000 µg/l of deltamethrin) and measured concentration. They hypothesized that deltamethrin and pyrethroids could have been either degraded or adsorbed on the surfaces at which it was in contact with, *e.g*. beakers or microtubes. In this essay particularly, these surfaces could have been microtubes, pipette tips, volumetric flasks, the flagon where the essay was done in and, also the glass tubs of the airway system. Unfortunately, deltamethrin measurements were not done and, therefore, one cannot conclude with certainty and with evidence that indeed, deltamethrin has been adsorbed. Nevertheless, even though there were many transfers of the deltamethrin, it is improbable that this fact, *per se* or with the added degradation of the deltamethrin, could cause no significant alterations in the biomarkers.

In aquaculture, deltamethrin treatments are applied via baths (Brooks et al. 2019). Deltamethrin has lower toxicity in mussels relatively to crustaceans. Nonetheless, this may lead to "high chemical body burden concentrations" (Brooks et al. 2019) and, in vertebrates that belong in a higher trophic level, it can increase biomagnification. This same study hypothesized that the rapid deltamethrin's elimination could have been due to the rapid metabolism of deltamethrin since *Mytilus edulis* promptly eliminates deltamethrin from their tissues, a hypothesis that should not be discharged.

According to Digka et al. (2018), the digestive gland is known for retaining a higher amount of microplastics, followed by the gills, but no significant differences were found in this bioassay, relatively to microplastics.

Köprücü et al. (2008) performed a study with different deltamethrin concentrations, including 800 and 1600 µg/L, during 24, 48, 72 and 96 hours. In this study, catalase and glutathione both on the digestive system and gills decreased, in the presence of deltamethrin. However, some differences were found in the essay, comparing to the bioassay performed in this dissertation.

Firstly, it was performed indeed, in a mussel, but rather a freshwater one (*Unio elongatulus*). Secondly, they used trout feed to feed the mussels (not indicating what kind of feed precisely). By performing a brief search on FAO website (FAO 2019), it was admitted that no algae were present on that feed, unlike this essay, where *T.chuii* was used. It is also noted that dead mussels were immediately removed during the study (Köprücü et al. 2008). On the bioassay, however, no mussels perished.

Lastly, the deltamethrin used in the study was not pure; having a purity of 2.5% dissolved in 97.5 % of acetone. On the essay, it was used approximately deltamethrin 91.74 % and acetone (8.25 %).

Collection of the mussels was done in Praia do Homem do Leme due to economic constraints.

It is believed that acute exposure to deltamethrin in the concentration of 1000 µg/L and a 0,3 mg/L to microplastics does not have acute effects in *Mytilus galloprovincialis*.

V. Conclusions and future perspectives

M. galloprovincialis collected in Vila Praia de Âncora were located in a place where metal elements or xenobiotics might have been present. According to the results in monitorization (*vide* III-Four sites in the Northern Coast of Portugal: Monitorization), it seems that the water in Vila Praia de Âncora could have been polluted in some form, maybe from xenobiotics or metals present in the moment it was collected. There were reports of illegal discharges three months before the collection was done (Jornal de Notícias; 2018 Jul 9; O Minho 2018 Jul 9) and SEPNA (the Portuguese Service for the Protection of Nature and the Environment) was contacted. It stated that the reported situation was due to a clog of the parish's irrigation canal, created when the canal itself was being cleaned. This situation caused a violent flow along a muddy path towards the Âncora river. SEPNA is still waiting for the water analysis results[6]. Nevertheless, disregarding this situation, the xenobiotic/metal elements trace was present in *M. galloprovincialis* at the time.

Carreço is located in the vicinity of farmland, and therefore organophosphorus contamination was not discarded, although other tests should have to be performed. It was also suspected that mussels here might have been suffering from a kind of inflammation, that could be due to hypoxia or metal contamination, but no definitive conclusion could come without the specific tests.

Results showed that *M. galloprovincialis* collected in Cabo do Mundo might have suffered from chronic hypoxia and petrochemical contamination. Evaluating the health of the whole ecosystem should also be interesting, particularly involving human health (the "One Health" approach), especially if in a broad collaboration with all the different fields of studies.

São Félix da Marinha mussels are believed to be healthiest of this monitorization.

Oceans have currents and other phenomenons that make its habitats be continually changing and exposed to outer perils (like pollution from affluents, petrochemical disasters and so forth). Monitorization work is necessary to evaluate an ecosystem's health and better protect it.

The bioassay performed could be used as a pre-bioassay for a chronic exposure study, in order to truly eliminate the hypothesis that deltamethrin might be toxic to mussels. The composition of the microplastics should be known and

[6] SEPNA. 2019. Personal Communication.

deltamethrin concentration measured. Nevertheless, it is believed that no more mussels should be sacrificed to perform another test since this bioassay is thought to be enough to discard any doubts of an acute exposition and there is plenteous research with deltamethrin and chronic exposure test.

However, one should be reminded that deltamethrin still has effects on other species (being aquatic or not) and it should still be carefully used. Moreover, microplastics hazard should not be ignored. Several studies are indicating that the scientific community is concerned about health problems they might be partially responsible for or have a significant impact on (Prata et al. 2019).

Although alarming signs are much concerning, there can still be found hope in Science. There have been several ideas to help fight this problem and, as an example, a young boy, winner of the Google Science Fair 2018/2019, found a way to extract microplastics from water (Fionn Ferreira 2019; Trevor Nace 2019 Jul 30).

The general public begins too to acknowledge the hazards of plastics and microplastics and therefore, although this situation might be irreversible, avoiding further damage to the planet and actively founding research and solutions is in human hands.

“We have the choice to use the gift of our life to make the world a better place-or not to bother”, Jane Goodall said.

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