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# Ecotoxicology of deep-sea environments: effects of suspended sediment under hyperbaric conditions


Rithielli Rodrigues Marassi

Mestrado em Ciências e Tecnologia do Ambiente – Área de Especialização em Tecnologias de Remediação Ambiental

Departamento de Geociências, Ambiente e Ordenamento do Território


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**Orientador**

Doutor Miguel Santos, Professor Auxiliar com Agregação, Faculdade de Ciências da Universidade do Porto/Centro Interdisciplinar de Investigação Marinha e Ambiental

**Coorientador**

Doutor Filipe Castro, Professor Auxiliar, Faculdade de Ciências da Universidade do Porto/Centro Interdisciplinar de Investigação Marinha e Ambiental


Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

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# Abstract

The ocean is characterized by a great diversity of species that create complex ecosystems. Besides this enormous biodiversity, it is also possible to find mineral deposits that contain chemical elements with value for industries, especially technological ones. Aspects such as reduced light, high pressure and low temperature are examples of the difficulties of studying the deep-sea and reproducing this environment. With the increasing interest in exploring mineral deposits in deep-sea areas, it is essential to develop studies addressing the impacts of this activity in the marine environment, thus enabling the creation of rules and guidelines for a sustainable exploitation of the deep-sea. One of the negative impacts that deep-sea mining activities can possible cause is the formation of sediment plumes that have the potential to affect the water quality and the organisms exposed.

Due to the challenges in harvesting, transporting, and maintaining deep-sea species in the laboratory, a subtidal species of filter-feeding bivalve was used in this study as a proxy to help understanding the impacts of sediment plumes in the marine environment. To simulate a deep-sea environment, a hyperbaric chamber patented by the Interdisciplinary Center for Marine and Environmental Research (CIIMAR) was used.

This study aims to evaluate the impacts of suspended sediments in the model species of clam, S*pisula solida*, under hyperbaric conditions. The clams were exposed to four different concentrations of sediment (0g/L, 1g/L, 2g/L and 4g/L) with 60% of fine sediment (63 to 125 µm), 30% of medium (125 to 250 µm) and 10% of coarse sediment (250 to 500 µm), at a pressure of 4 Bar in the hyperbaric chamber for 96h. A batch of clams sampled prior to the experiment was done to assess the basal levels of biochemical responses in this species. At the end of the experiment, a functional assay was performed to evaluate the filtration rate, and biochemical endpoints (Catalase, Glutathione s-transferase and Lipid peroxidation) were assessed using the gonad, digestive gland, and gills.

The filtration rate showed a decreasing trend with the increasing sediment concentration, with a significant decrease for the 2 and 4g/L treatments. LPO levels showed a fluctuation with an initial significant increase at 1g/L compared to control group followed by a significant decrease at the 4g/L treatment, in the gonad. GST activity showed a significant decrease at 1g/L comparing with control group. Differences between males and females were also observed but didn’t follow a pattern.

The results indicate significant impacts of suspended sediments in the exposed clams, under hyperbaric conditions. These findings provide additional insights to enhance guidelines and monitoring protocols to improve hazard assessment of deep-sea mining.

Key-words: deep-sea mining, biomarkers, filtration rate, *Spisula solida*, suspended sediments, hyperbaric chamber.

# Resumo

O oceano é caracterizado por uma grande diversidade de espécies animais, plantas e outros seres que criam um ambiente rico e um ecossistema complexo. Além da enorme biodiversidade, também é possível encontrar depósitos minerais que contêm elementos químicos com valor para as indústrias, principalmente as tecnológicas. Aspetos como luz reduzida, alta pressão e baixa temperatura são exemplos das dificuldades de estudar o mar profundo e reproduzir este cenário. Com o aumento do interesse em explorar os depósitos minerais do fundo do mar, é essencial o desenvolvimento de estudos sobre os impactos desta atividade na vida marinha, possibilitando assim a criação de regras para uma exploração sustentável do mar profundo. Entre os impactos negativos que a mineração em mar profundo pode causar, destaca-se a formação de plumas de sedimentos que têm o potencial de afetar não só a qualidade da água, mas também os organismos expostos.

Devido às dificuldades na colheita, transporte e manutenção de espécies de mar profundo em laboratório, foi utilizada uma espécie modelo, neste caso um bivalve filtrador subtidal, para permitir simular e compreender os impactos das plumas de sedimentos no ambiente marinho. Para simular um ambiente marinho profundo submetido a altas pressões, foi utilizada a câmara hiperbárica patenteada pelo Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR).

Este estudo teve como objetivo avaliar os impactos de sedimentos em suspensão numa espécie modelo de amêijoa, *Spisula solida*, em condições hiperbáricas. As amêijoas foram expostas a quatro concentrações diferentes de sedimento (0g/L, 1g/L, 2g/L e 4g/L) com 60% de sedimento fino (63 a 125 µm), 30% de médio (125 a 250 µm) e 10% de sedimento grosseiro (250 a 500 µm) à pressão de 4 Bar na câmara hiperbárica durante 96h. Foi também estudado um tratamento T0 constituído por amêijoas amostradas antes do início do ensaio para avaliar os níveis basais desta espécie. No final do ensaio, foi realizado um ensaio funcional e parâmetros bioquímicos foram analisados na gónada, glândula digestiva e nas brânquias.

Os resultados da taxa de filtração mostraram uma tendência de redução com o aumento da concentração de sedimentos. Os resultados dos níveis da LPO mostraram uma flutuação entre as concentrações, com um aumento significativo no tratamento 1g/L quando comparado com o grupo controlo e uma redução significativa no tratamento 4g/L. A atividade GST apresentou uma redução significativa no tratamento 1g/L comparado com o grupo

controlo. Foram também observadas algumas diferenças entre machos e fêmeas, mas não seguiram um padrão.

Os resultados das taxas de filtração e dos estudos bioquímicos indicam um impacto significativo dos sedimentos em suspensão nas amêijoas expostas, em condições hiperbáricas. Estes resultados trazem conhecimentos adicionais para melhorar as diretrizes e protocolos de monitorização para a avaliação dos perigos da exploração mineira em mar profundo.

Palavras-chave: mineração em mar profundo, biomarcadores, taxa de filtração, *Spisula solida*, sedimentos em suspensão, câmara hiperbárica.

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# Abbreviation list

| | |
|---|---|
| **BHT** | butylated hydroxytoluene |
| **CAT** | catalase |
| **Cd** | cadmium |
| **CDNB** | 2,4-Dinitrochlorobenzene |
| **CIIMAR** | Interdisciplinary Centre of Marine and Environmental Research |
| **°C** | Celsius |
| **Co** | cobalt |
| **Cu** | copper |
| **DNA** | deoxyribonucleic acid |
| **EDTA** | ethylenediaminetetraacetic acid |
| **EEZs** | economic exclusive zones |
| **Fe** | iron |
| **GPx** | glutathione peroxidase |
| **GSH** | glutathione |
| **GST** | glutathione S-transferase |
| **$H_2O$** | water |
| **$H_2O_2$** | hydrogen peroxide |
| **ISA** | International Seabed Authority |
| **LPO** | lipid peroxidation |
| **MDA** | malondialdehyde |
| **Mg** | magnesium |
| **mg** | milligrams |
| **mL** | milliliters |
| **mm** | millimeters |
| **NaOH** | sodium hydroxide |
| **Ni** | nickel |
| **nm** | nanometer |
| **$O_2$** | molecular oxygen |
| **Pb** | lead |
| **ROS** | reactive oxygen species |
| **rpm** | rotations per minute |
| **SOD** | superoxide dismutase |
| **TBA** | 2-thiobarbituric acid |

| | |
|---|---|
| **TCA** | trichloroacetic acid |
| **µ**L | microliters |
| **UNCLOS** | United Nations Convention on the Law of the Sea |
| **Zn** | zinc |

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# 1. Introduction

## 1.1 The deep-sea

The sea represents about 70% of the Earth's surface and most of this enormous volume of water (about 90%) is situated at depths over 200 meters with extreme conditions such as absence of light, diminished temperatures and high pressures (Colaço et al. 2017). These attributes characterize what is known as the deep-sea, the biggest biome on Earth, with particularities that make it different when compared to all others. Its significance is enormous, since it performs carbon sequestration, recovery of nutrients and is part of most biogeochemical cycles (Colaço et al. 2017).

The deep sea supports a great diversity of habitats (Kazanidis et al. 2020) as cold-water coral reefs (Roberts et al. 2006; Buhl-Mortensen et al. 2010; Henry and Roberts, 2017) and deep-sea sponge aggregations (Maldonado et al. 2017; Kazanidis et al. 2019), for example. In addition, specific habitats such as vents, seeps, seamounts and canyons give supports to the development of unique faunas in the deep-sea (Danovaro et al. 2017).

Although it establishes a large part of the earth's surface and is of enormous significance, the knowledge about deep-sea environments is limited. It is estimated that approximately only 1% has been directly observed (Santos et al. 2018), which indicates the need to develop more studies in this field to expand mapping and obtain a more profound information of marine resources before their exploitation, thus contributing to its preservation (Colaço et al. 2017).

## 1.2 Deep-sea mining

Nowadays, as numerous countries in the developing world invest massively in their industrialization, there is an ensuing increase in demand for natural resources such as nickel (Ni) for stainless steel production, cobalt (Co) for high-thickness battery assembly, and copper (Cu), which is utilized widely in the automotive industry (Martins et al. 2006). Due to this increased use of natural resources, there is a forecast of a decrease in the availability or even shortage of these mineral resources found ashore (Rattner, 1977), and so, the deep-sea turns into an appealing option for exploring strategic metals (Silva, 2008), such as Co, Ni, Cu, lead (Pb), zinc (Zn), cadmium (Cd) also iron (Fe) and magnesium (Mg) and earth rare elements (Sharma, 2015).

In general, there are three important types of marine deposits with potential industrial application: polymetallic sulfides, cobalt crusts and iron-manganese nodules. In every one of

these kinds of deposits, it's feasible to discover different attributes, like the area required for each mine site per activity (Sparenberg, 2019), and they also produce different effects and consequences, thus highlighting the complexity of their exploitation (Colaço et al. 2017).

Polymetallic sulfide deposits occur in plate separation zones and are formed through hydrothermal activity. These mineral deposits are created by the rising of the initially cold seawater that after percolating across the seafloor, is heated by geothermal energy, and begins to rise dissolving metals and sulfides of surrounding rocks (Boschen et al. 2013; Colaço et al. 2017). Cobalt crusts occur on rocky substrate surfaces, can be many centimeters thick, are located at shallower depths when compared to polymetallic nodules and are related to seamounts and seamount chains (Martins et al. 2006). Iron manganese nodules are formed over millions of years by the slow precipitation of metal compounds in seawater and are found on the seabed between 4000 and 6000 m in depth (Colaço et al. 2017).

The economic interest in mineral resources found at the bottom of the sea began around the 1960s, where studies were already being carried out regarding the feasibility of extracting these ores (Souza, 2000). Over the years, interests have diversified and many of the studies began to focus on oil exploration, including in Brazil (Dutra and Cecchi, 1995). However, several mining companies were also investing in the exploration of polymetallic nodules in the seabed (Lenoble, 1996).

Despite the lack of greater international agreements, economic interests in these resources continued to increase and questions were raised about the environmental damage that this type of activity could cause (Souza, 2000). After years of negotiations, the international community agreed on a set of principles and rules concerning the oceans, dealing with diverse issues and jurisdictions, which led to the United Nations Convention on the Law of the Sea (UNCLOS), that defines a detailed framework for the regulation of ocean spaces (Souza, 2000).

The International Seabed Authority (ISA) is responsible for the management and regulation of mining activities in international waters established under UNCLOS. It has the mission to enforce the rules, regulations and procedures with the objective of making the operationalization of these activities safer for all stakeholders, ensuring the preservation of the marine ecosystem (Gerber and Grogan, 2020).

In 2000, countries like Germany requested exploration areas in the Pacific Ocean. In the South Pacific, mining companies requested exploration areas in the Exclusive Economic Zones (EEZs) and Continental Platforms (Souza, 2000). According to article 55 of UNCLOS, the definition of EEZs is given as "the exclusive economic zone is an area beyond and

adjacent to the territorial sea..." (UNCLOS, 1982; Souza, 1999) and exploration licenses for mineral resources located in EEZs have been issued by the governments of Papua New Guinea and New Zealand, as well as more than 20 contracts awarded by the ISA in areas of the Pacific, Atlantic and Indian Oceans (Wedding et al. 2015).

## 1.3 Potential environmental impacts of deep-sea mining

Deep-sea mining is likely to have many negative impacts during its operation, including the installation and use of a collection vehicle to collect the nodules, a process that will most likely create a plume of sediments near the bottom, in addition to pumping the nodules to the surface and disposing the tailings, the by-products of hard rock mining operations creating a second plume near the ocean surface (Ma et al. 2017; Sparenberg, 2019).

In spite of the fact that a lot of mining organizations have followed with interest the improvement of research on the deposits of metallic sulfides on the seabed (Mello and Quental, 2000), there are yet many difficulties to solve in the regulatory, technical and environmental fields (Santos et al. 2018). Besides the challenges introduced, the concerns identified with the impacts that this kind of activity can cause in the marine ecosystem are diverse. Some potential impacts related to these operations are the transformation and destruction of the marine environment that can cause direct harm to fauna and flora, prompting a decrease in the quantity of species and an unbalanced environment (Clark, 1992).

As mentioned above, a potential adverse consequence of deep-sea mining is the development of sediment plumes, the resuspension of particles as a by-product of mining operations on the seabed (Souza, 2006), which can cause damage to marine life (Gomes et al. 2000).

Sediment plumes can cause disturbances at different levels as these particles can influence the breathing and filtration of organisms, for instance in benthic species, introducing a danger to the most sensitive environments like coral reefs and spawning and breeding regions (Amado Filho et al. 2008; Colaço et al. 2017). Another effect that these sediment plumes may present is related to its toxic effect, due to the associated high sorption and accumulation capacities of the particles, that can act as a compartment for accumulation of pollutants (Jesus et al. 2004) and if they get incorporated by the biota can lead to biochemical, physiological, behavioral and possible genetic alterations to marine species (Gomes et al. 2000).

## 1.4 The use of bivalves as bioindicators

Bivalves are invertebrate animals, predominantly sessile and filtering (Gonçalves et al. 2017), including clams, mussels and oysters, and can be used as bioindicators to assess the

marine environment (LaBrecque et al. 2004) since they have a wide geographical distribution, often appear in high density and are also easy to collect and monitor, and constitute a large fraction of the benthic communities in coastal areas (Cunningham, 1979; Rufino et al. 2010).

Further relevant features of these types of species are that, being filtering organisms, scavengers and bioaccumulators permits them to uptake and store contaminants in their tissues, where the digestive gland is the main site of metabolization and accumulation of xenobiotics (Zottis, 2005), thus generating cumulative effects in the trophic chain (Cunningham, 1979; Torres et al. 2002).

## 1.5 The species *Spisula solida*

The species *S. solida*, a subtidal clam species found between southern Iceland and the Norwegian Sea, to the Atlantic coast of the Iberian Peninsula, Morocco and Madeira (Tebble, 1976) was chosen as the model species to use due to its great geographic distribution and commercial importance in countries like Portugal, France and Morocco (Joaquim et al. 2008). *S. solida* is a bivalve digger occasionally found at low tide, but more commonly on the sublittoral of coastal areas (Sabatini, 2007). Although these species has the ability to live in different types of sediment, they are found mainly in fine sand habitats (Gaspar and Monteiro, 1999) in depths up to 50 m (Sabatini, 2007). *S. solida* may be seasonally influenced by hydrodynamics, due to the fact that in the winter, this species appears to be located furthest from the coast (Dolbeth et al. 2006).

The *S. solida* is gonocoric and there are no records of hermaphrodites. Males and females are distinguishable internally, since the color of the gonad in this species is reddish in females and orange-yellow in males (Gaspar and Monteiro, 1999). The reproductive cycle of *S. solida* is an annual cycle, consisting of a mature phase in winter and successive spawning beginning at the end of winter and lasting until spring and unleashed by an increase in the temperature of the surface seawater (Joaquim et al. 2008). They achieve sexual maturity in the first year of their life, regardless of size (Gaspar and Monteiro, 1999; Fahy et al. 2003).

## 1.6 Biomarkers as an indicator of environmental contamination

Biomarkers are defined as a biological response to a chemical or chemicals that measures the extent of exposure and sometimes the toxic effect as well and may vary from the molecular level to cellular responses to the physiological level with behavioral changes (Peakall and Walker, 1994), which means that a biomarker is capable of measuring a change in normal function of an organism in response to a contaminant (O'Donovan, 2018).

These biochemical responses can be used as sensitive early warning indicators and monitors for the potentially toxic effects of environmental contamination (O'Donovan, 2018). Biomarkers can be classified into two types, the first being exposure biomarkers since they indicate that the organism has been exposed to a toxin and the second as effect biomarkers, which measure the disturbance resulting from exposure (Van der Oost et al. 2003).

### 1.6.1 Oxidative stress

#### 1.6.1.1 Reactive oxygen species

Oxygen is an essential molecule for aerobic organisms, being used in the generation of energy through the electron transport chain in the mitochondria of eukaryotes, as well as in the cellular membrane of many bacteria, and in numerous fundamental metabolic pathways. On the other hand, its consumption can generate toxic substances at the intracellular and extracellular levels (Trevisan, 2008). Such toxic substances are produced during the transport of electrons, enzymatic reactions, auto-oxidation reactions, or even by the heme group of proteins, and are usually known as reactive oxygen species (ROS) (Halliwell and Gutteridge, 2015). Some of these ROS are free radicals, others are non-radical oxidizing agents, such as hydrogen peroxide ($H_2O_2$) (Trevisan, 2008).

The cells have a series of defenses, the so-called antioxidant defenses, which can diminish the effects of ROS created by the aerobic metabolism. Such defenses may be generated endogenously or obtained through diet (Trevisan, 2008). The term antioxidant can be considered as any substance that delays, prevents or removes the oxidative damage in a target molecule (Halliwell and Gutteridge, 2015). Some studies show that, even at low concentrations, ROS can cause high damage and acquire important cellular functions (Dröge, 2002). In contrast, the attack of ROS to biomolecules can generate cellular dysfunctions (Yu, 1994).

The modifications associated with the attack of ROS can be caused by its excessive formation and/or inefficiency in interception by antioxidant defenses, giving rise to oxidative stress (Trevisan, 2008). Oxidative stress is characterized by deviations in the intracellular dynamic equilibrium state between the antioxidant protections and the generation of ROS (Sies, 2000). The damages caused by the attack to biomolecules, in the event antioxidant defenses are not able to neutralize its effects, can be divided in three main categories, namely damages to proteins, lipids and DNA (Ramakrishnan et al. 2007).

### 1.6.1.2 Lipid peroxidation

Cell membranes are extremely susceptible to ROS-related peroxidation reactions. ROS, when reacting with lipids present in the membranes, start a lipoperoxidation process or lipid peroxidation, generating a series of reactions, among them the formation of malondialdehyde, which is quite toxic (Kappus, 1985).

The most common complications as a result of lipoperoxidation are related to disruption or destruction of cell membrane function and essential organelles, involving the transport process, maintenance of metabolite and ion gradient and signal transduction mediated by receptors (Meagher and FitzGerald, 2000).

### 1.6.1.3 Protein oxidation

The primary structure of a protein can be very variable, and it can be the target of numerous types of oxidative processes, which will give rise to different end products (Trevisan, 2008). Since proteins have large potential to combine with metal ions, and in case of subsequent exposure to $H_2O_2$ they can cause the Fenton reaction, and consequently, the formation of the hydroxyl radical. These can cause fragmentations of proteins, forming carbonyl groups (Halliwell and Gutteridge, 2015). These consequences on proteins can be given by the direct attack of ROS to its structure, or by molecules originated from oxidation processes (Trevisan, 2008).

### 1.6.1.4 Oxidative damage to DNA

Free radicals are related to ageing processes, cancer development, mutations and cellular death from chemical alterations, both in the nitrogenous bases, in the ribose of the DNA and in the breakage of its connections (Trevisan, 2008). Some nuclear proteins can, for example, suffer attacks from the hydroxyl radical which then performs cross-links with the DNA, causing failures in cell repair, replication, transcription and decondensation of chromatin (Dizdaroglu et al. 2002).

### 1.6.1.5 Antioxidant defenses

Biological systems defend themselves from aggression from ROS and other oxygen derivatives by converting these species to oxygen, by oxidation phenomena, or to water ($H_2O$) by reduction phenomena (Halliwell and Gutteridge, 2015). Such defenses mainly involve the enzyme superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), acting as detoxifiers of the anion superoxide, hydrogen peroxide and hydroperoxides, respectively (Trevisan, 2008).

CAT is a widely spread enzyme among aerobic organisms, being found mainly in membranous organelles called peroxisomes. In animals, it is located in most tissues, concentrating mainly in the liver and erythrocytes (Halliwell and Gutteridge, 2015). CAT acts on the decomposition of one $H_2O_2$ molecule into $H_2O$ and molecular oxygen ($O_2$) (Farber et al. 1990).

GPx represents the most important of the peroxidases and acts in the reduction of $H_2O_2$ to $H_2O$ and $O_2$, using as electron donor the reduced tripeptide thiolic glutathione (GSH). It also acts in the reduction of other organic peroxides, such as lipoperoxides resulting from lipid peroxidation, to their equivalent alcohols (Di Giulio et al. 1995).

Glutathione S-transferase (GST) is responsible for the conjugation of electrophilic xenobiotics to GSH, decreasing their toxicity, making them more hydrophilic, allowing the transport system to eliminate these conjugations to the extracellular environment, metabolized by the mercapturic acid route. GST, as an enzyme sensitive to exogenous compounds, has been widely used as a biomarker (Stegeman and Lech, 1991; Bucheli and Fent, 1995).

## 1.7. Objective

The central objective of this work is to study the effects of suspended sediments, as a stressor resulting from deep-sea mining activities, under hyperbaric conditions, in a model species of clam, *Spisula solida*, using a functional endpoint and biochemical markers indicative of oxidative stress. This will foster the understanding of the impacts of sediment plumes in the marine environment, thus contributing to improve risk assessment.

# 2. Materials and methods

## 2.1 Hyperbaric experiment

To perform the experiment directed at simulating the particular conditions of the deep-sea, specifically the increase in pressure, a hyperbaric chamber patented by CIIMAR was used. It is composed of stainless steel and has 45 cm in diameter, 101 cm in length and a capacity of 153 L.

The sediments used in this experiment, composed essentially of silica, were collected on sandy beaches off the west coast of Portugal above the high tide. They were processed in the laboratory to eliminate any organic matter or contaminant possibly sorbed to the particles. After this treatment, the sediments were sieved and separated following the range of diameters selected, 63 to 125 µm; 125 to 250 µm and 250 to 500 µm (Pinheiro et al. 2019). Based on other studies such as the results of a pre-test done to check the resuspension of the sediments, three concentrations (1 g/L, 2 g/L and 4 g/L) were used (Cheung and Shin, 2005; Tokumon et al. 2015; Yang et al. 2017; Pinheiro et al. 2019).

A variation of setup of acrylic tubes with a diameter of 6.3 cm developed in the work of Pinheiro et al. (2019), that enables the circulation of water and the use of 4 treatments in the same experiment, with 3 replicates per treatment, was introduced in the hyperbaric chamber to achieve true replication of the test conditions. A peristaltic pump associated to the hyperbaric chamber was used to set the pressure conditions and a chiller was used to maintain the water at a temperature of 16 ± 1°C with constant aeration during the experiment.

Adults of *S. solida* were collect in the north of the west coast of Portugal, between Póvoa de Varzim and Apúlia, transported to the laboratory and acclimatized for 4 days (96h) prior to the experiment. The clams were placed randomly in the tubes (21 organisms per replicate, n=252) and sediments were introduced in the defined concentrations (1 g/L, 2 g/L and 4 g/L) with 60% of fine sediment (63 to 125 µm), 30% of medium (125 to 250 µm) and 10% of coarse sediment (250 to 500 µm), except in the control group (3 tubes) where no sediment was added. A group, T0, consisting of clams sampled prior to the experiment was done to assess the basal levels of this species. All tubes were covered with mesh of 63 µm to prevent the sediments from getting into the hyperbaric chamber or into the other replicates (Pinheiro et al. 2019). The experiment, running for 96h, was done at a pressure condition of 4 Bar (mimicking 40 m in depth). No feeding was performed during this period.

### 2.1.1 Sampling

At the end of the experiment, the organisms were sampled. To study the filtration rate, 5 organisms per replicate (n=15) were used. For the biochemical studies, the digestive gland, gonad and gills of 8 organisms per replicate (n=24), were sampled, frozen in liquid nitrogen and stored at -80°C until analysis. In one replicate tube of the 1g/L treatment, a technical problem occurred and therefore this replicate was discarded. The sediments of each tube were also collected, centrifuged, dried and weighted to provide a comparison between the initial and final percentage (Pinheiro et al. 2019).

## 2.2 Filtration assay

For the feeding assay, the marine microalgae *Tetraselmis suecica* was chosen as the food source since it satisfies all the requisites of a good diet for filter feeders as is easily digestible and meets the nutritional requirements that this type of organisms needs (Fernandez-Reiriz et al. 2015). It is also commonly used as an introductory live food in aquaculture (Kermanshahi-Pour et al. 2014). To reach a proper concentration, *T. suecica* was cultured in 500 mL ballons with autoclaved sea water and Fabregas medium (495:5 mL, respectively) with constant aeration and a photoperiod of 16:8 light:dark. An improved Neubauer Chamber (MARIENFELD) under an inverted microscope (Nikon Eclipse TS100) was used to assess the algae concentration and then subsequently used as feed for the clams at the conclusion of the 96h experiment (Pinheiro et al. 2019).

After the chamber was opened, 5 organisms per treatment replicate were chosen at random and set in separate containers containing seawater and a steady airflow and left to acclimatize (Pinheiro et al. 2019). Following the 2h acclimatization period, to each container, approximately 30 million cells of a concentrated *T. suecica* solution was added and thereafter fed for 1h. A water sample of each container, post-feeding, was collected, fixed with Lugol's solution (CONDA Laboratories) and stored at 4°C until cell count.

The samples were vortexed prior to cell counting and the count, in duplicate, of the cells present in the 4 quadrants of the Neubauer chamber was performed. The final concentration of algae in each container was calculated according to equation 1 (Pestana et al. 2009), using the average cell count.

$$\boldsymbol{FR} = \frac{V}{nt}\left[(\mathrm{C} - C_0) - (\mathrm{C}' - C'_0)\right]$$

**Equation 1** - Filtration rate (FR) in mL/h; V is the volume of each container in mL; n represents the number of organisms per container; t is the number of hours of the experiment; C and $C_O$ are the natural logarithms of the initial and final concentration of algae in the containers, respectively; C' and C'O are the natural logarithms of the initial and final concentrations of algae in the controls.

## 2.3 Biomarkers

### 2.3.1 Protein quantification

To perform the protein quantification, the digestive glands, gonads and gills were weighted and homogenized in HB buffer [100 mM ($K_2HPO_4/KH_2PO_4$) 150 mM KCl, 1 mM DTT, 0.1 mM PMSF, 1 mM ethylenediaminetetraacetic acid (EDTA); pH 7.4] (Pinheiro et al. 2019), and then the protein quantification was performed according to the Lowry method (Lowry et al. 1951), and several aliquots were stored at -80ºC until biochemical analysis of CAT, GST and LPO.

### 2.3.2 GST

A reaction mixture was prepared containing glutathione S-transferase, reduced glutathione thiolic tripeptide and 2.4-Dinitrochlorobenzene (GST:GSH:CDNB, respectively), to measure GST activity according to (Habig et al. 1974), modified, where a 96 well microplate was used. Shortly, GST buffer was pipetted on the first three wells to create the blank and on the succeeding wells, the sample resulting from the Lowry method (section 2.3.1) was added in triplicate. To start the reaction, the reaction mixture was added to every well and then read in a microplate reader (Synergy HT, Biotek) at 340nm for 5 minutes in 20-second intervals.

### 2.3.3 Catalase

For CAT activity, a reaction mixture consisting of HB buffer and $H_2O_2$ at 30% was prepared. The samples were read in triplicate and the reaction mix was added to start the reaction. Absorbance was read using 96-well UV microplates in a microplate reader (Synergy HT, Biotek) at 240nm for 2 minutes, in 15 seconds intervals, and then the variation in absorbance per minute was calculated according to (Aebi, 1974).

### 2.3.4 Lipid peroxidation

Using the sample resulting from the Lowry method (section 2.3.1), 100% trichloroacetic acid (TCA) was added. After, samples were agitated and centrifuged (5000 rpm for 20 minutes at 4ºC), and the supernatant was removed, transferred to another tube, where a solution of EDTA 0.1M, 1% 2-tiobarbituric acid (TBA) 0.05M, sodium hydroxide (NaOH) and 0.025% butylated hydroxytoluene (BHT) was added. To guarantee the quality of the reaction, a blank and a buffer control were also prepared. The samples were boiled in the absence of light for 30 minutes and then left to cool, at room temperature, in the same condition. A 96-well microplate reader was used to read the absorbance at 532nm (Ferreira et al. 2008; Capela et al. 2016).

## 2.4 Statistical analysis

The data resulting from the functional and biochemical studies was checked for homogeneity of variances (Levene's test) and then analyzed by two-way ANOVA. Post-hoc comparisons were done using the Fisher's Least Difference (LSD). Significant differences were set at $p<0.05$ and all statistical analysis were computed with SPSS Statistics 26 (IBM Corp).

# 3. Results

## 3.1 Filtration rate

The results regarding the filtration rate (FR) of the clams exposed to suspended sediments are presented in Figure 1. FR of *S. solida*, expressed in mL/h, after 96h of exposure to suspended sediments at 4 Bar, showed a tendency to decrease with increasing sediment concentration, with a significantly reduction ($p < 0.05$) of the FR in the 2g/L and 4g/L treatments (the two highest concentrations used) compared to the control and the 1g/L treatment.

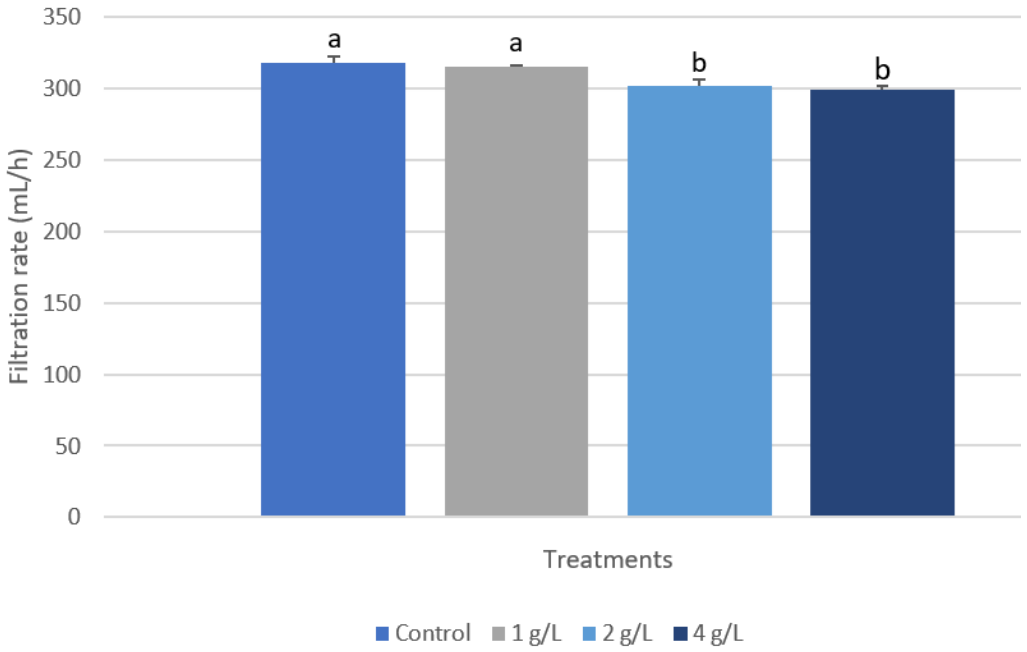


**Figure 1** - Filtration rate in *S. solida* exposed to four concentration of sediments (0, 1, 2 and 4 g/L) for 96h at 4 Bar and then fed with *T. suecica*. Error bars indicate standard errors and different letters (a, b) indicate significant differences between treatments, $p<0.05$; two-way ANOVA followed by the Fisher's least significant difference (LSD) post hoc test; n=14 (control), n=9 (1g/L), n=15 (2 g/L), n=13 (4 g/L).

The results regarding the FR from the clams exposed to 4 Bar, in males and females, are depicted in Figure 2. The FR showed a decrease between males of the 2g/L treatment and the control group.

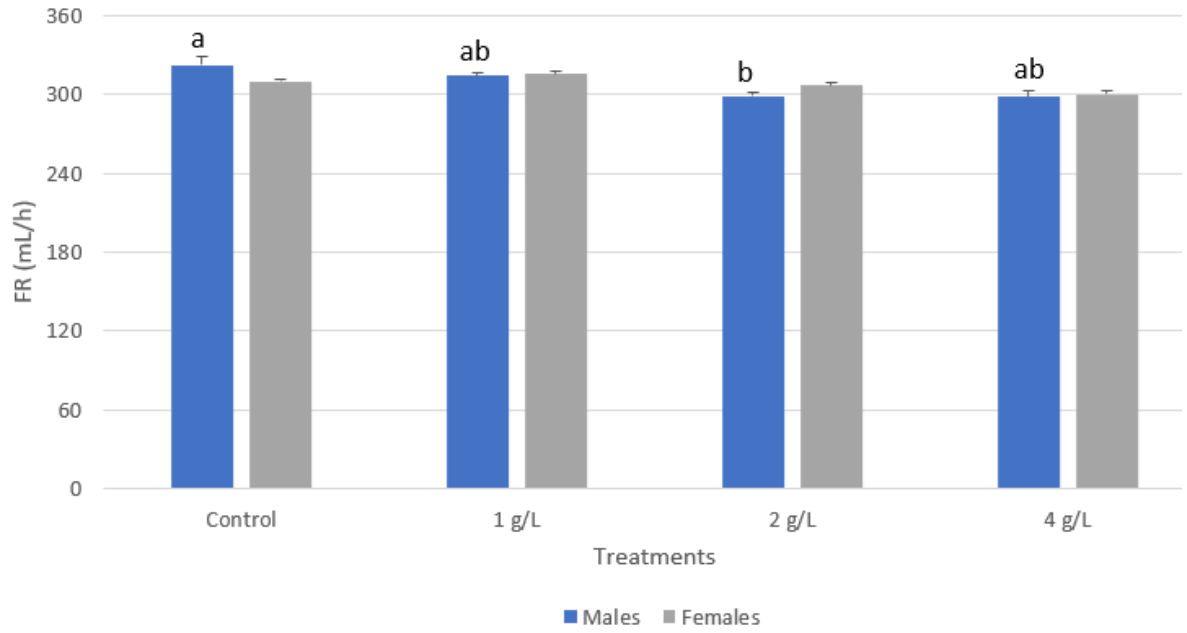


**Figure 2** - Filtration rate in males and females of *S. solida*, exposed to four concentration of sediments (0, 1, 2 and 4 g/L) for 96h at 4 Bar and then fed with *T. suecica*. Error bars indicate standard errors and different letters (a, b) indicate significant differences between treatments for males, $p<0.05$; two-way ANOVA followed by LSD post-hoc test. n=9 for males and n=5 for females (control), n=4 for males and n=5 for females (1g/L), n=9 for males and n=6 for females (2g/L), n=8 for males and n=5 for females (4g/L).

## 3.2 Collected sediment

The results of the sediment collected in each tube at the end the experiment are presented in Table 1, shown as a percentage of collected sediment in comparison with the starting amount of sediment settled in each tube (100%).

**Table 1** - Percentage of collected sediment at the end of 96h of experiment at 4 Bar.

| Treatments | Initial sediment (g) | Final sediment (g) | Remaining sediment (%) |
|---|---|---|---|
| 1g/L | 0,6889 | 0,5354 | 77,7165 |
| 2g/L | 1,3769 | 1,1952 | 86,8061 |
| 4g/L | 2,7582 | 2,1941 | 79,5461 |

## 3.3 Biochemical analysis

### 3.3.1 Comparative: Control group and T0 group

The results of the comparative of the control group (no sediment added under hyperbaric conditions) and the T0 group (no sediment added at atmospheric conditions in the beginning of the assay) to biomarkers responses are presented in Table 2.

**Table 2** - Comparative: control group and T0 group

| Treatments | Tissue | **CAT** (µmol/min/ mg protein) | **LPO** (nmol MDA/mg protein) | **GST** (nmol/min /mg protein) |
|---|---|---|---|---|
| T0 | Gonad | 16,440 | 0,0912 | 27,716 |
| Control | | 37,447 | 0,1059 | 63,598 |
| T0 | Digestive gland | | 0,1606 | 354,325 |
| Control | | | 0,1811 | 360,773 |
| T0 | Gills | 7,828 | 0,1049 | 7,633 |
| Control | | 19,376 | 0,0976 | 39,099 |

### 3.3.2 Catalase

The results of CAT activity after 96h of exposure of the clam *S. solida* to four different concentrations of sediments at 4 Bar are represented in Figure 3. CAT activity, expressed in µmol/min/mL protein, didn't showed significant differences between the control group and the treatments with different concentrations of sediment in both tissues.

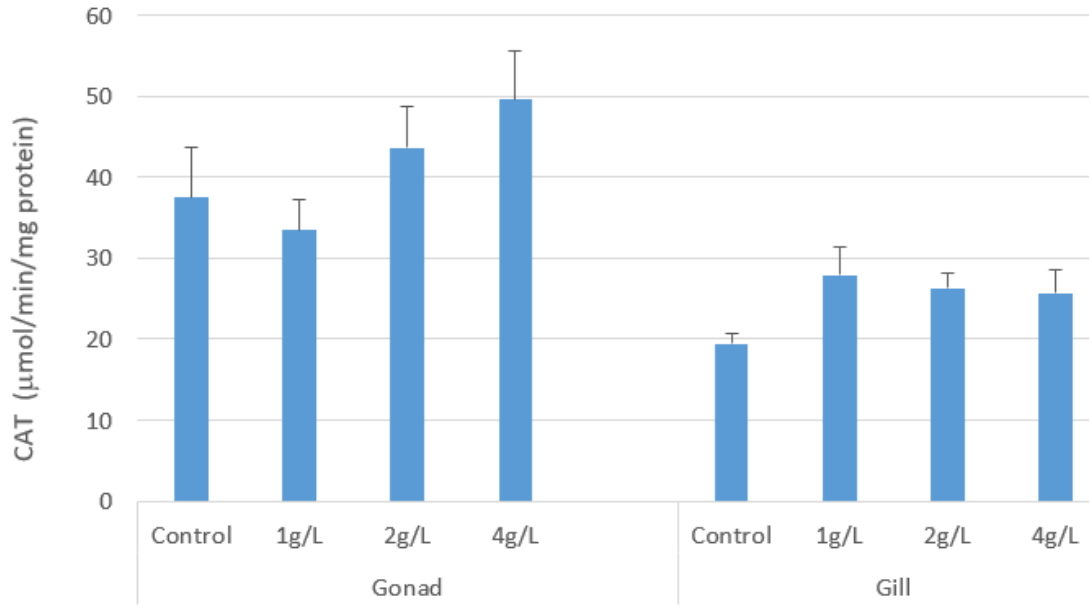


**Figure 3** - CAT activity in S. *solid*a exposed for 96h to four different concentrations of sediments at 4 Bar. Error bars indicate standard errors, p<0.05; two-way ANOVA followed by LSD post-hoc test, n=24 (control), n=16 (1g/L), n=24 (2g/L), n=24 (4g/L).

The results of CAT activity after 96h of exposure of the clam *S. solida* to four different concentrations of sediments at 4 Bar, in males and females, are presented in Figure 4. CAT activity, expressed in µmol/min/mL protein, showed no significant difference between males and females at the different treatments in both tissues.

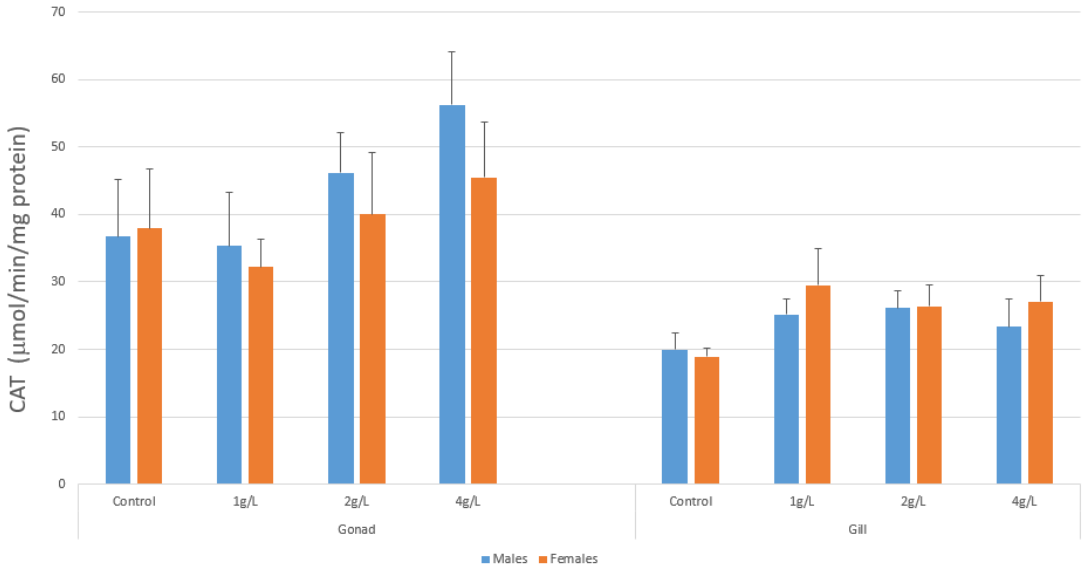


**Figure 4** - CAT activity in males and females of *S. solida* exposed for 96h to four different concentrations of sediments at 4 Bar. Error bars indicate standard errors, p<0.05; two-way ANOVA followed by LSD post-hoc test; n=14 for males and n=10 for females (control), n=6 for males and n=10 for females (1g/L), n=14 for males and n=10 for females (2g/L), n=9 for males and n=15 for females (4g/L).

### 3.3.3 Lipid peroxidation

Lipid peroxidation results after 96h of exposure of the clam *S. solida* to four different concentrations of sediments at 4 Bar are presented in Figure 5. LPO, expressed in nmol MDA/mg protein, showed significant differences in the gonad with the 1g/L treatment increasing significantly compared to the control group and then presented a significant decrease at the 4g/L treatment (the highest concentration used).

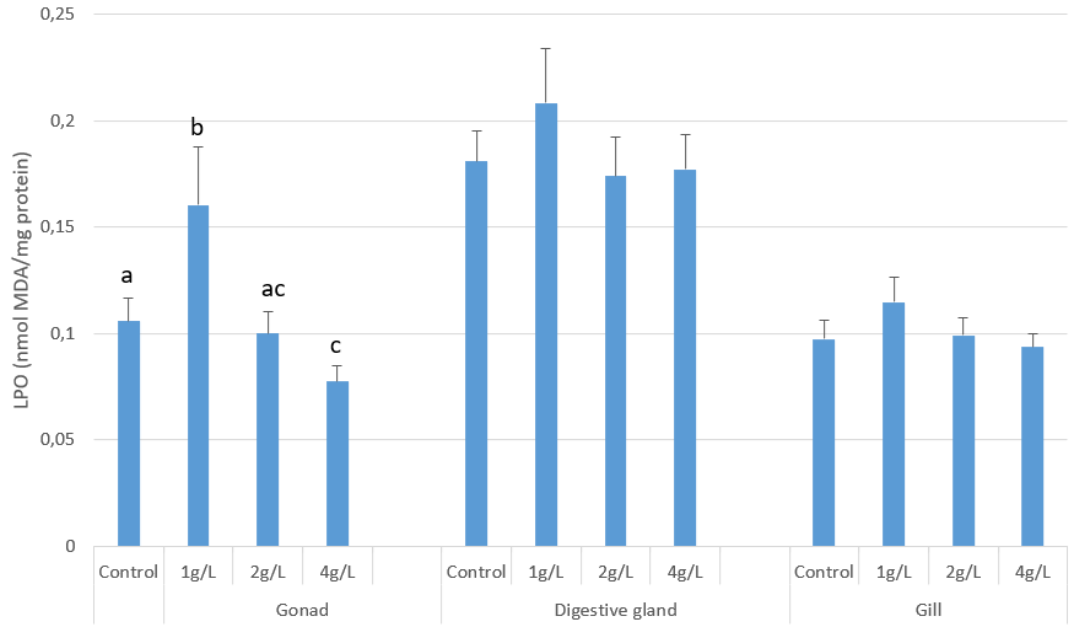


**Figure 5** - LPO levels in *S. solida* exposed for 96h to four different concentrations of sediments at 4 Bar. Error bars indicate standard errors different letters (a, b, c) indicate significant differences between treatments of the same organ , p<0.05; two-way ANOVA followed by LSD post-hoc test; n=24 (control), n=16 (1g/L), n=24 (2g/L), n=24 (4g/L).

Lipid peroxidation results after 96h of exposure of the clam *S. solida* to four different concentrations of sediments at 4 Bar, in males and females, are presented in Figure 6. LPO levels showed a significant increase at 1g/L in the gonad of females compared to the 2g/L and 4g/L treatment. The LPO activity also showed a significant increase at 1g/L in the gills of females compared to the control group and the others treatments, as well as, a difference between males and females at 1g/L.

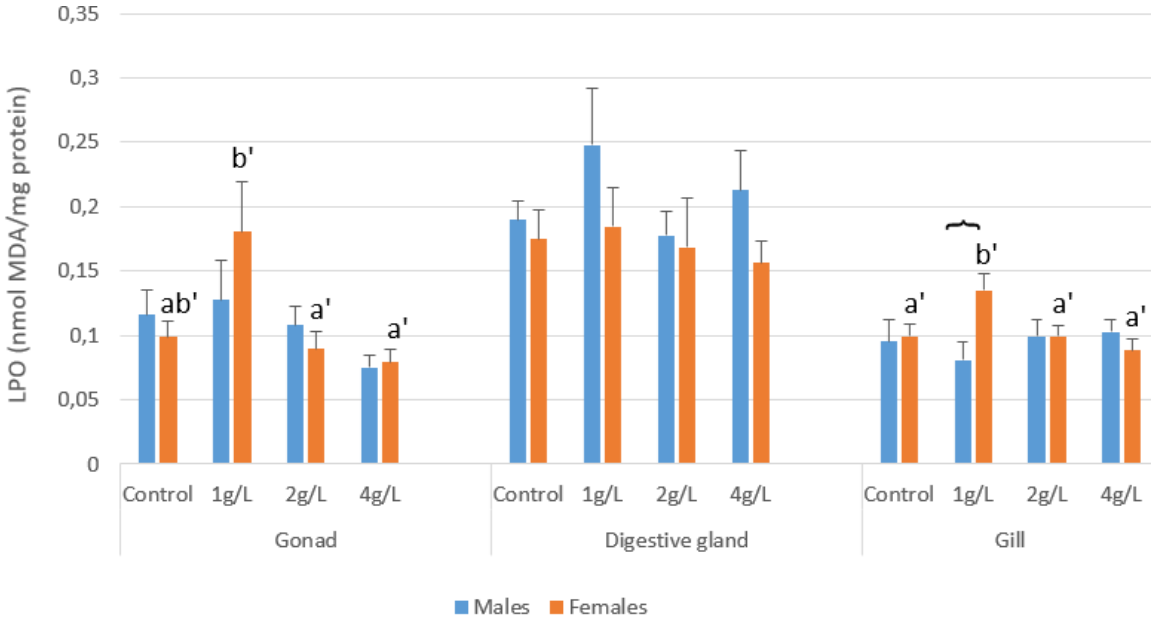


**Figure 6** - LPO levels in males and females of *S. solida* exposed for 96h to four different concentrations of sediments at 4 Bar. Error bars indicate standard errors, (a,b') indicate significant differences between treatments in females of the same organ and braces ( { ) indicate a significant difference between males and females of the same treament, $p<0.05$; two-way ANOVA followed by LSD post-hoc test; n=14 for males and n=10 for females (control), n=6 for males and n=10 for females (1g/L), n=14 for males and n=10 for females (2g/L), n=9 for males and n=15 for females (4g/L).

### 3.3.4 GST

The results of GST activity after 96h of exposure of the clam *S. solida* to four different concentrations of sediments at 4 Bar are presented in Figure 7. GST activity, expressed in nmol/min/mg protein, showed a significant decrease between the 1g/L treatment and the control group in the digestive gland.

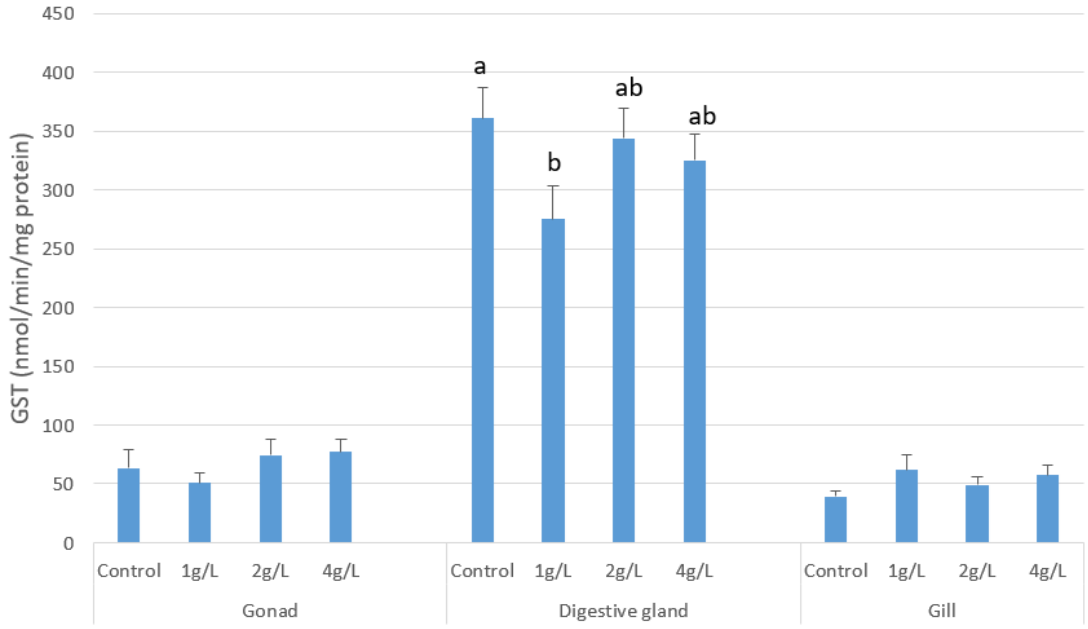


**Figure 7** - GST activity in *S. solida* exposed for 96h to four different concentrations of sediments at 4 Bar. Error bars indicate standard errors, different letters (a, b) indicate significant differences between treatments in the same organ, p<0.05; two-way ANOVA followed by the LSD post hoc test, n=24 (control), n=16 (1g/L), n=24 (2g/L), n=24 (4g/L).

The results of GST activity after 96h of exposure of the clam *S. solida* to four different concentrations of sediments at 4 Bar, in males and females, are presented in Figure 8. No significant differences between males and females to all tissues tested were observed.

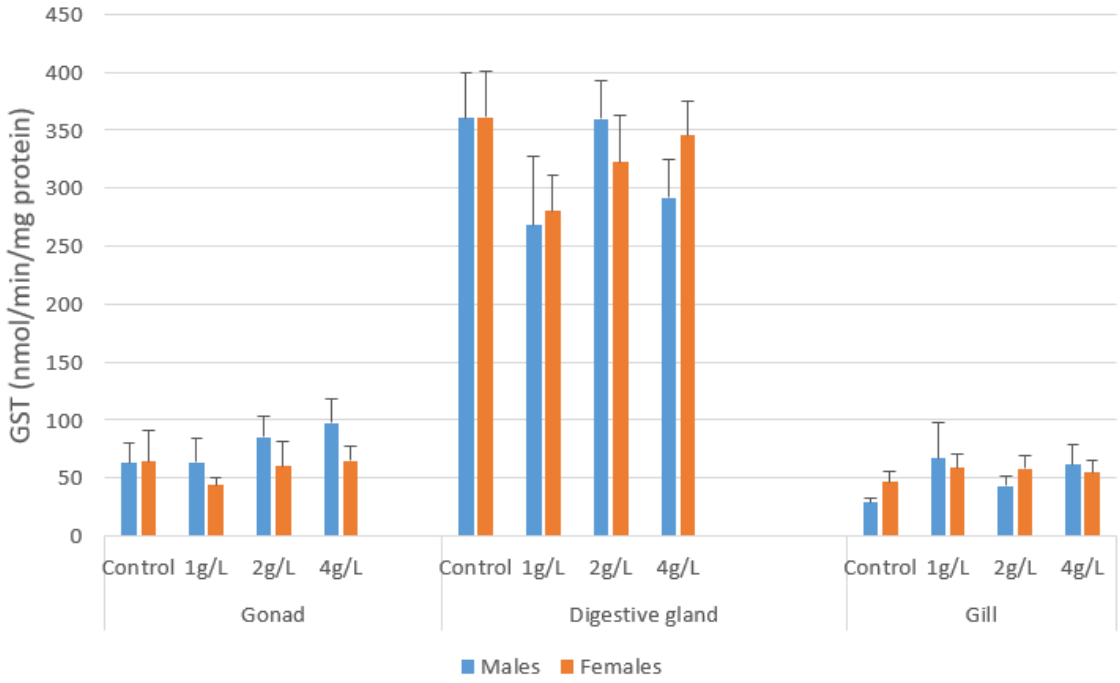


**Figure 8** - GST in males and females of *S. solida* exposed for 96h to four different concentrations of sediments at 4 Bar. Error bars indicate standard errors, p<0.05; two-way ANOVA followed by LSD post hoc test, n=14 for males and n=10 for females (control), n=6 for males and n=10 for females (1g/L), n=14 for males and n=10 for females (2g/L), n=9 for males and n=15 for females (4g/L).

# 4- Discussion

There is increase concern associated to physical effects of deep-sea mining, specifically about the systems required for this operation, the formation of sediment plumes through seabed activities, the discharge of waste resulting from pre-process sing of the minerals and the condition of the riser pipes (Salomon and Markus, 2018). It is expected that deep-sea mining activities will result in the formation of sediment plumes and these fine particles can move through the water column and can impact the water quality and the life of marine species (Pinheiro et al. 2019). Research has shown that suspended sediments can significantly influence the feeding and growth of suspension feeding bivalves (Winter, 1976; Widdows et al. 1979; Vahl, 1980; Kiorboe et al. 1980, 1981; Kiorboe and Mohlenberg, 1981) and an increase in concentrations of sediments made by silts and clay, for example, can make the pseudofaeces production increase consequently, the algal food actually ingested can be reduced and can also harm bivalve gills (Morse et al. 1982; Bricelji and Malouf, 1984; Robinson et al. 1984; Stevens, 1987; Navarro et al. 1992; Willows, 1992; Iglesias et al. 1996; Navarro and Widdows, 1997). The long-term exposure to rising concentrations of suspended particles can lead to a decrease in the energy available for growth and reproduction and cause destructive effects on local habitats (Ellis et al. 2002).

There are still many technological limitations to studying deep-sea scenarios (Santos et al. 2018). The system used in this work, which was designed in Pinheiro et al. (2019) is an innovative alternative under hyperbaric conditions because it allows a set of true replicates for each treatment due to the independency of each tube.

The use of a filter feeding species as a model represents a good approach to study the effects of suspended sediments (Pinheiro et al. 2019). Globally, there are approximately 20 thousand species (Pearse et al. 1987) of bivalves and due to this expressive number, it is a class of animals that offers a big diversity of adaptations to specific environmental conditions (Abele et al. 2009). Since they are filter feeding species, the filtration rate assessment is a convenient functional assay to estimate the effects caused by suspended sediments (Pinheiro et al. 2019) and has been used in several studies. In contrast to previous studies by Pinheiro et al. (2019, 2021), the species selected for the present study is characteristic from subtidal sandy habitats, and therefore we hypothesize that it could cope better with sediment disturbance then mussels. However, in the present study, the FR showed a decrease trend with increasing sediment concentration, and the two highest concentrations used (2g/L and 4g/L) showed a reduction when compared with the control group as well as the 1g/L group. The study of Sobral and Widdows (2000) investigated the feeding activity of the infaunal

bivalve *Ruditapes decussatus L*., comparable to the development of current velocities and concentrations of suspended particulate matter (SPM) and the results showed a decline trend with the increase of the concentration (from 10 to 300 mg $l^{-1}$). The experiment of Robinson et al. (1984) evaluates the effect of suspended clay on feeding and digestive efficiency of the surf clam *Spisula solidissima* and the results showed that levels of turbidity above 0.1 g. $l^{-1}$ leads to an increase in the algal food refused as pseudofeces and a decrease in the food actually ingested and broken down enzymatically. These results indicate that discharges at levels as low as 100 mg.$l^{-1}$ caused by anthropogenic turbidity-production can have negative effects on the surf clam populations, for example (Robinson, et al. 1984). The study of Pinheiro et al. (2019), that tested the effects of suspended sediments in 4 different concentrations (0, 1, 2 and 4 g/L) in a mussel species, *Mytilus galloprovincialis*, under hyperbaric conditions, showed that for all the pressures conditions studied, the FR was reduced with the increase of suspended sediment concentration. Comparing the results of this species of mussel, *M. galloprovincialis* and the clam *S. solida* used in the present study, it appears that clams are less sensitive to the exposure to suspended sediments since a significant decrease was only observed in the highest concentrations whereas in the mussel, the FR significantly decreased for all concentrations. As pointed out above, these differences between mussel and *S. solida* could be related with habitats preferences and tolerance to sediment disturbance. However, overall, the results of these studies are in accordance with the findings of this work, enhancing the idea of the negative effects that the increase of suspended sediments can have in the filtration activity of these organisms. The results of the collected sediment indicate that sediments seem to be either absorbed by the organisms and then expelled with the feces or became attached to the gills since for all treatments a percentage of sediment collected at the end was inferior to the percentage of sediment at the beginning of the experiment.

Oxidative stress reflects an unbalance between reactants, including reactive oxygen and nitrogen species, and antioxidants. The rise in reactive species leads to damage to lipoproteins, lipids, DNA, and proteins (Strobel et al. 2011). Considering that antioxidant enzymes perform an important role in protecting against these reactive oxygen species, it becomes an important tool to study antioxidant enzymes like CAT, GST (Pinheiro et al. 2019), and LPO as they represent the most frequently evaluated process in the investigation of oxidative stress (Strobel et al. 2011).

Antioxidant enzymes as CAT can have different responses when exposed to high-suspended solids in different tissues and species (Yang et al. 2017). Yang et al. studied the effect of suspended solids on the antioxidant enzymes activities in a bivalve *Sinonovacula constricta* and concluded that the antioxidant enzymes in *S. constricta* showed an adaptation

to the environmental changes as water turbidity (Suzuki et al. 2018). In this present study, CAT activity did not differ significant in comparison with the control treatment, although a trend towards an increase was observed in the two treatments with the highest sediment concentration.

Results regarding LPO levels showed a fluctuation between the concentrations, as well as a significant increase in the 1g/L comparing with the control group and then a significant decrease in the 4g/L treatment in the gonad. These results are in accordance with other works published, for example the results obtained in the work of Pinheiro et al. (2019) that also showed differences in LPO levels between the different concentrations and pressures tested.

Other studies reported fluctuations in CAT and LPO related to the time of exposure. These differences might be related to the time of activation of the antioxidant system (Aouini et al. 2018). An increase in CAT activity indicates an attempt to protect the cell against oxidative stress due to elevated ROS production (Wang et al. 2012). During the oxidative stress, ROS produced is known to affect cell membrane lipids where there is an increase in lipid peroxidation due to the formation of malondialdehyde (MDA) (Viarengo et al. 2000). Consequently, the significant differences observed in LPO levels can be related to the fluctuations in CAT activity.

Various studies in mussels indicate differences in GST activity when exposed to some stress condition, as organic contaminants for example, that can lead to increase or decrease in this enzymatic activity (Michel et al. 1993; Fitzpatrick et al. 1997; Akcha et al. 2000; Gowland et al. 2002; Lima et al. 2007; Solé et al. 2007; Boccheti et al. 2008; Banni et al. 2010; Fernández et al. 2012, Capello et al. 2013). Boccheti et al. (2008) in a study of harbor-caged mussels during dredging showed GST activity with biphasic responses with an initial increase and a posterior decrease. Tsangaris et al. (2014) reported the impacts of chemical contaminants related with dumping of dredged urban river sediments and the results showed a decrease in GST activity in caged mussels in the local of disposal and neighboring area (Tsangaris et al. 2014). GST activity, in the present study, showed a significant decrease between the 1g/L treatment and the control group, which indicates that this concentration affects GST activity in the digestive gland of both males and females.

A comparison between the T0 (atmospheric pressure) and control group (4 Bar) showed that for all biomarkers responses (CAT, GST and LPO), the values were higher in the tissues of clams exposed to hyperbaric conditions (control group), except for LPO in the gills. Considering that both groups had no sediment, the differences observed may be related to the pressure change or the time laps between both groups.

Some studies report that antioxidant defenses and the effects of oxidative stress in marine species are influenced by the reproduction cycle and sex (Viarengo et al. 1991; Wilhelm Filho et al. 2001; Gismondi et al. 2012; Xiu et al. 2015). A study of mussels from the Arcachon Bay reported that seasonal variability and individual characteristics as sex, may represent a significant confounding element that potentially masks the response of oxidative stress biomarkers when exposed to pollutants (Devier et al. 2005). Although gender differences were also observed in the present study for some endpoints, which was carried out during the reproductive season, they didn't follow a recognizable pattern.

# 5- Conclusion

Regarding results from the functional and biochemical endpoints performed in this study, namely the filtration rate, and biomarkers CAT, LPO and GST, they showed that suspended sediments can have a significant effect in the model species *S. solida* under hyperbaric conditions. The use of a hyperbaric chamber to simulate a deep-sea pressure represents an important variable to be considered to estimate the impacts of a deep-sea mining scenario, due to the high pressures of this environment.

Based on the findings of the present study, other aspects should be studied in the future, such as the effects that long-term exposure to suspended sediments can have on marine species, considering that in a real scenario of deep-sea mining, the necessary operations will last for longer periods of time. Furthermore, due to the adsorption and accumulation capacity of the particles, toxicity effects of metals present in the sediments should also be studied to provide a better understanding of the possible damage contaminants can cause in conjunction with the particles themselves. These findings will provide additional insights to improve guidelines and monitoring protocols into hazard assessment of deep-sea mining.

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