

***Evaluation of the impact of an oil spill on the coast of Peniche  
using the bioindicator Paracentrotus lividus (Lamarck, 1816).***

Monique Santos Sarly da Silva

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**Monique Santos Sarly da Silva**

Dissertation for the master's degree in Biotechnology of Marine Resources.

Dissertation of Master performed under the orientation of Doctor Sílvia Gonçalves and  
co-orientation of Doctor Ana Pombo.

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Title: Evaluation of the impact of an oil spill on the coast of Peniche using the bioindicator *Paracentrotus lividus* (Lamarck, 1816).

Título: Avaliação do impacto de um derrame de fuelóleo na costa de Peniche utilizando o bioindicador *Paracentrotus lividus* (Lamarck, 1816).

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*“Aprendi que se depende sempre, de tanta, muita, diferente gente... Toda pessoa sempre é as marcas das lições diárias de outras tantas pessoas. E é tão bonito quando a gente entende que a gente é tanta gente onde quer que a gente vá. E é tão bonito quando a gente sente que nunca está sozinho por mais que pense estar (...)”*

*Caminhos do coração – Gonzaguinha*

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Seguimos juntos (as)!

## Resumo

A contaminação de águas costeiras por poluentes pode ter impactos em diferentes aspetos do sucesso reprodutivo e no desempenho de diversos organismos marinhos podendo, conseqüentemente, afetar a manutenção dos ecossistemas. Neste sentido, muitos trabalhos de monitorização ambiental têm sido desenvolvidos com o intuito de estudar os efeitos da presença de contaminantes nas áreas costeiras utilizando organismos bioindicadores.

Na costa de Peniche, em julho de 2017, ocorreu acidentalmente uma descarga de fuel óleo na Praia do Abalo, proveniente do sistema de alimentação de uma caldeira de uma empresa. Perante este cenário, o principal objetivo do presente estudo foi contribuir para a avaliação do impacto ambiental desse derrame, usando o ouriço-do-mar *Paracentrotus lividus* (Lamarck, 1816) como indicador ambiental da presença de contaminantes. Visando este objetivo, 30 ouriços-do-mar foram recolhidos na Costa Rochosa do Impacto e numa Costa Rochosa de Referência nos meses de julho, agosto e setembro de 2017. Foram realizadas análises para quantificação dos elementos Cd, Pb, Ni, Fe, Mn, Zn e Cu nas gónadas de ouriços-do-mar por espectrometria de absorção atómica, análises biométricas, histológicas e bioquímicas para investigar se o derrame afetou as respostas fisiológicas e bioquímicas dos animais em estudo. Foram também realizadas análises para detetar a presença de anomalias, lesões ou outras alterações histopatológicas na aparência das gónadas que possam ser indicativas de um cenário de poluição ambiental.

As variáveis biométricas estudadas variaram significativamente entre as estações de amostragem e entre os meses de estudo, sendo maioritariamente mais elevadas, na Costa Rochosa Impactada, exceto o Índice Gonadossomatico, que foi significativamente maior na Costa Rochosa de Referência. Os resultados demonstraram que houve presença de contaminantes nas duas estações de amostragem ao longo dos meses estudados, com exceção do Cd em setembro, o qual não foi detetado em nenhuma das praias. O estudo identificou também um efeito significativo da interação entre os locais e os meses para as concentrações de Cd obtidas. Além disso, os resultados demonstram que as concentrações médias dos metais Zn e Cd foram significativamente menores nas gónadas masculinas do que nas gónadas femininas dos ouriços do mar. A maioria dos índices histopatológicos registados não variou significativamente entre as estações de amostragem, apresentando valores muito

semelhantes, exceto para os índices IHPA dilatação da membrana e a IHPA atrofia, que em julho na Costa Rochosa Impactada foram significativamente maiores que na Costa Rochosa de Referência. Cd e Cu apresentaram correlação positiva com as IHPAs reabsorção, dilatação e atrofia. Observou-se ainda um atraso no desenvolvimento do ciclo gametogénico dos ouriços do mar, sobretudo na Costa Rochosa Impactada. Por fim, observaram-se correlações negativas entre os metais Cd, Cu, Ni e o conteúdo em ácidos gordos das gónadas.

Diante dos resultados observados neste estudo, não foram observadas evidências fortes e diretas de contaminação em *P. lividus* pelo derrame de nafta ocorrido em julho de 2017, embora tenham sido detetados efeitos ligeiros na função reprodutiva. Supõe-se, portanto, que o evento não tenha produzido impactos negativos fortes no ecossistema em questão, uma vez que os possíveis efeitos prejudiciais desse acidente foram atenuados com sucesso com as medidas de controle imediato tomadas pela empresa envolvida e pelos órgãos ambientais responsáveis. Ainda assim, o presente estudo contribui com um conjunto de dados e resultados que podem servir, no futuro, como referência para estudos ecotoxicológicos utilizando *Paracentrotus lividus* como indicador ambiental na costa de Portugal.

**Palavras-chave:** *Paracentrotus lividus*, bioindicador, nafta, avaliação ambiental, metais traços, respostas fisiológicas, respostas bioquímicas, biologia reprodutiva.

## Abstract

Pollutant-contaminated coastal waters can impact different aspects of the reproductive success and performance of various marine organisms and, consequently, may affect ecosystem maintenance. In this sense, many environmental monitoring works have been developed in order to study the effects of the presence of contaminants in coastal areas using bioindicator organisms.

On the coast of Peniche, in July 2017, there was an accidental fuel oil spill at Abalo Beach, which originated in a company's boiler feed system. Given this scenario, the main objective of this study was to contribute to the assessment of the environmental impact of this accidental discharge, using *Paracentrotus lividus* (Lamarck, 1816) as an environmental indicator of the presence of contaminants. For this purpose, 30 sea urchins were collected at the Impacted Shore (Abalo) and at a Reference Shore in July, August and September of 2017. The presence of the trace metals Cd, Pb, Ni, Fe, Mn, Zn and Cu in sea urchin gonads was quantified by atomic absorption spectrometry, and several biometric, histological and biochemical analyses were performed in order to investigate whether the spill affected the physiological and biochemical responses of the animals at those two locations. In addition, analyses to detect the presence of anomalies, lesions or other histopathological changes in the appearance of the gonads, that may be indicative of an environmental pollution scenario, were also performed.

The biometric variables studied varied significantly between sampling stations and between the months of study, being mostly higher at the Impacted Shore, except for Gonadosomatic Index, which was significantly higher at the Reference Shore. The results showed that contaminants were present in both sampling stations over the months studied, except for September regarding the metal Cd, which was not detected in either sampling site. Also, the concentrations of Cd were significantly influenced by both the Shore and the Months of study. Moreover, significantly different concentrations of Zn and Cd were observed between male gonads and female gonads of sea urchins. Most of the histopathological indexes recorded did not vary significantly between the rocky shores, presenting very similar values, except for the IHPAs dilation and atrophy, which in July at the Impacted Shore were significantly higher than at the Reference Shore. Cd and Cu

exhibited a positive correlation with the IHPAs reabsorption, dilation and atrophy. The results also suggest a delay in the development of the gametogenic cycle of the sea urchins at IS. Finally, significant correlations between the metals Cd, Cu and Ni and the fatty acid content of the gonads were observed.

As a whole, according to the results observed in this study, strong and direct evidences of contamination in *P. lividus* by the oil spill that occurred in July 2017 were not observed, although slight effects were detected in the reproductive function. Therefore, it can be assumed that this accident has not produced strong negative impacts on the ecosystem in question, since the possible harmful effects of this accident were successfully mitigated with the immediate control measures taken by the company involved and by the responsible environmental agencies. Nevertheless, this study brings together a set of data and results that may serve, in the future, as a reference for ecotoxicological studies using *Paracentrotus lividus* as an environmental indicator on the coast of Portugal.

**Keywords:** *Paracentrotus lividus*, bioindicator, naphtha, environmental assessment, trace metals, physiological responses, biochemical responses, reproductive biology.

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## List of Abbreviations & Acronyms

Cd – Chemical element Cadmium  
Pb - Chemical element Lead  
Ni - Chemical element Nickel  
Cu - Chemical element Copper  
Fe - Chemical element Iron  
Zn - Chemical element Zinc  
Mn – Chemical element Manganese  
PAHs - Polycyclic aromatic hydrocarbons  
EC50 - Half maximal effective concentration  
GSTs - Glutathione transferases  
AChE – Acetylcholinesterase  
EEZ - Exclusive Economic Zone  
IS – Impacted Shore  
RS – Reference Shore  
DC – Diameter of carapace  
GW - Gonads weight  
GSI - Gonadosomatic index  
WW - Total wet weight  
mg - Milligrams  
ml – Milliliter  
g - Grams  
mg/l – Milligrams per liter  
g/l – Grams per liter  
mol/l – Mol per litre  
µl – Microliter  
µg/l – Microgram per liter  
µm – Micrometer  
mg/kg - Milligram per kilo  
rpm - Rotations per minute  
AAS - Atomic Absorption Spectrometry

GFAAS - Graphite Furnace Atomic Absorption Spectrometry  
HCL - Hollow cathode lamps  
NP - Nutritive phagocytes  
OV - Vitellogenin oocyte  
OR - Remnant mature oocytes  
IPHA - Index of pathological alterations  
LLP - lipofuscin-like pigments  
IHPA Dilat - Index of membrane dilation  
IHPA Reabs - Index of sex cell resorption  
IHPA LLP – Index of lipofuscin-like pigments accumulation  
IHPA Hyp - Index of hypertrophy in nutritive phagocytes  
IHPA Atro - Index of atrophy in nutritive phagocytes  
FA - Fatty acids  
SFA - Saturated fatty acids  
MUFA - Monounsaturated fatty acids  
PUFA - Polyunsaturated fatty acids  
C14 – Myristic acid  
C15 - Pentadecanoic acid,  
C16 – Palmitic acid  
C16:1 - Palmitoleic acid  
C18 – Stearic acids  
C18:1 trans - Elaidic acid  
C18:1 cis - Oleic acid  
C18:2n6 - Linoleic acid  
C18:3n6 - Gamma-linolenic acid  
C18:4n3 - Stearidonic acid  
C20:4n6 – Arachidonic acid  
C20:1n9 - Gondoic acid  
C20:1n11 - Gadoleic acid  
C20:2n6 – Eicosadienoic acid  
C20:3n6 - Homo- $\gamma$ -Linolenic acid  
C21 - Henicosanoic acid  
C22:1n9 - Erucic acid

EPA - Eicosapentaenoic acid

ALA - Alpha linolenic acid

NO - Nitric oxide

CoA – Coenzyme A

PCA - Principal Component Analysis

AChE – Acetylcholinesterase

MYP - Yolk protein

## **1. INTRODUCTION**

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## **1. Introduction**

### **1.1 Marine Environments and Pollution**

Marine ecosystems are the result of millions of years of several evolutionary processes and represent the largest reservoirs of water on Earth, covering over 70% of the planet's surface. Because they are the largest and oldest of the Earth's ecosystems, they have almost the double of the number of organisms, serving as shelter for a high variety of species (Boeuf, 2011; Gouletquer *et al.*, 2014). The marine domain is extremely important for maintaining life on the planet, since their ecosystems carry out about 50% of global primary production, have important ecological functions (such as control over climate) and they have resources which are essential for the social and economic development of the world, mainly for coastal communities (Cicin-Sain *et al.*, 2015; Aslan *et al.*, 2018).

In the marine domain, ecosystems are composed basically of habitats ranging from the seas to the coastal zones. In the coastal zone there are several habitats with unique characteristics, and ecological and commercial importance such as coral reefs, mangroves and intertidal systems (rocky, sandy, and muddy shores) (Baker *et al.*, 2009). These ecosystems have a high biodiversity value, can play a key role in mitigating global climate change by its ability to store carbon and are responsible for much of the maintenance of productivity (especially fisheries) (Torres and Hanley, 2016). Coastal populations interact with these systems in different forms, mainly through artisanal fishing. Despite their importance, indiscriminate use of their resources and services by humanity is a fact and it has great direct and indirect impacts in those environments (Halpern *et al.*, 2008).

In the last decades, with increasing population growth and the consequent increase in human activities, coastal marine waters have entered an intense process of pollution (Waldichuk, 1977; Dong, 2017). The World Health Organization (2000) defines coastal pollution as “the introduction by man, directly or indirectly, of substances or energy into the marine environment, including estuaries, which results or is likely to result in such deleterious effects such as harm to living resources and marine life, hazards to human health, hindrance to marine activities, including fishing and other legitimate uses of the sea, impairment of quality for use of sea water and reduction of amenities”. The reasons why

pollution occurs can be diverse, however the most frequent causes are associated to plastic debris, sewage and industrial effluents, oil spill and “no-point source”; this last refers to a source of pollution that issues from widely distributed or pervasive environmental elements. Aslan *et al.* (2018) affirms that since the sea became the final recipient of these elements, besides organic matter, many contaminants such as hydrocarbons, inorganic and chemical elements can be found accumulated in sediments or tissues of organisms (Martinho, 2016; Dong, 2017). Some of these substances are biodegradable while others are persistent. Their cumulative impact to the coastal marine environment over a long period could be quite harmful. This scenario can have devastating consequences for marine life and habitats on which marine organisms depend on (Elliott, 2003; Mearns *et al.*, 2013).

The accumulation of contaminants in the marine environment can lead to a range of consequences in different levels (Gómez *et al.*, 2004; Lohmann & Belkin, 2014). Some pollutants can be extremely toxic or fatal to some aquatic organisms during a short period of exposure, even at low concentrations; on the other hand, other pollutants may present a strenuous effect, that is, they do not kill organisms immediately, however they can cause chronic sub-lethal effects such as growth retardation, physiological stresses, and reproductive failures (Waldichuk, 1977; Dong, 2017; Hylland *et al.*, 2017; Tornero and Hanke, 2017). Within this context, humans are also susceptible to toxicity through the consumption of contaminated seafood, having their health compromised with, for example, neurological development deficits, and disturbances of the hormone system (Waldichuk, 1977).

The issue of marine environments pollution is serious and has made it increasingly worrisome as it is rising at an alarming rhythm. Solving this problem of pollution in the oceans is a difficult and comprehensive task, since it is necessary to understand which are the main causes and the main effects (Cross *et al.*, 1985).

## **1.2 Marine Pollution by Oil Products**

Among the environmental problems that strongly affect the health of oceans, pollution by oil or its derivatives stands out (Moore & Dwyer, 1974; Martinho, 2016). Marine oil pollution comes mainly from oil spill, offshore oil production, marine transport and urban

pollution (ITOPF, 2011). According to the World Energy Balance (2018), millions of tons of oil are spilled into the sea annually; these numbers tend to become larger as the oil industry expands.

The impact of oil or its derivatives on marine fauna is relative, depending on the viscosity, toxicity, amount of oil and the sensitivity of different animals to contact with the substance (IPIECA, 1995). Crude oil is composed by a complex mixture of organic compounds, basically consisting of hydrocarbons (PAHs), some heterocyclic compounds and some heavy metals (Dupuis & Ucán-Marín, 2015); mainly due to its composition and properties, the consequences of marine contamination can be diverse and drastic. Because it is a hydrophobic substance, the oil does not mix with water and it forms a film on it; thus, there is a blockage of light incidence necessary for photosynthesis by algae and other photosynthetic organisms (Moore & Dwyer, 1974). This impact affects the entire marine ecosystem and causes damage to the environment, such as direct death of organisms, alterations in the food chain and interference with the reproduction rates of animals (Lee and Page, 1997).

Heavy metals and PAHs contained in oil spill represent a significant source of contamination, being highly toxic to organisms (Curl & O'Donnell, 1977; Vikas & Dwarakish, 2015; Lu *et al.*, 2018). Metals can be classified into two different classes: essential (manganese, copper, iron, zinc) and non-essential (nickel, cadmium and lead) (USFDA, 1993; Burger *et al.*, 2002; Veiga, 2015). This classification is associated with the biological functions that these elements perform in organisms. Essential metals play important roles in the metabolic processes of organisms when minimally present; on the other hand, non-essential metals have high toxicity and generally do not play any important role in organisms (Jakimska *et al.*, 2011). The absorption or direct contact of some metals with the tissues of organisms can lead to damage and disturbance of cell walls and cellular functions at the molecular level. If the concentration and duration of exposure to these toxic contaminants is high enough, the organisms can undergo potential physiological damage, with structural changes at the cellular level that can culminate in death (Curl & O'Donnell, 1977; Soualili *et al.*, 2008; Vikas & Dwarakish, 2015; Lu *et al.*, 2018).

There are several serious cases of oil spills around the world. A study carried out by Fernández *et al.* (2006) evaluated the toxicity of the soluble fraction of fuel oil spilled on the embryo development of the sea urchin *Paracentrotus lividus* (Lamarck, 1816) when an oil tanker sank in November 2002 on the coast of Galicia. Analyses of PAHs and four metals (copper, cadmium, lead and zinc) were conducted. The study observed that embryo development was strongly inhibited when associated with a fuel oil content in water and the effective concentration which provoked a delay in the successful embryogenesis of 50% of the population (EC50) was 2.3% of fuel oil. Some studies of the composition of samples collected in the oil spill area showed an increase of Zn, Cu and Pb concentration in seawater after the spill (CSIC, 2003). In this work, despite the observed metal concentrations were below the median effective concentrations reported, the authors did not rule out the possibility of a synergistic effect. When combined with some organic substances, metals can form highly toxic metal-organic complexes (Veiga, 2015); this event has often been observed for different chemical mixtures and marine organisms, including the sea urchin *Paracentrotus lividus* (Lamarck, 1816) (Vanegas *et al.*, 1997; Fernández & Beiras, 2001).

Another serious accident happened in December 2000 on the Portuguese coast, in which a ship ran aground and spilled a large quantity of fuel oil into the sea. Sometime after the oil spill, mussels collected at stations near the ship had higher and lower values of glutathione transferases (GSTs) and acetylcholinesterase (AChE) activity, respectively (Moreira *et al.*, 2004). GSTs are enzymes that act on the biotransformation of xenobiotics (Beiras, 2018). In turn, acetylcholinesterase is an enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine and the completion of transmission of nerve impulses in cholinergic synapses; its inhibition has important implications in the health of organisms (Castellanos *et al.*, 2018). In this sense, besides demonstrating the efficacy of these biomarkers as indicators of exposure to this kind of pollution, these results confirm the contamination by the oil. Similarly, in January 2017 an Indian coastal city named Chennai was impacted by a large oil spill caused by a collision that occurred between two cargo ships, spreading tons of heavy oil in the Bay of Bengala. Subsequent studies indicated that samples collected in the spill area showed relatively high levels of heavy metals and highly toxic polycyclic aromatic hydrocarbons (PAHs) (Han *et al.*, 2018).

Portugal is a country with almost 1000 square kilometres of coast which, together with the archipelagos of Azores and Madeira, form the third largest Exclusive Economic Zone (EEZ) in the European Union. Because of all this extension, the exposure to the potential risks of accidents is large and there is an urgent need to develop techniques for environmental monitoring and forecasting of future damages (Borges, 2015). Disaster planning for oil spills requires learning from past events. However, it is always a challenge as the consequences depend on the geographic, ecological, social and temporal contexts in which the disaster occurs. Therefore, it is important to increasingly understand the mechanisms involved and the potential consequences to the ecosystem caused by the spill.

### **1.3 Bioindicators as a tool for the monitoring and evaluation of marine pollution**

In the last years, many environmental quality evaluation works have been carried out, using bioindicator organisms as a tool (Branco *et al.*, 2008; Soualili *et al.*, 2008; Chiarelli & Roccheri, 2014). According to McGeoch (1998), the term bioindicator refers to organisms that are resistant to some levels of contamination, that is, they do not die when exposed to toxic agents and derivatives; instead, they provide assertive information about the environmental conditions of the places where they live (Gonçalves *et al.*, 2013). This happens because the response of each organism is strongly influenced by the physical, chemical and biological conditions of the environment (*e.g.* temperature, humidity, winds and radiation) as well as by physiological, structural, nutritional and morphological conditions. In short, when the environment is contaminated, the bioindicator organism responds by changing some aspect(s) of its physiology; these changes, in turn, can be measured and may represent significant disturbances in the environment in which these organisms live (Market *et al.*, 2003).

Marine invertebrates have been widely used as bioindicators in environmental monitoring under different levels of anthropic impact and in assessments of ecosystem health (Rainbow, 2002). Among the features that justify the frequent choice of invertebrates as biological indicators, the facts that these organisms have long life cycles, being sessile or with low mobility, are easy to sample with relatively low costs and are sensitive to different concentrations of environmental pollutants, providing a wide range of responses to different

levels of environmental contamination, stand out (Hodkinson and Jackson, 2005). Furthermore, invertebrates occupy a key position as intermediate consumers in the pelagic and benthic food chains of aquatic ecosystems; once affected, even if in the long term, the ecosystem would be impacted (Chiarelli & Roccheri, 2014).

#### **1.4 The Sea urchin *Paracentrotus lividus* as a bioindicator**

Several studies indicate that sea urchins living in or exposed to water contaminated by hydrocarbons or metals, can present alterations associated with their reproductive, physiological and morphological aspects, which vary according to the levels of pollution (Danis *et al.*, 2005; Branco *et al.*, 2008; Soualili *et al.*, 2008; Schäfer and Kohler, 2009; Chiarelli & Roccheri, 2014). The sea urchin *Paracentrotus lividus*, in special, has been qualified as an excellent bioindicator species of contaminants at sea. This qualification is justified by its tolerance to physiological pressure, resistance to high levels of salinity, temperature, and desiccation or food shortage for long periods of time (Allain, 1975; Hereu, 2005). In addition, *P. lividus* is a widely distributed and abundant species, establishing very well in specific regions, thus allowing an analysis of micro regions. Finally, they are animals easy to collect, to handle and to maintain in the laboratory. Its value as a bioindicator for heavy metals and polycyclic aromatic hydrocarbons contaminations has been documented in several laboratory and field studies (Warnau *et al.*, 1995a, 1996, 1998; Geffard *et al.*, 2001; Soualili *et al.*, 2008; Rocha *et al.*, 2018; Ternengo *et al.*, 2018).

*Paracentrotus lividus* (Figure 1) is a species of sea urchin in the Parechinidae family, which lives in rocky substrates and in seagrass meadows, from shallow water to about 20 m depth (Tenuzzo *et al.*, 2012). These organisms feed mainly on aquatic vascular plants and algae but may also feed on debris in the water column (Boudouresque & Verlaque, 2001). This species has a natural and wide distribution in the rocky bottoms of the shores of the eastern Atlantic, from Ireland to the south of Morocco and across the Mediterranean Sea. In countries such as France and Japan, *P. lividus* gonads are widely consumed (Fernandez & Boudouresque, 2000; Boudouresque & Verlaque, 2001), and aspects such as color, size, texture and granularity of the gonads greatly influence their trade and consumption (Blount & Worthington, 2002; Pearce *et al.*, 2002). In the winter and spring months, the gonads reach a larger volume, a better texture and flavor, and thus these animals are harvested more

intensely (Ouréns *et al.*, 2013). In recent years, its commercial exploitation has grown, and the wild populations have decreased drastically in some areas (Ceccherelli *et al.*, 2011; Pais *et al.*, 2011; Bertocci *et al.*, 2014; Sartori *et al.*, 2016).



**Figure 1:** The sea urchin *Paracentrotus lividus*.

Like most echinoids, *P. lividus* are gonochoristic organisms; that is, they have separate sexes. These animals are devoid of sexual dimorphism and perform external fertilization, releasing simultaneously their gametes in the water. According to Boudouresque and Verlaque (2001), in a general way, reproduction occurs once or twice a year; however, it can vary among geographic areas depending on the environmental conditions.

The gonads of *P. lividus* are considered a source of proteins, lipids, fatty acids and minerals (Volpe *et al.*, 2018). This whole nutritional apparatus is closely associated with the gender and the different gametogenic stages of the organism. At the beginning of the gametogenic process, there is a higher nutritional need and macrophagic cells - called nutritive phagocytes - synthesize proteins and other essential nutrients to provide energy and to promote the growth and maturation of the gametes (Carboni, 2013). As the gametes are released, the nutritional need decreases. Aspects of reproduction and gametogenic cycle can be altered by unfavorable conditions and limitation of food supply (Lozano *et al.*, 1995).

*Paracentrotus lividus* is abundant on the Portuguese coast, colonizing different biotopes (Girard *et al.*, 2012; Jacinto *et al.*, 2013; Domínguez *et al.*, 2015). Their gonads make it a highly appreciated seafood in the country and, as previously presented, this echinoid is used as an excellent bioindicator of pollution in the marine environment. Parallel to these facts,

the Lusitanian coast has been increasingly exposed to anthropogenic impacts, mainly due to the occupation of areas close to coastal zones. In this sense, it is important to understand the importance of this species as a bioindicator of pollution in the habitats where they live. Thus, analyses performed on *P. lividus* can represent useful tools to evaluate the effects of pollutants in marine environments and when the results demonstrate a relationship with the concentration of these pollutants, the interpretation of toxicities and exposure conditions becomes clearer.

### **1.5 Objectives of the study**

The increasing installation of human activities in coastal ecosystems has arguably a strong environmental impact, which is represented not only by the increasing degradation of coastal zones but also by the increased risk of serious environmental damage. In addressing this issue, the need for environmental assessment studies is recognized in order to ensure the permanence of the biota and to analyse the impacts that these problems may have on the long and short term for human life. Environmental assessment studies, therefore, are relevant tools that can aid in the management and prevention of environmental accidents, having also a fundamental role in planning and as a support in decision-making processes. In short, works such as these make it possible to understand the sensitivity of ecosystems to changes, the resulting impacts on biodiversity, and the risks to public health directly or indirectly related to environmental quality in those areas.

In July 2017, an accidental spill of 3 tons of naphtha occurred in Abalo's Beach, on the coast of Peniche (Portugal). The fuel oil spill was originated in the system of feeding a boiler of a company. In this scenario, the main objective of the present study is to contribute to the environmental impact assessment of this oil spill by using *Paracentrotus lividus* as a bioindicator of the presence of contaminants. To achieve the main purpose of this work, the following specific objectives are proposed:

a) To perform analysis for the quantification of the elements Cd, Pb, Ni, Fe, Mn, Zn, Cu in the gonads of the sea urchins, in order to assess the degree of contamination derived from the oil spill.

b) To perform biometric, histological and biochemical analysis to investigate if the spill affected the following physiological and biochemical responses in *P. lividus*:

- The size and weight of the individuals (carapace diameter and individual wet weight);
- The gonadal somatic index; the development of the gonads and the stages of gametogenesis;
- The characterization and quantification of total lipids, proteins and fatty acids;

c) To detect the presence of anomalies, lesions or other histopathological changes in the appearance of the gonads that could be indicative of an environmental pollution scenario.

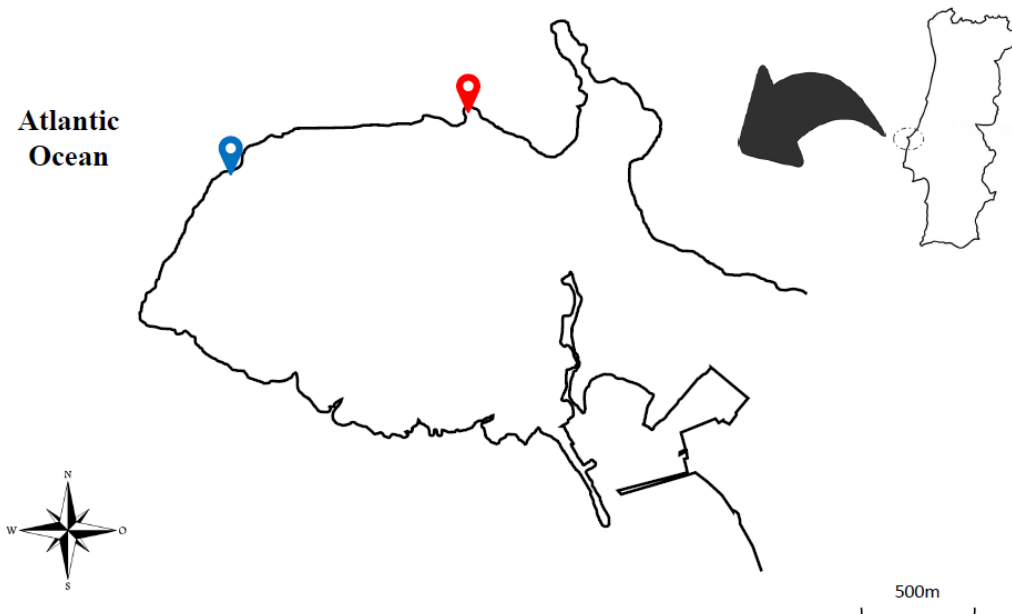
## 2. MATERIALS AND METHODS



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## 2. Materials and methods

### 2.1 Study Area

This study was performed on 2 rocky shores of the coastal zone of Peniche (Portugal). The specimens of *P. lividus* were collected at two distinct sampling areas: the first one, Abalo's Beach (Impacted Shore - IS -  $39^{\circ}22'12.69''\text{N};009^{\circ}23'7.07''\text{W}$ ), the central point where the fuel oil spill happened; the second area was not directly impacted by the spill, functioning in the present study as a reference area for comparison (Reference Shore - RS -  $39^{\circ}22'02.4''\text{N};9^{\circ}24'08.07''\text{W}$ ). The reference area is located 1.5 km to the west of the Impacted Shore (Figure 2).



**Figure 2:** Map of Peniche with the indication of the study sites (  Impacted Shore,  $39^{\circ}22'12.69''\text{N};009^{\circ}23'7.07''\text{W}$ ;  Reference Shore,  $39^{\circ}22'02.4''\text{N};9^{\circ}24'08.07''\text{W}$ ) where the specimens of *Paracentrotus lividus* were sampled.

Abalo's beach is characterized by a very irregular topography, presenting a very heterogeneous relief, due to the existence of large rocky fragments with a diverse morphology. In this beach, three distinct zones can be considered and easily discernible by their specific content: the supralittoral zone, the eulittoral zone and the sublittoral zone, as in most rocky shores of the world. The rocky shore of Abalo has a low hydrodynamics, is very fragmented and has also a small sandy area. Abalo's beach is located next to an area where there are a series of industrial activities and industrial companies, with intense

movement. In addition, it is common to observe in the area shellfish harvesters distributed by the rocky shores at low tide; all these features make this site more susceptible to human impacts (Figure 3a).

The area used as a reference in this work, on the other hand, has exposed rocky shores, formed by smooth seawalls, which present a strong hydrodynamism due to the high impact of waves; therefore, this area possibly has less habitat diversity and a high rate of primary productivity due to an high nutrients flux (Satyam & Thiruchitrambalam, 2018). There is no sandy area in this location and the site's accessibility is difficult compared to the Abalo's Beach (Figure 3b).



**Figure 3:** Sea urchins collection stations. a) Abalo's Beach, the main point of the oil spill; b) Reference Shore, located 1.5 km away from the rocky shore hit by the oil spill.

## 2.2 Collection of Sea Urchins and Determination of Biometric Data

On each rocky shore, 30 sea urchins were collected once a month during the months of July (immediately after the spill), August and September of 2017. The individuals were

collected manually and at random in the morning, during low tide of spring tides. As the tidal level was in the infralittoral zone, it was possible to perform the activities between the rocks and in the several intertidal pools left by the ebbing tide. After collection, the animals were transported to the aquaculture laboratory of MARE-IPLeiria - Marine and Environmental Sciences Centre, *Campus* of the Polytechnic of Leiria, inside buckets with marine water. In the laboratory, the horizontal diameter of the carapace (DC;  $\pm 0.1$  mm accuracy) of each specimen was measured with the help of a caliper. Also, the total wet weight (WW;  $\pm 0.01$  g) and the gonads weight (GW;  $\pm 0.01$  g) of each individual were obtained using an analytical balance (AE ADAM PGL 3002, Milton Keynes, England) (Figure 4). With this information, it was possible to calculate the gonad somatic index (GSI) from the following formula (Marsh *et al.*, 2013):

$$\text{Gonad somatic index (GSI)} = \left( \frac{\text{Gonad weight}}{\text{Total weight of individual}} \right) \times 100$$



**Figure 4:** Material used to determine biometric data and to remove the gonads from the sea urchins.

All 30 individuals (from each month) were histologically analysed; for that purpose, one of the five gonads in each individual was removed, fixed and preserved in 4% buffered formalin for further histological analysis (James *et al.*, 2018). The other four gonads were stored in individual tubes at  $-80^{\circ}\text{C}$  and later lyophilized for biochemical analysis and for the determination of the presence of metals.

## 2.3 Analysis of the Concentration of Metals

### 2.3.1 Acid digestion of biological samples by microwave radiation

For the determination of the concentration of the metals cadmium (Cd), lead (Pb), nickel (Ni), iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu), 16 sea urchins (8 males and 8 females), from each sampling moment and from each rocky shore, were randomly selected. The samples of the gonads of these sea urchins were first acid digested. Approximately 100 mg of *P. lividus* gonadal samples were accurately weighed into a Teflon microwave digestion tube. Then, the samples were digested using 10 ml of HNO<sub>3</sub> (69.6%, AnalaR NORMAPUR, VWR pro lab chemicals, France) in a high-performance microwave digestion system (Milestone connect, MA182-001 Ethos up, Italy) (Figure 5). After being digested, the samples were transferred to a 50 ml plastic bottle and stored in a freezer until analysis by Atomic Absorption Spectrometry.



**Figure 5:** High-performance microwave digestion system (Milestone connect, MA182-001 Ethos up, Italy).

### 2.3.2 Analysis of metals by Atomic Absorption Spectrometry

The concentrations of non-essential (Cd, Ni and Pb) and essential (Fe, Mn, Zn and Cu) metals in the samples of the gonads were determined by Atomic Absorption Spectrometry (AAS) (Thermo Scientific™ iCE™ 3500, Thermo Unicam, Portugal) (Figure 6). The two

types of atomizers most used in AAS are the flame and the graphite furnace. The choice between one technique and another is directed according to the levels of metals expected in the samples. Atomic Absorption Spectrometry by flame is the technique most used for analysis at milligrams per liter levels, while the Atomic Absorption Spectrometry with electrothermal atomization in a graphite furnace (GFAAS) is used to quantify at low concentrations (micrograms per liter) (García & Báez, 2012). In this sense, Fe, Zn and Mn concentration in gonads were determined using flame atomic absorption spectrometry, using Acetylene and compressed air, while the quantifications of Cd, Cu, Ni, and Pb were carried out by AAS with graphite furnace (Thermo Scientific™ iCE™ 3500 Atomic Absorption spectrometer, Thermo Unicam, Portugal), using Argon.

Standard solutions of Cadmium (Cadmium standard solution (1000±0.0002 g/l AA Panreac Quimica SLU, European Union); Lead (AAS Standard Lead 1000 mg/l in nitric acid 2%, AVS TITRINORM, VWR international Haasrode, Belgium); Nickel (Nickel standard solution traceable to SRM from NIST Ni(NO<sub>3</sub>)<sub>2</sub> in HNO<sub>3</sub> 0.5 mol/l, 1000 mg/l Ni contains nickel (II) nitrate Certipur®, Merck KGaA, Darmstadt Germany EMD Milipore Corporation); Iron (Iron standard solution traceable to SRM from NIST Fe(NO<sub>3</sub>)<sub>2</sub> in HNO<sub>3</sub> 0.5 mol/l 1000 mg/l); Manganese (Manganese standard solution traceable to SRM from NIST Mn(NO<sub>3</sub>)<sub>2</sub> in HNO<sub>3</sub> 0.5 mol/l 1000 mg/l Mn Certipur®, Merck KGaA, Darmstadt Germany EMD Milipore Corporation); Zinc (Zinc standard solution traceable to SRM from NIST Zn(NO<sub>3</sub>)<sub>2</sub> in HNO<sub>3</sub> 5 mol/l 1000 mg/l Zn Certipur®, Merck KGaA, Darmstadt Germany EMD Milipore Corporation); and Copper (Copper standard solution traceable to SRM from NIST Cu(NO<sub>3</sub>)<sub>2</sub> in HNO<sub>3</sub> 0.5 mol/l 1000 mg/l Cu Certipur®, Merck KGaA, Darmstadt Germany EMD Milipore Corporation) were used to construct the calibration curve for each metal and all standards and reagents were diluted using ultra-pure water.

For GFAAS, a volume of 20 µl of magnesium nitrate (Magnesium Matrix Modifier, Magnesium nitrate, Matrix Modifier Solution, 1% Mg (NO<sub>3</sub>)<sub>2</sub> in 2% HNO<sub>3</sub> Specture, ThermoFisher (Kandel) GmbH, Karlsruhe, Germany) was added in each sample aliquot. The matrix modifier is added to samples to reduce analyte loss by decreasing its volatility or increasing matrix volatility during the decomposition reaction which occurs due to high temperatures (Voth, 1983). Magnesium Nitrate was added in all metals analysed in this work except Nickel.

As radiation source for the analysis of each metal, hollow cathode lamps (HCL) were used, where each cathode consists of a different element. The detection limits of the techniques used were 0.008 µg/l for Cd, 0.4 µg/l for Cu, 0.1 µg/l for Fe, 0.06 µg/l for Pb, 0.035 µg/l for Mn, 0.3 µg/l for Ni and 0.1 µg/l for Zn for this equipment.



**Figure 6:** Atomic Absorption Spectrometer (Thermo Scientific™ iCE™ 3500 Atomic Absorption spectrometer, Thermo Unicam, Portugal).

The determination of metal concentrations was performed from the method of standard additions which is a quantitative method generally used when the sample of interest has several components that result in matrix effects, so that additional components can reduce or enhance the analyte absorbance signal (Smith, 1983). Samples of sea urchin gonads were determined on a dry weight basis, as milligrams per kilogram, and the determination of concentrations was performed with 3 replicates. In all quantifications, a blank (1% HNO<sub>3</sub>) was subtracted and mean values and standard deviations were calculated (Pedro *et al.*, 2013).

To verify the accuracy of the method, a known amount of analyte was added to the natural test sample matrix and its response was measured (recovered) in the assay compared to an identical elevation in the standard diluent. The objective of this procedure was to evaluate if the methodology used in this work was valid, thus allowing the repeatability of the analysis.

## 2.4 Histological Analysis

For histological analysis, the gonads were fixed in 4% buffered formalin for 24h and then inserted into a solution of 70% ethanol (also during 24h). Subsequently, three steps were followed: dehydration, clarification and impregnation. The gonads were processed in a tissue processor (Leica TP 1020, Nussloch, Germany), in which they passed through different ethanol solutions with increasing concentrations. After going through the dehydration step, the gonads were clarified in order to remove the dehydrating agent by replacing it with a liquid miscible with the impregnation medium (liquid paraffin). The intermediate agent used in the diaphanization was xylol. Lastly, gonads were impregnated in paraffin, which gave structure to their tissues. After being processed, the gonads were included in paraffin to obtain blocks that posteriorly allowed obtaining sections without destruction of the tissues. For this, the tissues were embedded in a medium which supports them: paraffin at 60°C. With the material included in paraffin, microtomy was performed on an Accu-Cut® SRM™ 200 Rotary Microtome obtaining 5 µm thickness sections. The sections were dried and preserved in a kiln at 37°C (Binder, Tuttlingen, Germany) for 24 hours, until subjected to the staining technique. The staining was performed according to the Hematoxylin-Eosin staining technique.

For completion of the gonadal tissue preparations, the Coverquick 2000 Path® assembling medium (San Francisco, USA) was used and the slides were dried for 24 hours at room temperature. Finally, after obtaining the slides, the gonads of *Paracentrotus lividus* were observed under a composite optical microscope (Leica DM 2000 LED, Wetzlar, Germany), photographed using a microscope camera (Leica® MC170 5MP HD) and the combined LAS V4.4.0 software (Leica Application Suite) for monitor display (Leica Microsystems GmbH). From there, it was possible to determine the sex of individuals, which allowed the calculation of the sex ratio and to characterize the different stages of gametogenesis (Santos *et al.*, 2019).

The characterization of the gametogenic cycle of sea urchins was performed according to Byrne (1990) and Spirlet *et al.* (1998). The growth pattern of both testicles and ovaries has been divided into six stages and were used to document the major events of the

individuals' spermatogenic and oogenic cycle. The stages were identified based on the following characteristics:

- Stage I - Early Phase: In this phase, the gonad lumen is completely filled with nutritive phagocytes (NP) - the non-germinal accessory cells - which vary in size and colour. It is possible to observe the presence of primary oocytes along the ovarian/testicular ascinal wall, which in turn is covered by a thin basophilic layer. Eventually, the presence of remnant oocytes, not expelled in the last spawning, can be observed and are later reabsorbed by NP.
- Stage II - Growth: It is from this stage that cell growth gradually begins. The lumen of the gonad is occupied by nourishing phagocytes, and the pre-yolk oocytes, which are still along the ascending wall of the ovary/testis, begin to grow as they absorb nutrients supplied by nourishing phagocytes (NPs). The female gamete assumes the typical form of vitellogenin oocyte (OV). In males, sperm begin to protrude centrally and the basophilic layer increases.
- Stage III - Pre-maturation: In this phase there is a reduction in the presence of NPs and an increase in oocytes in number and size. It is still possible to observe pre-yolk oocytes attached to the gonadal tissue wall, surrounded by nutritive phagocytes (NPs). As vitellogenesis occurs, there is also a migration of mature oocytes to the centre of the acino. At this stage vitellogenesis is a continuous process and oocytes in all states are present. When primary oocytes reach their maximum size, they immediately mature and the eggs accumulate in the ovarian lumen. In males, premature testicles contain spermatocyte columns along the ascinal wall and sperm accumulates in the lumen.
- Stage IV - Maturation: A large number of mature oocytes (90  $\mu\text{m}$  in diameter) occupy the lumen of the acini. Eventually, nutritive phagocytes can be observed forming a very thin layer near the ascinal wall, as well as some small vitellogenin oocytes (10 to 60  $\mu\text{m}$  diameter) present in the ascinal wall, indicating that the vitellogenin process has not yet been completed. In males, the mature testes are full of sperm and the nutritive phagocytes are limited to the ascinal wall.

- Stage V - Posture: At this stage, the acini contracts and is emptied, however remnant mature oocytes (OR) can eventually be observed, which will later be reabsorbed by nutritive phagocytes (NPs). In females there are spaces between the unreleased oocytes. In males, this stage has a very similar aspect to stage IV, differing only by the presence of spaces in the lumen and the smaller amount of sperm. Also, the ascinal wall looks thin and sperm may be present in the gonoduct.
- Stage VI – Spent stage: At this stage, the ovaries have thin ascinal walls, lose their internal structure leading to disorganization, and it is possible to observe mature oocytes and pre-vitelline oocytes, which have detached from the ascinal wall. Any oocytes present in the ovary at this stage will probably be reabsorbed. One may also observe a meshwork of nutritive phagocytes around the periphery that may have begun to sequester reserves for the next oogenic cycle. In males, thin ascinal walls and a meshwork of nutritive phagocytes are observed on the periphery of the testis, as well as spaces created by the absence of sperm.

During the identification of the gametogenic stages of the sea urchins, careful observations to detect the presence of anomalies, lesions or other histopathological changes in their appearance that could be indicative of an environmental pollution scenario, were also performed. Thus, an index of histopathological alterations (IPHA) was determined by analysing the gonads lesions and classifying these lesions on a scale from 0 (no lesions) to 3 (severe) in the observed slides (Vaschenko *et al.*, 2012). The changes observed were: oocyte resorption, accumulation of lipofuscin-like pigments (LLP), nutritive phagocyte hypertrophy or atrophy. High amounts of lipofuscin are easily observed in extracellular spaces and in the oocytes of some individuals, since the colour of large globular inclusions are from gold–yellow to brown–yellow. For the identification of hypertrophy of nutritive phagocytes (NPs), some accumulation of larger cells is observed due to the increased synthesis of their basic constituents and their volume. NPs atrophy, in turn, is found when smaller cells are identified as a result of the decreased nutrition, metabolism, and synthesis needed to renew their structures. Finally, resorption of oocytes (phagocytosing of atretic oocytes by NPs) is found when a higher volume density of atretic oocytes are observed. To

calculate the IPHA, the total score of the different pathologies observed in the gonads was divided by the number of individuals analysed (Vaschenko *et al.*, 2012).

## **2.5. Biochemical Analysis**

For the biochemical analysis, 12 individuals (6 males and 6 females) were randomly chosen for each month. All the samples were homogenized (Ystral ® D-79282, Ballrechten-dottingen, Germany) and all following analyses were performed in duplicates.

### **2.5.1. Total lipids**

The determination of the total lipids present in the gonads was performed based on the protocol of Bligh and Dyer (1959). About 20 mg of each gonad sample were weighed into eppendorf's in an analytical balance (Sartorius); then 500 µl of chloroform (CHCl<sub>3</sub>, Prolabo ® VWR, Lyndhurst, South Africa), methanol (CH<sub>3</sub>OH, HiPerSolv. CHROMANORN, Prolabo ® VWR, Fontanay-Sous-Bois, France) and ultra-pure water (18,02 MΩ, HiPerSolv. CHROMANORN, Prolabo ® VWR, Fontanay-Sous-Bois, France) were added. After addition of these reagents, with the objective of separating the layers, centrifugation was carried out at 2000g during 10 minutes at 4 °C (Eppendorf Centrifuge 5810 R, Billerica, EUA). Then, 100 µl of the organic phase were withdrawn into glass tubes. For the determination of the amount of lipids, a standard curve was prepared using a stock solution of known initial concentration of tripalmitin (C<sub>51</sub>H<sub>98</sub>O<sub>6</sub>, Alfa Aesar, Karlsruhe, Germany) in chloroform (3.2 mg/ml<sup>-1</sup>). Thereafter, 500 µl of sulfuric acid (95% H<sub>2</sub>SO<sub>4</sub>, Chem-Lab, Zedelgem, Belgium) was added in both standards and samples. Finally, all tubes were placed in a 200 °C kiln (Memmert oven, Schwabach, Germany) for 15 minutes. After removal from the kiln, and after cooling, 1.5 ml of ultrapure water was added to all tubes. Finally, each sample was divided into 4 x 300 µl into a microplate. A plate reader (Synergy H1 Hybrid Reader Biotek ® Winooski, USA) was used for reading the plates and absorbance was read at 375 nanometres.

### 2.5.2. Proteins

Protein determination in the gonad samples was performed according to the method of Lowry *et al.* (1951). To perform this extraction, approximately 0.02 to 0.03g of *P. lividus* gonad samples were weighed into glass tubes, using an analytical balance (Sartorius). 500 µl of saccharide solution in EDTA was added and then the tubes were placed on the shaker for 5 minutes (130 rpm). Thereafter, 4.5 ml of N/100 Sodium Hydroxide (NaOH Prolabo® VWR, Belgium) was added to make a total of 5 ml. The tubes were shaken by inversion (5x) and then they were placed in the refrigerator for 30 minutes. After this time, a filtration was performed by the pleat filter. 200 µl of the filtrate were transferred to glass tubes, and then 4.8 ml of N/100 Sodium Hydroxide were added to make a total of 5 ml. Once this was done, it was stirred again by inversion (5x).

For the determination of proteins, 300 µl were transferred into Eppendorf tubes. Parallel to this, two blanks were prepared with 300 µl of N/100 Sodium Hydroxide and standards. The standards were made from a solution of bovine albumin 1 mg/ml (CAS-No 90604-29-8, USA) and ultra-pure water. Thereafter, all samples, blanks and standards were treated with an alkaline solution of copper (1 ml in each tube), vortexed and incubated at 37 ° C for 3 minutes in the oven (Memmert oven, Schwabach, Germany). Then, 100 µl of the Folin-Colciseau reagent (diluted 1:1) was added and the tubes were vortexed again and incubated under the same conditions. Finally, 200 µl were transferred to wells in microplates and the reading was carried out at 750 nanometres in a plate reader (Synergy H1 Hybrid Reader Biotek ® Winooski, USA).

### 2.5.3. Fatty acids

For the quantification of fatty acids, the protocol established by Lepage and Roy (1986) was followed. Samples were weighed into glass tubes on an analytical balance (Sartorius, Göttingen, Germany). 2 ml of a solution of methanol (CH<sub>3</sub>OH) + sulfuric acid (2%) (H<sub>2</sub>SO<sub>4</sub>, Merk, Darmstadt, Germany) were added to each sample and then these were placed in 80 °C bath for 120 minutes. After the samples cooled, 1 ml of ultrapure water (18.02 MΩ, HiPerSolv CHROMANORN, Prolabo ® VWR, Fontanay-Sous-Bois, France) and 2 ml of heptane (C<sub>7</sub>H<sub>16</sub>, Fisher Scientific, Bishop Meadow Road, England) were added. Finally, the

samples were centrifuged (Eppendorf Centrifuge 5810 R, Billerica, USA) at 1500 g, for 5 minutes for subsequent collection of the organic phase (1 ml of supernatant) and transfer to vials. The samples in the vials were analyzed by Gas Chromatography (GC) (Finnigan TraceGC ultra Massachusetts, USA), according to Masood *et al.* (2005). Fatty acid characterization was then performed using two commercial standards (Polyunsaturated Fatty Acid Mix No. 3 analytical standard from menhaden fish oil [PUFA n-3], Supelco®, Bellefonte, USA; Supelco 37 component FAME mix, Sigma-Aldrich®, St. Louis, USA).

## **2.6. Data Analysis**

The statistical techniques used in this study were performed with the statistical software IBM® SPSS® Statistics 26, except for the PERMANOVA, which was performed in PRIMER7. In the statistical analysis of the data, the significance level  $\alpha = 0.05$  was assumed. Prior to any statistical analysis, all data were tested for normal distribution (using the non-parametric test Kolmogorov-Smirnov) and homogeneity of variance (Levene's test) and, when necessary, the data that did not meet these assumptions were transformed. When the transformations did not remove the heterogeneity, the analyses were performed on the untransformed data (whenever  $n > 30$ ), since analysis of variance is quite robust to departures from their assumptions (Underwood, 1997). On the other hand, if none of the assumptions of normality and homogeneity were met for samples with  $n < 30$ , Kruskal-Wallis nonparametric tests were performed.

Depending on the nature of the data - that is, whether they were parametric or non-parametric - Student's t-tests and Mann-Whitney test were conducted to compare means of variables between the two sampling stations and between the sex of individuals. Given this, Student's t-tests for independent samples were conducted to compare means of biometric and histological variables, whereas Mann-Whitney tests were performed to evaluate the differences between the means of biochemical variables and metal concentrations.

Kruskal-Wallis nonparametric tests were conducted to evaluate the variation between the months of study on the concentrations of metals in the gonads and on some physiological and biochemical responses (the percentage of lipids, proteins and fatty acids in the gonads) of the organisms.

Two-Way ANOVA or PERMANOVA analyses (according to data normality and heterogeneity) were used to test the effects of the sampling stations and the animals' sex, as well as the interaction between the 2 factors, on biometrics responses and gonadal lesions. The significant effects detected were then subjected to post-hoc tests: (i) Tukey HSD to analyse the individual effects of the factors and (ii) Bonferroni tests to analyse the significant interactions between the factors.

To test if the presence of metals on the gonads of *P. lividus* had any influence on their biological variables, Spearman correlations were performed between the bioaccumulated metals in the gonads and the biometrics, index of histopathological lesions and biochemical variables ( $p < 0.05$ ).

Finally, the variation of *P. lividus* sex ratio over time was evaluated by performing a chi-square test for the reproductive biology component of the species.

### **3. RESULTS**

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### 3. Results

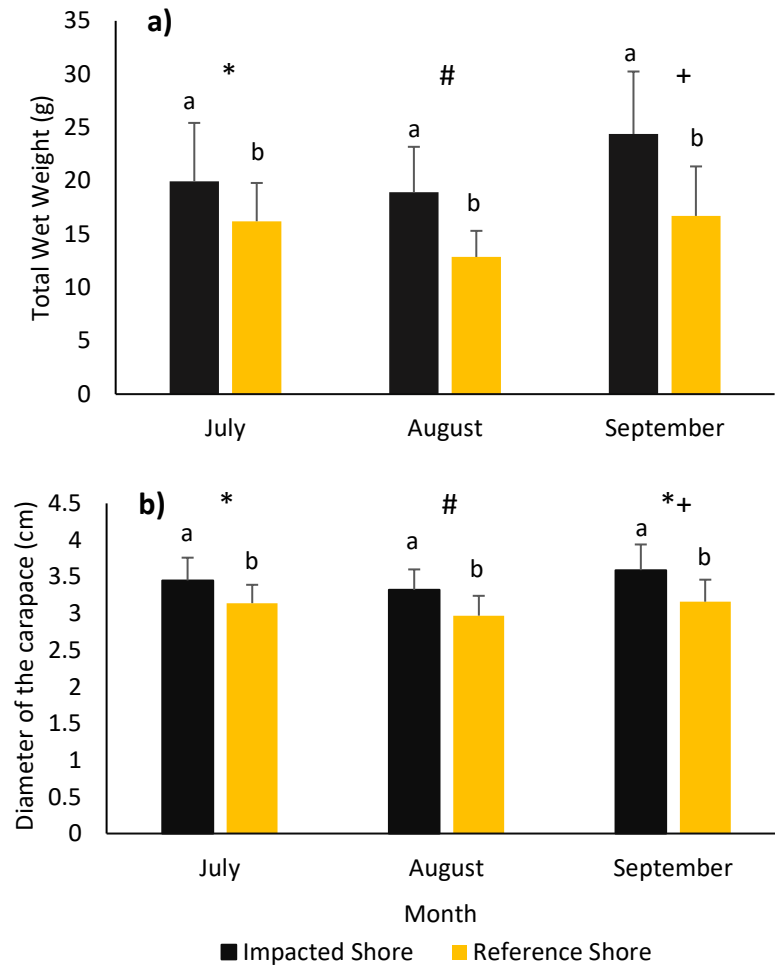
#### 3.1. Biometric data

The comparison of the biometric variables between the 2 rocky shores revealed that the sea urchins from the Impacted Shore, epicentre of the spill, were significantly larger and heavier than those from the Reference Shore (DC:  $3.45 \pm 0.33$  cm; WW:  $21.08 \pm 5.71$  g *versus* DC:  $3.09 \pm 0.28$  cm; WW:  $15.26 \pm 4.02$  g, respectively (Figure 7a, 7b and Appendix I). The independent-samples t-test did not reveal significant differences between the observed values for the gonadal weight ( $p = 0.394$ ), however, higher values were observed on the specimens from the IS ( $1.66 \pm 0.35$  g) compared to the RS ( $1.46 \pm 0.37$  g) (Figure 8a). On the other hand, individuals collected at the IS had significantly lower gonadosomatic index values ( $7.49 \pm 0.72$  %) than individuals collected at the RS ( $9.50 \pm 0.28$  %) (Figure 8b and Appendix I).

Two-way ANOVA procedures revealed that WW was significantly influenced by an interaction between the two rocky shores and the months of sampling ( $p = 0.042$ , Appendix I). The Bonferroni post-hoc test, in turn, indicated that this interaction was statistically different for WW over the three months of sampling and between the rocky shores. In the month of September the sea urchins presented the highest WW values (IS:  $24.39 \pm 5.86$  g; RS:  $16.71 \pm 4.64$  g); followed by July, in which the animals presented intermediate values (IS:  $19.94 \pm 5.49$  g; RS:  $16.21 \pm 3.59$  g) and, lastly, August, in which the sea urchins presented the lowest WW (IS:  $18.93 \pm 4.26$  g; RS:  $12.86 \pm 2.45$  g). In all cases, the sea urchins collected at the IS showed higher mean WW values than the animals collected at RS (*Bonferroni test*;  $p < 0.05$ ) (Figure 7a and Appendix I).

The biometric variables DC, GW and GSI also varied significantly between the months of sampling, as revealed by the Two-Way ANOVA analysis (Appendix I). The sea urchins with the highest DC values were recorded in September (IS:  $3.59 \pm 0.35$  cm; RS:  $3.16 \pm 0.30$  cm) while the smallest values were observed in August (IS:  $3.32 \pm 0.28$  cm; RS:  $2.97 \pm 0.27$  cm), being statistically different from each other (*Tukey HSD test*;  $p < 0.05$ ). The month of July presented intermediate values (IS:  $3.45 \pm 0.31$  cm; RS:  $3.14 \pm 0.25$  cm), being statistically different from August (*Tukey HSD test*;  $p < 0.05$ ), but

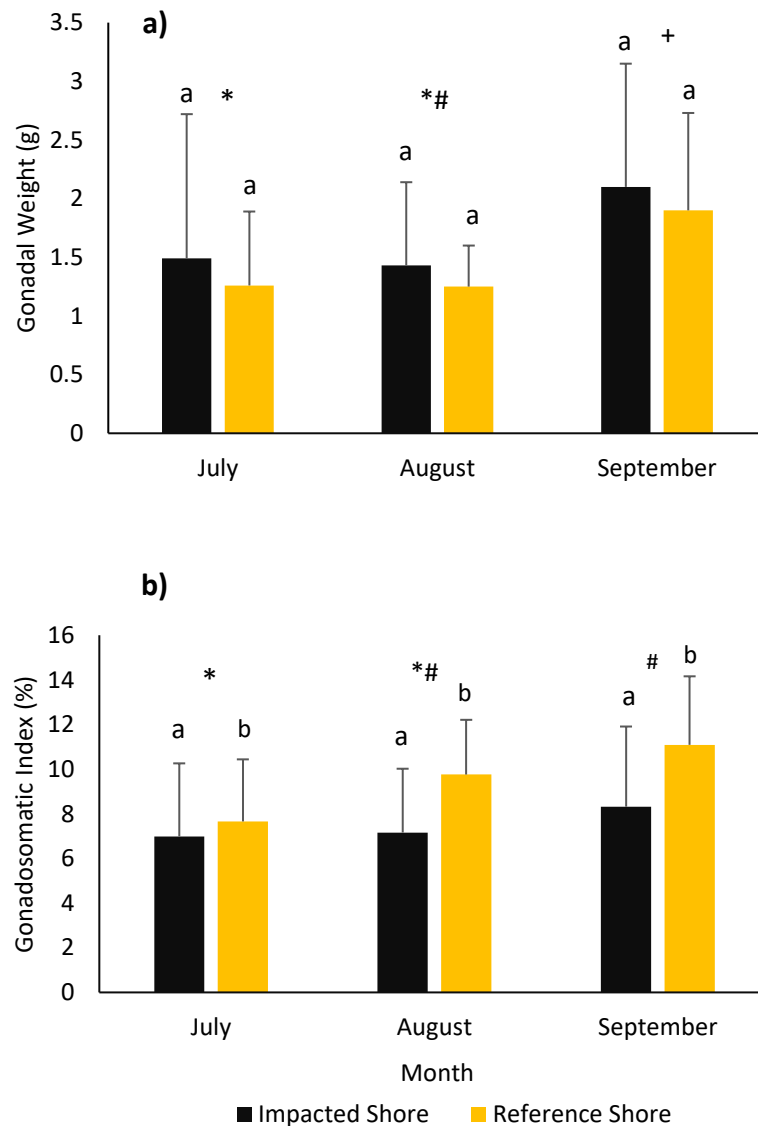
not statistically different from September (*Tukey HSD* test;  $p > 0.05$ ) (Figure 7b and Appendix I).



**Figure 7:** Total wet weight (WW) and diameter of the carapace (DC) average values of *Paracentrotus lividus* at the Impacted Shore (IS) and at the Reference Shore (RS) (Peniche, Portugal) collected in July, August and September of 2017, after an oil spill event. a) Total wet weight in grams (g); b) Carapace Diameter in centimetres (cm). All values were expressed as mean  $\pm$  standard deviation. Different letters (a and b) indicate significant differences between sites and different symbols (\*, #, +) indicate significant differences within months (*Tukey HSD* test;  $p < 0.05$ ).

In September, the gonadal weight of sea urchins reached the highest values (IS:  $2.10 \pm 1.05$  g; RS:  $1.90 \pm 0.83$  g), being statistically different from July (IS:  $1.49 \pm 1.23$  g; RS:  $1.26 \pm 0.63$  g) and August (IS:  $1.43 \pm 0.71$  g; RS:  $1.25 \pm 0.35$  g) (*Tukey HSD* test;  $p < 0.05$ ) (Figure 8a and Appendix I). Similarly, the highest records of GSI were observed in September (IS:  $8.32 \pm 3.59$  %; RS:  $11.09 \pm 3.07$  %), being statistically different from the month of July, in which the lowest values were recorded (IS:  $6.99 \pm 3.27$  %; RS:  $6.99 \pm 3.27$  %). The values observed in August (IS:  $7.16 \pm 2.86$  %; RS:  $9.76 \pm 2.45$  %) were

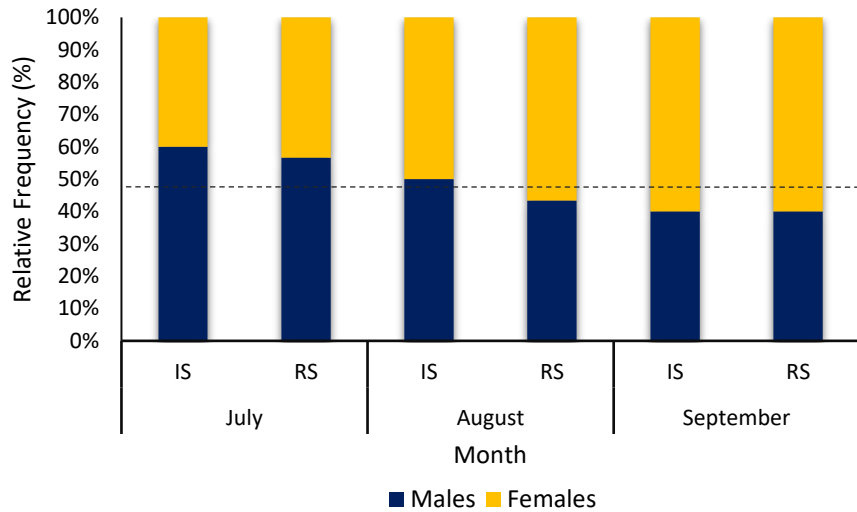
not statistically different from those observed in July and September (*Tukey HSD* test;  $p > 0.05$ ) (Figure 8b and Appendix I).



**Figure 8:** Gonadal Weight (GW) and Gonadosomatic Index (GSI) average values of *Paracentrotus lividus* at the Impacted Shore (IS) and at the Reference Shore (RS) (Peniche, Portugal) collected in July, August and September of 2017, after an oil spill event. a) Gonadal Weight (GW) in grams (g); b) Gonadosomatic Index (GSI) in percentage (%). All values were expressed as mean  $\pm$  standard deviation. Different letters (a and b) indicate significant differences between sites and different symbols (\*, #, +) indicate significant differences within months (*Tukey HSD* test;  $p < 0.05$ ).

The sex ratio of the individuals collected at the two sampling areas did not show statistically significant fluctuations over the months ( $X^2(2) = 3.513$ ;  $p > 0.05$ ), although occasional cases of 1:1 ratio deviations were identified. The relative frequency of females compared to males

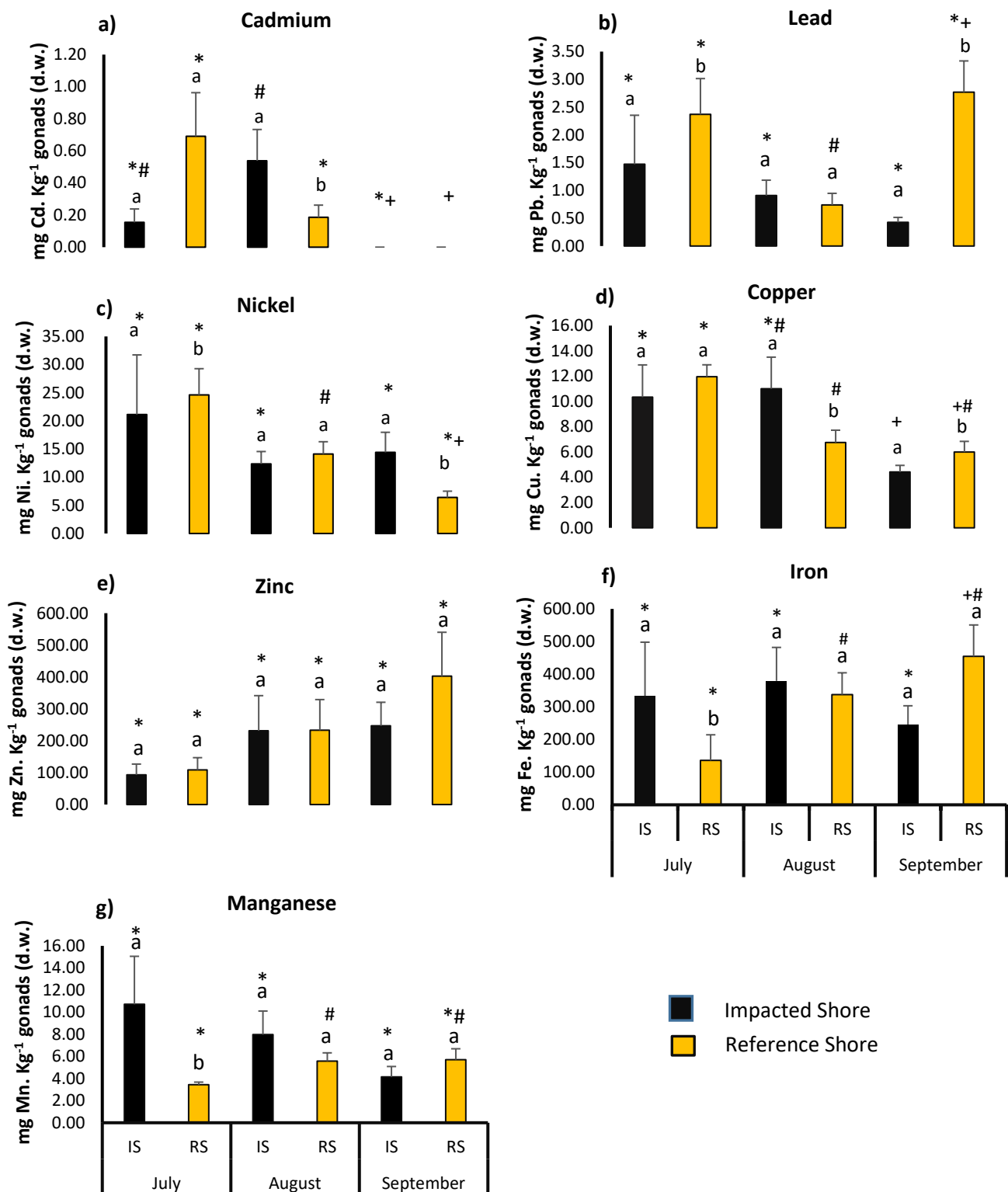
increased from July to September and this pattern was coherent between the 2 sampling stations (Figure 9).



**Figure 9:** Relative frequency of *Paracentrotus lividus* males and females at the Impacted Shore (IS) and at the Reference Shore (RS) (Peniche, Portugal) collected in July, August and September of 2017, following an oil spill event.

### 3.2. Presence of Metals

Concentrations of the seven metals were detected in *P. lividus* gonads from the two sampling sites during almost the entire period of study (July, August and September as shown in Figure 10). Among the essential metals studied in this research, Zn and Fe were the elements which exhibited the highest average concentrations ( $402.624 \pm 145.80$  mg/kg and  $454.70 \pm 101.99$  mg/kg, respectively), registered in September at the RS. By comparison, the lowest concentrations of both metals were found in individuals with average concentrations of  $93.14 \pm 35.75$  mg/kg for Zn at the IS and  $136.09 \pm 83.17$  mg/kg for Fe at the RS, both in July. Alternatively, regarding to the non-essential metals, the lowest concentrations were observed for Cd, which was not detected in any of the sampling stations in the month of September, and Pb with average concentrations of  $0.43 \pm 0.09$  mg/kg in September at IS. The highest concentrations of these metals, in contrast, were recorded in July at RS for Cd ( $0.69 \pm 0.29$  mg/kg) and in September at RS for Pb ( $2.77 \pm 0.60$  mg/kg).



**Figure 10:** Metal concentrations (in milligram per kilogram, dry weight) on the gonads of *Paracentrotus lividus* at the Impacted Shore (IS) and at the Reference Shore (RS) (Peniche, Portugal) collected in July, August and September of 2017, after an oil spill event. a) Cadmium b) Lead, c) Nickel, d) Copper, e) Zinc, f) Iron and g) Manganese. All values were expressed as mean  $\pm$  standard error. Different letters (a and b) indicate significant differences between sites and different symbols (\*, #, +) indicate significant differences within months (Tukey HSD test;  $p < 0.05$ ).

Results obtained from Mann-Whitney U test showed significant differences in the concentration of some metals studied between the different sampling stations in each month. In July, the concentrations of the essential metals Fe and Mn found in *P. lividus* gonads were statistically different between the two study sites, being significantly higher at IS ( $333.62 \pm 174.42$  mg/kg and  $10.72 \pm 4.60$  mg/kg, respectively) than the concentrations found at RS ( $136.09 \pm 83.17$  mg/kg and  $3.44 \pm 0.25$  mg/kg, respectively) (*Mann-Whitney test*; Fe:  $U = 57.500$ ; Mn:  $U = 35.000$ ,  $p < 0.05$ ). Still in July, Pb and Ni concentrations presented statistical differences between the rocky shores, being higher at RS ( $2.37 \pm 0.68$  mg/kg and  $24.62 \pm 4.94$  mg/kg, respectively) compared to IS ( $1.47 \pm 0.93$  mg/kg and  $21.13 \pm 44.93$  mg/kg, respectively) (*Mann-Whitney test*; Pb:  $U = 72.000$ ; Ni:  $U = 60.000$ ,  $p < 0.05$ ). In the month of August, among all metals studied, only Cu presented statistically different values between the sampling stations, presenting higher concentrations at IS ( $11.00 \pm 2.65$  mg/kg) compared to the concentrations found at RS ( $6.75 \pm 1.03$  mg/kg) (*Mann-Whitney test*  $U = 75.000$ ,  $p < 0.05$ ). Finally, in September, only the average values obtained for Pb and Ni were statistically different between the two rocky shores. For Pb, the highest average concentrations were observed at RS ( $2.77 \pm 0.60$  mg/kg) in comparison to IS ( $0.43 \pm 0.09$  mg/kg); in contrast, the highest average Ni concentrations were obtained at IS ( $14.42 \pm 3.77$  mg/kg) comparatively to RS ( $6.41 \pm 1.18$  mg/kg) (*Mann-Whitney test*; Pb:  $U = 35.000$  ; Ni:  $U = 76.500$ ,  $p < 0.05$ ) (Figure 10).

Kruskal Wallis tests performed indicated that the average contents of some analyzed metals presented significant variation over the sampling months in the two collection stations. At IS, the elements which showed statistically significant variations between months was the non-essential metal Cd and the essential metal Cu. In this sampling station, Cd concentrations detected in *P. lividus* gonads collected in July and August were  $0.15 \pm 0.09$  mg/kg and  $0.54 \pm 0.21$  mg/kg, respectively; between the mean levels found for these two months no significant differences were observed. However, as mentioned before, the analyses did not indicate the presence of Cd in the sea urchins' gonads in September, explaining the significant variation from August to September obtained (Appendix II). Regarding to Cu, the highest average concentrations were registered in August ( $11.00 \pm 2.65$  mg/kg) whereas the lowest concentrations were observed in September ( $4.42 \pm 0.55$  mg/kg). In July, the average content of this metal was  $10.34 \pm 2.70$  mg/kg; the

statistical analyzes identified significant differences between July and September and September and August for this element (Appendix II).

For the sea urchins from RS, Kruskal Wallis results showed that almost all the trace metal concentrations obtained showed significant variation between the months of sampling, except for the essential metal Zn. Regarding non-essential metals, it was possible to observe that the average values found for Cd in July ( $0.69 \pm 0.29$  mg/kg) and August ( $0.19 \pm 0.08$  mg/kg), were statistically different from the month of September (Appendix II). The Ni element, in turn, presented its highest average concentrations in July ( $24.62 \pm 4.94$  mg/kg) and its lowest average concentrations in September ( $6.41 \pm 1.18$  mg/kg); in August the average content of this metal was  $14.12 \pm 2.32$  mg/kg. Like the results of Cd, the values found for Ni exhibited significant variations between July and September and August and September. Lastly, regarding to Pb levels, the concentrations found in the sea urchin gonads collected in July ( $2.37 \pm 0.68$  mg/kg) and September ( $2.77 \pm 0.60$  mg/kg) were statistically higher than the average levels found in August ( $0.74 \pm 0.22$  mg/kg). On the other hand, with respect to essential metals, only Cu, Fe and Mn values showed statistically significant differences between the months. The average concentrations for these metals found in July ( $11.96 \pm 1.00$  mg/kg for Cu and  $136.09 \pm 83.17$  mg/kg for Fe) were statistically different from those found in September ( $6.0 \pm 0.89$  mg/kg and  $454.70 \pm 101.99$  mg/kg, respectively) and they were also different from August ( $6.75 \pm 1.03$  mg/kg and  $337.53 \pm 70.89$  mg/kg). For Mn, the sea urchins gonads had average concentrations that differed statistically between July ( $3.44 \pm 0.99$  mg/kg) and August ( $5.58 \pm 3.17$  mg/kg). In September the average concentration of this metal was  $5.70 \pm 4.19$  mg/kg, however it did not differ significantly from the other months (Appendix II).

Complementarily, the PERMANOVA results revealed that Cd concentrations found in *P. lividus* gonads were significantly influenced by the rocky shores and by the months of sampling ( $\rho$  perm = 0.014). More specifically, the Pairwise Method Comparisons tests indicated that the concentrations registered at the IS in the months of July and September ( $\rho$  perm = 0.043) and August and September ( $\rho$  perm = 0.003) were statistically different. In the same way, at the RS, the mean values observed in the months of July and September ( $\rho$  perm = 0.026) and August and September ( $\rho$  perm = 0.006) differed statistically.

The results of this study also showed that some of the trace elements analyzed, tend to bioaccumulate preferentially in the sea urchin gonads according to the gender of the individuals. The highest concentrations recorded in male gonads refer to the essential element Fe. In contrast, the highest mean values of trace metals found in females were also for an essential metal, Zn. The metals with the lowest concentrations recorded in the gonads of male sea urchins were Cd and Zn, while in females were Cd and Pb (Table I). In fact, Mann-Whitney U tests revealed that the average concentrations of Cd and Zn were the only ones that differed significantly and between genders. The results highlight the fact that Zn and Cd levels were significantly higher in female gonads ( $425.92 \pm 65.78$  mg/kg and  $0.47 \pm 0.12$  mg/kg, respectively) than in male gonads ( $12.67 \pm 8.87$  mg/kg and  $0.06 \pm 0.02$  mg/kg, respectively) (*Mann-Whitney test*;  $p < 0.05$ ).

In a more specific analysis, it was possible to evaluate the average values of trace metals obtained in gonads from the two sampling stations in each month. At IS, in the month of July, Cd was the only non-essential metal that presented average concentrations statistically different between genders, with female gonads exhibiting higher concentrations ( $0.31 \pm 0.16$  mg/kg) than male gonads (in which the presence of this metal was not detected, Table I). As for the essential metals group, Zn and Fe were the only ones that presented statistical differences in the mean values found in males and females. Zn exhibited a similar pattern to that observed for Cd, in which female gonads had higher mean concentrations ( $180.45 \pm 57.13$  mg/kg) than those found in male gonads ( $5.82 \pm 5.82$  mg/kg); however, unlike the pattern observed in Zn and Cd, females had significantly lower mean values ( $115.09 \pm 40.63$  mg/kg) than males ( $552.14 \pm 339.24$  mg/kg) for Fe. In August, only the elements Zn and Cd showed statistically significant differences between genders, with the same pattern found in the previous month, in which males (Cd:  $0.09 \pm 0.09$  mg/kg and Zn was not detected) had lowest concentrations than females (Cd:  $0.99 \pm 0.34$  mg/kg and Zn:  $462.70 \pm 207.62$  mg/kg). Finally, in September, only the average Zn concentrations were statistically different between female and male gonads.

Regarding to the average values of the elements observed at RS in July, besides Zn, statistically significant differences were detected in the average concentrations of Cu between genders, with females presenting lower values ( $11.14 \pm 0.63$  mg/kg as opposed to

12.78 ± 1.75 mg/kg found in males, Table I). In August, only the average concentrations found for Zn varied between genders, being higher in females (417.02 ± 179.78 mg/kg) than in males (49.20 ± 49.20 mg/kg). Lastly, in September, Zn stands out again among the essential metals, exhibiting statistically significant variation at the mean concentrations obtained between males (no presence detected) and females (805.28 ± 211.64 mg/kg). In the group of non-essential metals, on the other hand, only Ni showed statistically different mean values, following an opposite pattern to the one observed for the average concentrations of Zn, in which the female gonads presented lower values (4.06 ± 0.44 mg/kg) than those found in the male gonads (8.76 ± 2.04 mg/kg).

**Table I:** Concentrations of Cd, Pb, Cu, Zn, Ni, Fe and Mn in the gonads of males and females of *Paracentrotus lividus* collected in the Impacted Shore and in the Reference Shore at Peniche (Portugal) in July, August and September of 2017, following an oil spill event. All values were expressed as mean  $\pm$  standard error.

	July				August				September			
	Impacted Shore (mg/kg dry wt)		Reference Shore (mg/kg dry wt)		Impacted Shore (mg/kg dry wt)		Reference Shore (mg/kg dry wt)		Impacted Shore (mg/kg dry wt)		Reference Shore (mg/kg dry wt)	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
<b>Cd</b>	< LOD	0.31 $\pm$ 0.16	0.18 $\pm$ 0.13	1.20 $\pm$ 0.52	0.09 $\pm$ 0.09	0.99 $\pm$ 0.34	0.08 $\pm$ 0.07	0.29 $\pm$ 0.14	< LOD	< LOD	< LOD	< LOD
<b>Pb</b>	2.28 $\pm$ 1.83	0.66 $\pm$ 0.47	2.60 $\pm$ 0.59	2.14 $\pm$ 1.28	1.15 $\pm$ 0.59	0.70 $\pm$ 0.18	0.39 $\pm$ 0.18	1.09 $\pm$ 0.38	0.46 $\pm$ 0.15	0.39 $\pm$ 0.13	2.32 $\pm$ 0.83	3.22 $\pm$ 0.89
<b>Cu</b>	8.61 $\pm$ 5.02	12.07 $\pm$ 2.29	12.78 $\pm$ 0.71	11.14 $\pm$ 1.89	12.34 $\pm$ 5.35	9.67 $\pm$ 0.97	8.71 $\pm$ 1.75	4.79 $\pm$ 0.63	5.11 $\pm$ 0.90	3.73 $\pm$ 0.59	4.79 $\pm$ 0.87	7.20 $\pm$ 1.50
<b>Zn</b>	5.82 $\pm$ 5.82	180.45 $\pm$ 57.13	< LOD	217.25 $\pm$ 60.96	< LOD	462.70 $\pm$ 207.62	49.20 $\pm$ 49.20	417.02 $\pm$ 179.78	20.97 $\pm$ 20.97	472.85 $\pm$ 105.81	< LOD	805.28 $\pm$ 211.64
<b>Ni</b>	16.00 $\pm$ 7.87	26.27 $\pm$ 21.71	27.95 $\pm$ 8.54	21.28 $\pm$ 5.34	8.85 $\pm$ 1.24	15.88 $\pm$ 4.26	13.64 $\pm$ 2.36	14.59 $\pm$ 4.16	11.06 $\pm$ 3.47	17.78 $\pm$ 6.76	8.76 $\pm$ 2.04	4.06 $\pm$ 0.44
<b>Mn</b>	16.51 $\pm$ 8.96	4.93 $\pm$ 0.90	3.31 $\pm$ 0.24	3.57 $\pm$ 0.44	6.61 $\pm$ 3.36	9.35 $\pm$ 3.16	6.18 $\pm$ 0.82	4.97 $\pm$ 1.38	5.62 $\pm$ 1.65	2.68 $\pm$ 0.96	5.71 $\pm$ 1.17	5.69 $\pm$ 1.83
<b>Fe</b>	552.14 $\pm$ 33.92	115.09 $\pm$ 40.63	245.60 $\pm$ 161.58	26.58 $\pm$ 10.34	266.48 $\pm$ 127.93	492.76 $\pm$ 174.91	358.63 $\pm$ 74.99	316.43 $\pm$ 125.64	335.02 $\pm$ 76.42	156.93 $\pm$ 88.10	460.19 $\pm$ 119.01	449.22 $\pm$ 174.37

### 3.3. Histology

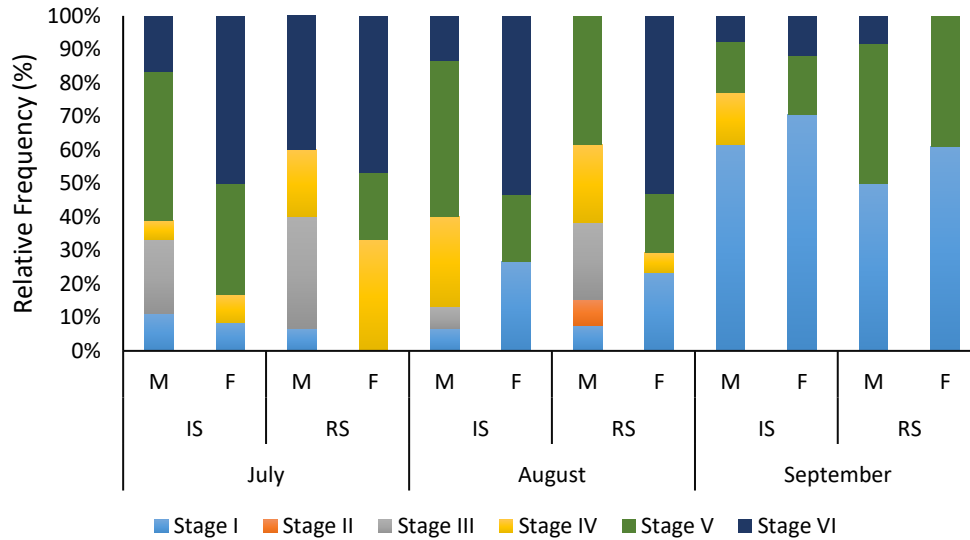
#### 3.3.1 Gametogenic cycle

During the histological analysis, all stages of gametogenic development were observed in the sea urchins collected. The relative frequencies of the different stages of the gametogenic cycle of *P. lividus* revealed to be heterogeneous over the three months of study, being statistically different ( $X^2(10) = 65.618$ ;  $p < 0.05$ ), so that at least two and up to five gonadal stages can be described in the monthly samples (Figure 11).

The individuals collected in July and August, in both sampling stations, were mostly at the end of the reproductive season (with spawning and post-spawning gonads in stages V and VI). Sea urchins in early growth usually appeared in September, although it was also possible to observe early-stage individuals in July and August. The Kruskal-Wallis test was performed to evaluate the influence of months at different gametogenic stages and it was observed that only Stage I showed statistically significant differences between the months of sampling (*Kruskal-Wallis*  $H_2 = 8.320$ ;  $p < 0.05$ ). In fact, stage I (initial growth), in which resorption of remaining gametes occurs, was predominant in September and statistically significant differences were detected between the relative frequency in September as opposed to the other months (*Pairwise Method Comparisons*;  $p < 0.05$ ). Statistically significant differences were not detected in the relative frequency of the other stages over the months (*Kruskal-Wallis test*;  $p > 0.05$ ). In September it was also possible to observe a considerable number of individuals in stages V and VI. At this point, sea urchins usually prepare to close their reproductive cycle and start a new one. It is also noted the low incidence of individuals in Stage II (Growth), which was detected only in August at the Reference Shore.

The analysis of the gametogenic stages showed a synchronized reproductive pattern between sexes (Figure 11). A clear and complete reproductive cycle was evident in both sampling stations and for both sexes, with a spawning peak in August. Spent or recovering gonads were observed in all sampling months, but in September almost the entire population was in these “immature” stages. Spawning periods were evidenced by a decrease in the amount of mature gametes in the gonads, with free spaces appearing between the gametes.

The differentiation of gametes and the presence of mature gonads were synchronous among males and females (Figure 11).



**Figure 11:** Variation on the gametogenic stages of *Paracentrotus lividus* at the Impacted Shore (IS) and at the Reference Shore (RS) (Peniche, Portugal) collected in July, August and September of 2017, after an oil spill event. I - Initial; II - Growth; III - Premature; IV - Matures; V - Posture; VI - Post-posture.

### 3.3.2 Identification of histopathological lesions

The analysis of histopathological lesions showed various levels of expression of pathological changes in the ovaries and in the testes of *P. lividus* from the two different sampling stations during July, August and September of 2017. Resorption of oocytes, dilation of ascinal wall, the presence of LLP in different gonadal compartments, hypertrophy of NPs or atrophy of NPs, were observed in at least one individual of each sampling station and each sampling moment analysed.

In July, the month in which the oil spill occurred, statistically significant differences were observed in the histopathological indices dilatation and atrophy of the gonads, with higher values in the Impacted Shore (1.63 and 0.77, respectively) than in the Reference Shore (0.90 and 0.20, respectively) (IHPA dilatation: *Independent samples test*  $t(58) = 2.89$ ;  $p < 0.05$ ; IHPA atrophy: *Mann-Whitney test*  $U = 334$ ;  $p < 0.05$ ). In contrast, in September, there was a significant difference between the LLP indices, being these values higher at RS (0.50) (*Mann-Whitney test*  $U = 348$ ;  $p < 0.05$ ; Table II).

A Kruskal-Wallis test and the One-Way ANOVA procedure were performed, depending on data normality, on each histological lesion indexes found to identify differences between the different months in the two sampling stations. At the Impacted Shore, IHPA resorption, IHPA dilation and IHPA LLP showed significant differences over the time (*Kruskal-Wallis*  $H_2 = 32.12$ ,  $p < 0.05$ ;  $H_2 = 40.52$ ,  $p < 0.05$ ;  $H_2 = 8.8$ ,  $p < 0.05$ , respectively). All these indexes followed the same pattern, being more evident in July and less evident in September. Comparisons by the Pairwise Method showed that there were statistically significant differences between the month of July compared to the month of August and September ( $p < 0.05$ ). In contrast, at the Reference Shore, only the hypertrophy (One-Way ANOVA: [F 2.87 = 3.39], MF: 3.878;  $p$ -value  $< 0.05$ ), dilation and resorption rates were significantly different over the months of study (*Kruskal-Wallis*  $H_2 = 14.06$ ,  $p < 0.05$ ;  $H_2 = 28.79$ ,  $p < 0.05$ , respectively).

Statistical tests were also performed to verify the existence of statistical differences between males and females in the two sampling stations in each month of collection. No significant differences were found between average IHPA dilatation, IHPA resorption and IHPA LLP in NPs of male sea urchins from both stations. The only histopathological indices which showed statistically significant differences between genders were IHPA hypertrophy in July at IS, in which there was a higher incidence of this lesion in males (1.30) than in females (0.47) (*Independent samples test*  $t_{(28)} = -2.50$ ;  $p < 0.05$ ), and IHPA atrophy in August, both at IS as in RS, in which the opposite was observed, with males having a lower incidence of this injury (0.07) compared to females (0.50) (IS: *Mann-Whitney test*  $U = 80$ ;  $p < 0.05$ ; RS: *Mann-Whitney test*  $U = 65$ ;  $p < 0.05$ ) (Table II).

**Table II:** Index of histopathological alterations (IHPA) in the gonads of *Paracentrotus lividus* collected in the different sampling areas: Impacted Shore (IS) and Reference Shore (RS) (Peniche, Portugal) in July, August and September of 2017, after an oil spill event. Different letters (a and b) indicate significant differences between sites, different roman numbers (i and ii) indicate significant differences between sexes and different symbols (#, \*, +) indicate significant differences within months (*Tukey HSD test*;  $p < 0.05$ ).

Month	Sampling station	Sex	IHPA dilat	IHPA reabs	IHPA llp	IHPA hyper	IHPA atro
July	IS	M	0.93 i <sub>a</sub>	1.03 i <sub>a</sub>	0.57 i <sub>a</sub>	1.30 i <sub>a</sub>	0.27 i <sub>a</sub>
		F	0.70 i <sub>#</sub>	0.77 i <sub>*</sub>	0.20 i <sub>*</sub>	0.47 ii <sub>*</sub>	0.50 i <sub>*</sub>
	RS	M	0.40 i <sub>b</sub>	0.63 i <sub>a</sub>	0.50 i <sub>a</sub>	0.70 i <sub>a</sub>	0.00 i <sub>b</sub>
		F	0.50 i	0.73 i	0.43 i	0.73 i	0.20 i
August	IS	M	0.37 i <sub>a</sub>	0.47 i <sub>a</sub>	0.33 i <sub>a</sub>	0.63 i <sub>a</sub>	0.07 i <sub>a</sub>
		F	0.13 i <sub>#</sub>	0.50 i <sub>#</sub>	0.17 i <sub>+</sub>	0.70 i <sub>#</sub>	0.50 ii <sub>*</sub>
	RS	M	0.23 i <sub>a</sub>	0.17 i <sub>a</sub>	0.43 i <sub>a</sub>	0.33 i <sub>a</sub>	0.07 i <sub>a</sub>
		F	0.20 i	0.63 ii	0.13 i	0.43 i	0.67 ii
September	IS	M	0.20 i <sub>a</sub>	0.07 i <sub>a</sub>	0.07 i <sub>a</sub>	0.70 i <sub>a</sub>	0.10 i <sub>a</sub>
		F	0.10 i <sub>#</sub>	0.00 i <sub>#</sub>	0.07 i <sub>#</sub>	0.60 i <sub>#</sub>	0.20 i <sub>#</sub>
	RS	M	0.07 i <sub>a</sub>	0.00 i <sub>a</sub>	0.40 i <sub>b</sub>	0.60 i <sub>*</sub>	0.17 i <sub>#</sub>
		F	0.10 i <sub>a</sub>	0.03 i <sub>a</sub>	0.10 i <sub>b</sub>	0.73 i <sub>a</sub>	0.30 i <sub>a</sub>

IHPA dilat = Index of membrane dilation; IHPA reabs = Index of sex cell resorption; IHPA llp = Index of lipofuscin-like pigments accumulation; IHPA hyper = Index of hypertrophy in nutritive phagocytes; IHPA atro = Index of atrophy in nutritive phagocytes.

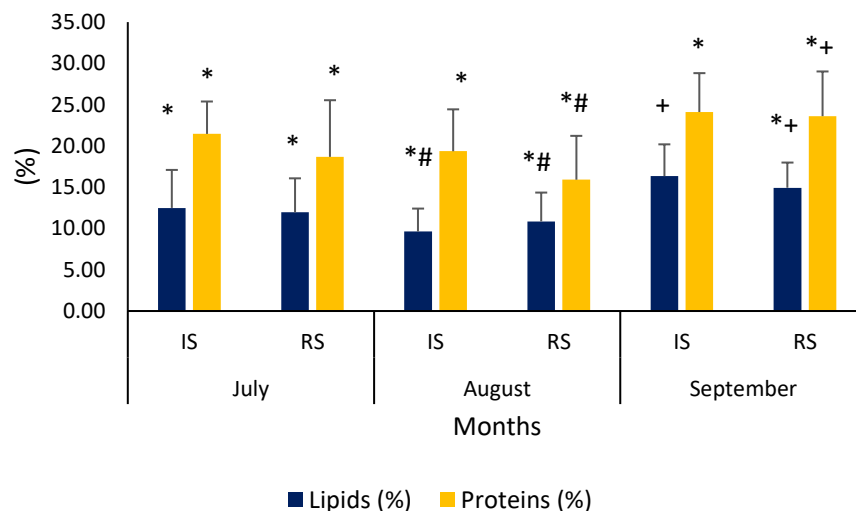
### 3.4. Biochemical Analyses

#### 3.4.1 Total lipids, proteins and fatty acids in the gonads of *Paracentrotus lividus*.

The analysis of the percentages of total lipids and proteins in the gonads of *P. lividus* showed, in general, that changes in lipid levels throughout the study period were less obvious than those of proteins (Figure 12). Moreover, statistically significant differences between the sampling stations were not detected between the average values of the molecules in question during the three months of study.

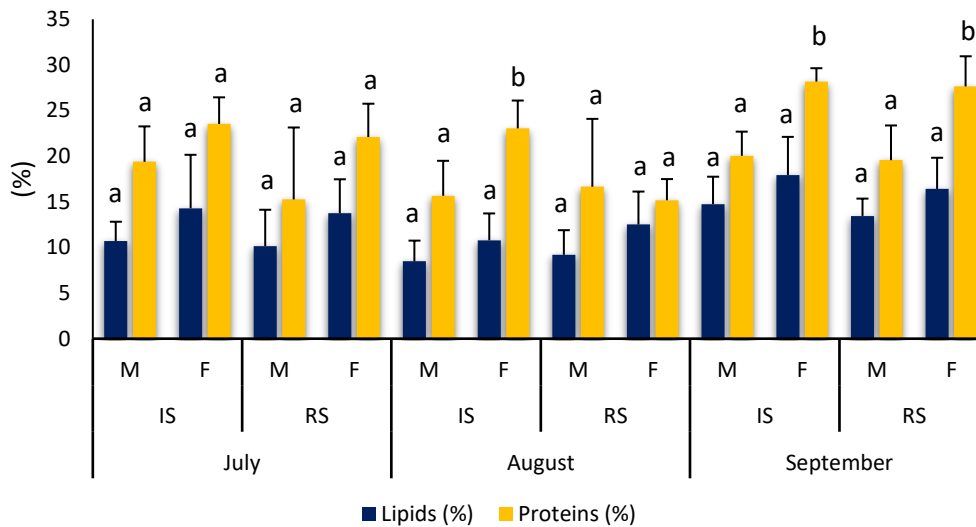
In the temporal context, a significant variation in lipid content of the gonads was observed at the Impacted Shore, while the protein values found were similar over the months (Appendix III). Regarding lipid levels, individuals collected in September presented significantly higher values ( $16.34 \pm 3.84\%$ ) when compared to the values from sea urchins collected in July ( $12.49 \pm 4.60\%$ ) and August ( $9.63 \pm 2.27\%$ ). Although statistically significant differences were not found in the values between months, protein levels showed basically the same distribution pattern over the months: the highest average percentages were obtained in September ( $24.11 \pm 4.70\%$ ), followed by July, where the protein content found in IS was  $21.45 \pm 3.92\%$  and, finally, its minimum level was observed in the month of August ( $19.35 \pm 5.07\%$ ; Figure 12).

At the RS, the values of both biochemical parameters seemed to follow the same variation pattern, with higher values in September and lower values in August (Figure 12). The lipid content values found in the sea urchins in each month were as follows: July ( $11.95 \pm 4.12\%$ ), August ( $10.85 \pm 3.49\%$ ) and September ( $14.93 \pm 3.05\%$ ). Similarly, the average values of proteins in the three months were: July ( $18.67 \pm 6.86\%$ ), August ( $15.91 \pm 5.31\%$ ) and September ( $23.60 \pm 5.41\%$ ). However, only August and September showed statistically significant differences for the two variables in RS (Appendix III).



**Figure 12:** Percentage of lipids and proteins in *Paracentrotus lividus* gonads at the Impacted Shore (IS) and at the Reference Shore (RS) (Peniche, Portugal) collected in July, August and September of 2017, after an oil spill event. Different symbols (\*, #, +) indicate significant differences within months (Tukey HSD test;  $p < 0.05$ ).

Mann Whitney tests were performed to identify significant variations in lipid and protein content found in *P. lividus* between males and females. The results indicated considerable differences in the protein content observed between the sexes of the individuals collected in August and September, in both sampling stations (Figure 13). The average values of protein content observed in July at the IS showed no statistical differences between sexes (*Mann-Whitney test*  $U = 6.00$ ;  $p > 0.05$ ). Nevertheless, in August and September at the IS, females had significantly higher protein values compared to males (August: *Mann-Whitney test*  $U = 3.00$ ;  $p < 0.05$ ; September: *Mann-Whitney test*  $U = 0.000$ ;  $p < 0.05$ ). In the RS, the average protein of the different sexes was not statistically different in July and August (July: *Mann-Whitney test*  $U = 7.000$ ;  $p > 0.05$ ; August: *Mann-Whitney test*  $U = 16.000$ ;  $p > 0.05$ ); but in September it presented a significant variation (*Mann-Whitney test*  $U = 0.000$ ;  $p < 0.05$ ). As for the lipid content, statistically significant differences between males and females were not found in the months of sampling (*Mann-Whitney test*;  $p > 0.05$ ).



**Figure 13:** Percentage of lipids and proteins in female and male gonads of *Paracentrotus lividus* gonads at the Impacted Shore (IS) and at the Reference Shore (RS) (Peniche, Portugal) collected in July, August and September of 2017, after an oil spill event. Different letters (a and b) indicate significant differences between males and females at each sampling station (Tukey HSD test;  $p < 0.05$ ).

The analysis of the fatty acids (FA) present in the gonads of both sexes revealed that polyunsaturated fatty acids (PUFA) were the dominant FA class, followed by saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) (Table III). The three main saturated fatty acids were myristic (C14), palmitic (C16) and stearic (C18) acids, although their levels, especially C14 and C18, were different between males and females. Higher percentages of

these fatty acids were found in the ovaries when compared to the testes. Six monounsaturated fatty acids (MUFA) were identified in the sea urchin's gonads: palmitoleic acid (C16:1), elaidic acid (C18:1 trans), oleic acid (C18:1 cis), gadoleic acid (C20:1n11), gondoic acid (C20:1n9) and erucic acid (C22:1n9). The main MUFA was gadoleic acid (C20:1n11) with values ranging between 4.73 and 6.98%, while the lowest percentage of MUFAs was erucic acid (C22:1n9) whose levels did not reach 1%. The palmitoleic acid (C16:1) percentage was very low and hardly exceeded 2%.

The two major polyunsaturated fatty acids (PUFA) in the ovaries and testes were arachidonic (C20:4n6) and eicosapentaenoic acid/EPA (C20:5n3) (Table III). Overall, the percentage of linoleic acid (C18:2n6), arachidonic acid (C20:4n-6) and EPA was higher in males than in females, while the two essential fatty acids alpha linolenic acid (ALA) and stearidonic acid (C18:4n3) showed higher levels in females. Eicosadienoic acid (C20:2n6) and gamma-linolenic acid (C18:3n6) did not present evident differences between males and females.

The analyzes using Mann-Whitney test indicated statistical differences in some of the fatty acids identified in the sea urchin gonads between the two sampling stations. In July, *P. lividus* specimens collected at the IS presented higher values of pentadecanoic acid (C15), stearic acid (C18), elaidic acid (C18:1 trans) and linoleic acid (C18:2n6) than the sea urchins collected from the RS (*Mann-Whitney test*, C15: U = 30.000; C18: U = 25.000; C18:1 trans: U = 23.000 and C18:2n6: U = 18.500,  $p < 0.05$ ). On the other hand, the values of EPA were significantly lower at IS as compared to RS (*Mann-Whitney test*, EPA: U = 34.000,  $p < 0.05$ ). In September, the alpha linolenic acid (ALA), stearidonic acid (C18:4n3), arachidonic acid (C20:4n6) and eicosadienoic acid (C20:2n6) were higher at the IS than at the RS (*Mann-Whitney test*; ALA: U = 32.000; C18:4n3: U = 35.000; C20:4n6: U = 37.000, C20:2n6: U = 26.500,  $p < 0.05$ ). The palmitic acid (C16) on the contrary, was more expressed in individuals collected at RS (*Mann-Whitney test*, C16: U = 37.000,  $p < 0.05$ ). Statistically significant differences were not identified between the percentages of fatty acids found in individuals on both rocky shores in August (*Mann-Whitney test*;  $p > 0.05$ ).

Mann-Whitney tests were also performed to verify the existence of statistical differences in the percentages found regarding the different FA identified in the sexes of the individuals

during the months of sampling in the two study sites. The results revealed that in July, males and females presented similar percentages of FA, without evident differences in the two sampling stations (IS and RS) (*Mann-Whitney test*;  $p > 0.05$ ). In August at the IS, myristic acid (C14), pentadecanoic acid (C15), and palmitoleic acid (C16:1) showed higher values in females than males, while eicosadienoic (C20:2n6) and erucic (C22:1n9) acids showed significantly higher percentages in male sea urchins as compared to females (*Mann-Whitney test*; C14:  $U = 5.000$ ; C15:  $U = 4.000$ ; C16:1:  $U = 3.000$ ; C20:2n6:  $U = 5.500$ ; C22:1n9:  $U = 5.000$ ,  $p < 0.05$ ). In September, females had significantly and statistically more palmitic acids (C16), elaidic acid (C18:1trans) and homo- $\gamma$ -Linolenic acid (C20:3n6) than males (*Mann-Whitney test*; C16:  $U = 5.000$ ; C18:1 trans:  $U = 4.000$ ; C20:3n6:  $U = 4.000$ ,  $p < 0.05$ ). In contrast, the percentages of arachidonic acid (C20:4n6) and EPA were evidently higher in males than in females (*Mann-Whitney test*; C20:4n6:  $U = 2.000$ ; EPA:  $U = 2.000$ ,  $p < 0.05$ ). At the RS, in August, females had significantly higher ALA values than males (*Mann-Whitney test*;  $U = 4.000$ ,  $p < 0.05$ ). In September, significant differences were also detected: females showed statistically higher values of myristic (C14), pentadecanoic (C15), alpha linolenic (ALA) and stearidonic (C18:4n3) acids than males; in contrast, higher percentages of stearic (C18) and arachidonic acid (C20:4n6) were observed in male gonads compared to females gonads (*Mann-Whitney test*; C14:  $U = 1.000$ ; C15:  $U = 2.000$ ; ALA:  $U = 2.000$ ; C18:4n3:  $U = 1.000$ ; C18:  $U = 1.000$ ; C20:4n6:  $U = 5.000$ ;  $p < 0.05$ ).

Sea urchins varied over the months in their gonadal content relative to some of the fatty acids identified in both rocky shores. At IS, significant statistical differences were identified in the values of the acids C14, C16:1, C18, ALA, C18:4n3, C20:4n6 and C22:1n9 between July and September and between August and September (*Kruskal-Wallis* C14:  $H_2 = 14.158$ , C16:1:  $H_2 = 11.721$ ; C18:  $H_2 = 18.677$ ; ALA:  $H_2 = 20.348$ ; C18:4n3:  $H_2 = 12.991$ ; C20:4n6:  $H_2 = 8.271$ ; C22:1n9:  $H_2 = 11.475$ ,  $p < 0.05$ ) (*Tukey HSD test*;  $p < 0.05$ ). Similarly, at RS, the collected animals had statistically different values of the acids C16, C16:1, C18 between July and September and between August and September (*Kruskal-Wallis* C16:  $H_2 = 7.389$ ; C16:1:  $H_2 = 11.766$ ; C18:  $H_2 = 15.910$ ,  $p < 0.005$ ) (*Tukey HSD test*;  $p < 0.05$ ). In addition, the values found for C18:1 trans in September were statistically different from the values found in July and August; and C22:1n9 differed statistically between July and September (*Kruskal-Wallis* C18:1 trans:  $H_2 = 7.738$  C22:1n9:  $H_2 = 7.288$ ,  $p < 0.005$ ; *Tukey HSD test*;  $p < 0.05$ ).

PERMANOVA procedures were performed to test the influence of the sampling stations and the sexes of the animals (individually and combined) and to test the influence of months and rocky shores (individually and combined) on the fatty acids studied in the animals (Tables IV and V). The results demonstrated that: (i) the different sampling stations and the sexes, individually, influenced the percentage of some FA's in the gonads; (ii) the two factors combined (sampling stations and sexes) only influenced the percentage of C20:3n6 in the gonads of the sea urchins (Table IV); and (iii) the different months and sampling stations, individually, influenced several acids, while the two factors combined (sampling stations and months) only influenced the acids C18:2n6 and ALA (Table V).

**Table III:** Gonad fatty acid composition (%) of *Paracentrotus lividus* males and females collected at the Impacted Shore and the Reference Shore (Peniche, Portugal) in July, August and September of 2017, after an oil spill event. Values are mean  $\pm$  standard deviation.

	July				August				September			
	Impacted Shore		Reference Shore		Impacted Shore		Reference Shore		Impacted Shore		Reference Shore	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
<i>Saturated (SFA)</i>												
<b>C14:0</b>	5.91 $\pm$ 1.70	6.54 $\pm$ 2.04	5.74 $\pm$ 3.05	7.94 $\pm$ 2.13	5.31 $\pm$ 1.43	7.67 $\pm$ 1.72	5.52 $\pm$ 2.01	6.89 $\pm$ 2.15	9.05 $\pm$ 2.81	10.59 $\pm$ 1.06	6.70 $\pm$ 1.44	11.13 $\pm$ 1.91
<b>C15:0</b>	0.81 $\pm$ 0.35	1.22 $\pm$ 0.38	0.48 $\pm$ 0.32	0.68 $\pm$ 0.35	0.51 $\pm$ 0.36	1.26 $\pm$ 0.70	1.42 $\pm$ 1.04	0.55 $\pm$ 0.09	3.31 $\pm$ 6.88	0.64 $\pm$ 0.10	0.53 $\pm$ 0.08	0.72 $\pm$ 0.14
<b>C16:0</b>	17.50 $\pm$ 1.24	15.73 $\pm$ 2.53	17.19 $\pm$ 4.49	19.21 $\pm$ 2.67	16.23 $\pm$ 2.93	20.36 $\pm$ 3.63	17.23 $\pm$ 1.93	17.49 $\pm$ 5.92	15.34 $\pm$ 7.20	19.99 $\pm$ 0.78	19.76 $\pm$ 3.07	22.00 $\pm$ 0.70
<b>C18:0</b>	11.55 $\pm$ 4.28	10.26 $\pm$ 3.11	8.57 $\pm$ 5.29	5.98 $\pm$ 1.58	9.74 $\pm$ 4.38	11.76 $\pm$ 9.27	10.21 $\pm$ 3.57	7.57 $\pm$ 2.63	4.78 $\pm$ 1.03	4.29 $\pm$ 0.42	5.50 $\pm$ 0.74	3.82 $\pm$ 0.89
<b>C21:0</b>	0.70 $\pm$ 0.25	0.63 $\pm$ 0.10	0.68 $\pm$ 0.27	0.94 $\pm$ 0.36	0.81 $\pm$ 0.19	0.74 $\pm$ 0.28	0.77 $\pm$ 0.19	0.71 $\pm$ 0.19	0.82 $\pm$ 0.13	0.85 $\pm$ 0.22	1.01 $\pm$ 0.20	1.00 $\pm$ 0.40
<i>MUFA</i>												
<b>C16:1</b>	0.92 $\pm$ 0.44	0.90 $\pm$ 0.78	0.63 $\pm$ 0.52	1.04 $\pm$ 0.59	0.43 $\pm$ 0.29	1.28 $\pm$ 0.71	0.61 $\pm$ 0.42	1.38 $\pm$ 0.97	1.55 $\pm$ 0.95	2.59 $\pm$ 0.26	1.52 $\pm$ 0.71	2.03 $\pm$ 0.20
<b>C18:1 trans</b>	2.23 $\pm$ 1.07	1.92 $\pm$ 0.68	1.37 $\pm$ 0.81	0.94 $\pm$ 0.20	1.23 $\pm$ 0.42	2.61 $\pm$ 1.76	2.19 $\pm$ 1.20	2.44 $\pm$ 1.27	1.14 $\pm$ 0.35	2.21 $\pm$ 1.06	1.30 $\pm$ 0.58	1.58 $\pm$ 1.38
<b>C18:1 cis</b>	4.18 $\pm$ 1.00	3.38 $\pm$ 1.22	4.02 $\pm$ 0.92	4.36 $\pm$ 0.95	3.67 $\pm$ 1.19	4.43 $\pm$ 0.81	3.96 $\pm$ 0.79	5.43 $\pm$ 2.24	3.86 $\pm$ 0.26	3.88 $\pm$ 0.41	4.55 $\pm$ 0.98	3.36 $\pm$ 1.62
<b>C20:1n-11</b>	5.56 $\pm$ 1.51	4.92 $\pm$ 1.61	6.13 $\pm$ 2.23	6.95 $\pm$ 1.67	6.67 $\pm$ 1.67	5.37 $\pm$ 1.28	5.96 $\pm$ 1.02	4.73 $\pm$ 1.71	6.78 $\pm$ 0.95	5.83 $\pm$ 0.58	6.98 $\pm$ 1.93	5.34 $\pm$ 0.82
<b>C20:1n-9</b>	5.92 $\pm$ 2.88	4.30 $\pm$ 1.53	5.61 $\pm$ 3.81	7.37 $\pm$ 1.19	4.75 $\pm$ 1.59	6.28 $\pm$ 2.16	4.71 $\pm$ 1.53	6.53 $\pm$ 1.46	5.46 $\pm$ 0.51	5.85 $\pm$ 0.81	6.23 $\pm$ 1.40	6.56 $\pm$ 1.06
<b>C22:1n-9</b>	0.41 $\pm$ 0.18	0.49 $\pm$ 0.15	0.54 $\pm$ 0.30	0.49 $\pm$ 0.15	0.59 $\pm$ 0.17	0.40 $\pm$ 0.06	0.65 $\pm$ 0.22	0.56 $\pm$ 0.19	0.67 $\pm$ 0.22	0.76 $\pm$ 0.14	0.81 $\pm$ 0.34	0.77 $\pm$ 0.18

**Table III (continued)**

	July				August				September			
	Impacted Shore		Reference Shore		Impacted Shore		Reference Shore		Impacted Shore		Reference Shore	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
<i><b>ω6 - PUFA</b></i>												
<b>C18:2n-6</b>	1.71 ± 0.68	1.57 ± 0.14	1.08 ± 0.44	0.81 ± 0.17	1.45 ± 0.48	1.10 ± 0.26	1.47 ± 0.62	1.71 ± 1.18	1.14 ± 0.21	1.35 ± 0.33	1.18 ± 0.27	1.07 ± 0.32
<b>C18:3n-6</b>	0.42 ± 0.13	0.46 ± 0.18	0.47 ± 0.26	0.57 ± 0.16	0.47 ± 0.17	0.56 ± 0.24	0.39 ± 0.14	0.39 ± 0.25	0.59 ± 0.20	0.47 ± 0.07	0.50 ± 0.14	0.49 ± 0.06
<b>C20:2n-6</b>	0.55 ± 0.31	0.65 ± 0.18	0.43 ± 0.25	0.49 ± 0.29	0.80 ± 0.26	0.48 ± 0.11	0.72 ± 0.20	0.51 ± 0.16	0.87 ± 0.30	0.68 ± 0.10	0.60 ± 0.19	0.48 ± 0.09
<b>C20:3n-6</b>	0.53 ± 0.47	1.15 ± 0.56	0.50 ± 0.31	0.38 ± 0.25	0.70 ± 0.44	0.61 ± 0.32	0.75 ± 0.47	0.44 ± 0.32	0.46 ± 0.23	0.82 ± 0.14	0.46 ± 0.21	0.51 ± 0.29
<b>C20:4n-6</b>	9.10 ± 3.00	8.85 ± 1.71	8.60 ± 3.45	9.23 ± 2.43	11.99 ± 3.42	8.52 ± 2.51	10.24 ± 2.45	8.86 ± 1.69	13.40 ± 1.90	10.69 ± 0.71	11.90 ± 2.45	8.69 ± 1.15
<b>ALA</b>	0.54 ± 0.31	0.67 ± 0.37	0.64 ± 0.54	1.00 ± 0.64	0.62 ± 0.29	0.95 ± 0.37	0.80 ± 0.49	1.74 ± 0.79	1.39 ± 0.38	1.84 ± 0.28	0.84 ± 0.24	1.56 ± 0.36
<i><b>ω3 - PUFA</b></i>												
<b>C18:4n-3</b>	3.81 ± 1.14	4.22 ± 1.30	3.26 ± 1.93	2.83 ± 1.37	3.29 ± 1.35	3.89 ± 2.23	3.41 ± 1.81	5.04 ± 2.45	5.57 ± 2.55	8.14 ± 0.69	2.50 ± 1.93	7.15 ± 0.74
<b>(EPA) C20:5n-3</b>	14.67 ± 3.04	14.55 ± 2.09	17.90 ± 7.60	20.31 ± 4.47	18.17 ± 5.18	14.58 ± 4.87	18.81 ± 5.20	17.53 ± 3.52	19.93 ± 2.14	15.61 ± 1.48	22.76 ± 4.41	17.94 ± 1.94
<b>ΣSFA</b>	36.47 ± 7.24	34.38 ± 6.34	2.65 ± 6.88	34.76 ± 7.55	32.59 ± 6.61	41.78 ± 8.13	35.16 ± 6.84	33.21 ± 6.91	33.30 ± 5.70	36.36 ± 8.17	33.50 ± 7.79	38.67 ± 9.01
<b>ΣMUFA</b>	19.22 ± 2.36	15.91 ± 1.83	18.28 ± 2.53	21.15 ± 3.14	17.33 ± 2.55	20.38 ± 2.34	18.07 ± 2.21	21.07 ± 2.39	19.45 ± 2.52	21.11 ± 2.05	21.40 ± 2.71	19.65 ± 2.27

**Table III (continued)**

	July				August				September			
	Impacted Shore		Reference Shore		Impacted Shore		Reference Shore		Impacted Shore		Reference Shore	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
<b>ΣPUFA</b>	31.34 ± 5.26	32.13 ± 5.12	32.88 ± 6.23	35.63 ± 7.06	37.49 ± 6.68	30.69 ± 5.14	36.59 ± 6.62	36.23 ± 6.02	43.34 ± 7.34	39.60 ± 5.78	40.74 ± 8.11	37.90 ± 6.24
<b>Σω3</b>	18.49 ± 7.68	18.77 ± 7.31	21.16 ± 10.35	23.15 ± 12.36	21.46 ± 10.52	18.47 ± 7.56	22.22 ± 10.89	22.57 ± 8.83	25.49 ± 10.15	23.74 ± 5.28	25.26 ± 14.33	25.09 ± 7.63
<b>Σω6</b>	12.86 ± 3.44	13.35 ± 3.27	11.72 ± 3.26	12.48 ± 3.51	16.03 ± 4.58	12.23 ± 3.18	14.37 ± 3.86	13.65 ± 3.29	17.85 ± 5.12	15.86 ± 3.97	15.48 ± 4.57	12.80 ± 3.24

**Table IV:** PERMANOVA tests results for the fatty acids content in gonads of *Paracentrotus lividus* collected in the sampling sites at Peniche (Portugal) during the study period. Only the variables that presented significant results were represented ( $\rho$ -value < 0.05). The effects of different sampling stations (Impacted Shore and Reference Shore) and the different sexes (Male and Female) were considered as factors. The following symbols stand for: df - degrees of freedom; MS - Mean Square.

PERMANOVA					
	Dependent variable	df	MS	F-statistic	$\rho$ -value
<b>Rocky Shore</b>	C20:2n6	1	0.3255	6.1837	0.014
	C20:3n6	1	0.7474	5.6844	0.019
	EPA	1	157.45	8.5377	0.005
<b>Sex</b>	C14	1	78.250	12.918	0.000
	C16	1	66.618	4.6519	0.032
	C16:1	1	6.3601	10.098	0.002
	ALA	1	4.317	13.817	0.000
	C18:4n3	1	44.376	9.365	0.004
	C20:1n11	1	12.165	5.155	0.030
	C20:2n6	1	0.234	4.457	0.036
	C20:4n6	1	53.898	8.184	0.006
<b>Rocky Shore*Sex</b>	C20:3n6	1	0.800	6.089	0.017

**Table V:** PERMANOVA and Pairwise Method Comparisons tests results for the fatty acids content in gonads of *Paracentrotus lividus* collected in the sampling sites at Peniche (Portugal) during the study period. Only the variables that presented significant results were represented ( $\rho$ -value < 0.05). The effects of different sampling stations (Impacted Shore and Reference Shore) and different months (July, August and September) were considered as factors. The following symbols stand for: df - degrees of freedom; MS - Mean Square.

PERMANOVA					
Factor	Dependent variable	df	MS	F-statistic	$\rho$ -value
<b>Rocky Shore</b>	C20:2n6	1	0.325	6.180	0.015
	C20:3n6	1	0.747	5.243	0.024
	EPA	1	157.45	8.357	0.004
<b>Month</b>	C14	2	68.892	12.942	0.000
	C16:1	2	8.475	17.651	0.000
	C18	2	192.05	12.705	0.000
	C18:4n3	2	36.784	8.859	0.000
	C21	2	0.241	4.081	0.023

**Table V (continued)**

<b>Factor</b>	<b>Dependent variable</b>	<b>df</b>	<b>MS</b>	<b>F-statistic</b>	<b>ρ-value</b>
	C20:4n6	2	29.896	4.522	0.014
	C22:1n9	2	0.4792	11.844	0.000
<b>Rocky Shore*Month</b>	C18:2n6	2	1.559	6.232	0.003
	ALA	2	1.279	4.74	0.010
<b>Pairwise Method Comparisons</b>					
<b>Factor</b>	<b>Dependent variable</b>	<b>Condition</b>		<b>ρ-value</b>	
<b>Month</b>		Comparison:			
	C14	July and September		0.003	
		August and September		0.000	
	C16:1	July and September		0.000	
		August and September		0.000	
	C18	July and September		0.000	
		August and September		0.000	
	C18:4n3	July and September		0.000	
		August and September		0.004	
	C21	July and September		0.017	
		August and September		0.017	
	C20:4n6	July and September		0.004	
	C22:1n9	July and September		0.000	
		August and September		0.001	
<b>Rocky Shore*Month</b>	C18:2n6				
Within Impacted Shore		July and September		0.0231	
Within Reference Shore		July and August		0.028	
	ALA				
Within Impacted Shore		July and September		0.000	
		August and September		0.000	

### 3.5 The influence of the presence of metals on the biological data

The effect of the presence of different contaminants on *P. lividus* gonads was evaluated by the Spearman correlation technique ( $\rho_{\text{spearman}}$ ) and significant relationships were found ( $p$  value  $<0.05$ ;  $\rho_{\text{spearman}} > 0.5$ ).

Regarding the biometric variables, the GW and the GSI of sea urchins were negatively and weakly correlated with Cd (Table VI). Similarly, there was a negative but moderate correlation between Pb and the individual's WW. Also, a negative and weak correlation was found between Ni and the total weight of the individuals. Cu showed a weak and negative correlation with the individual's DC. Finally, the GSI exhibited a positive but only weak correlation with Fe (Table VI). As expected, the GW and the GSI were positively and strongly correlated with each other. The DC of the animals showed a moderate correlation with the weight of the gonad; in turn, the weight of the gonad showed a positive, albeit weak, correlation with the individual's total weight. In addition, significant positive correlations were also observed between different metals: Zn and Cd, Cd and Cu, Pb and Mn, and lastly, the strongest among all observed, Fe and Mn (Table VI).

**Table VI:** Spearman correlation matrix ( $\rho_{\text{spearman}}$ ) for metal concentrations [Cd], [Pb], [Ni], [Zn], [Cu], [Fe] and [Mn], and biometric variables in gonads of *Paracentrotus lividus*, namely the diameter of the carapace (DC), total wet weight (WW), gonadal weight (GW) and gonadosomatic index (GSI). Significant correlations with  $p < 0.05$  are highlighted in bold (N = 96).

	DC	WW	GW	GSI	[Cd]	[Pb]	[Ni]	[Zn]	[Cu]	[Fe]	[Mn]
DC	1.0										
WW	<b>0.471</b>	1.0									
GW	<b>0.528</b>	<b>0.277</b>	1.0								
GSI	-0.003	-0.035	<b>0.765</b>	1.0							
[Cd]	-0.069	0.069	<b>-0.225</b>	<b>-0.236</b>	1.0						
[Pb]	-0.175	<b>-0.441</b>	-0.081	0.010	0.092	1.0					
[Ni]	-0.186	<b>-0.015</b>	-0.166	-0.092	0.192	0.125	1.0				
[Zn]	0.104	0.043	0.020	-0.026	<b>0.353</b>	0.007	-0.138	1.0			
[Cu]	<b>-0.207</b>	-0.144	-0.195	-0.076	<b>0.260</b>	0.139	<b>0.306</b>	-0.016	1.0		
[Fe]	-0.119	-0.106	0.106	<b>0.243</b>	-0.092	0.064	0.128	-0.190	0.034	1.0	
[Mn]	-0.130	-0.132	0.003	0.120	0.037	<b>0.224</b>	0.182	-0.129	0.146	<b>0.611</b>	1.0

In the context of the histological lesions, Cd showed a positive correlation with three of the five lesions studied in this work: IHPA dilation, IHPA atrophy and IPHA reabs (Table VII). Cd was positively and weakly correlated with the first two indexes and showed a positive but moderate relationship with IPHA reabs. Similarly, a weak positive correlation was observed between Cu with IHPA reabs. Zn, in turn, exhibited a poorly negative correlation with IPHA LLP, just as Fe exhibited a weakly negative correlation with IPHA reabs and IHPA hyper. Lastly, the metal Mn was negatively and weakly correlated with IPHA hyper.

Spearman's correlation technique was also applied between the concentrations of metals and lipid and protein contents. The results showed a weak and negative correlation between Ni and the protein content found in the animal's gonads (Table VIII). Moreover, Zn showed a moderate positive correlation with protein levels and a weak positive correlation with lipid levels. Finally, protein and lipid contents also showed a positive and weak correlation.

Lastly, regarding the interactions between metals and fatty acids, it was observed that the metals Cd, Zn, Cu, Ni and Mn exhibited correlations with FA's. Zn and Cu were the metals that showed the highest number of correlations with the FA's identified in sea urchins' gonads and C18 was the acid which presented the highest number of correlations with the different metals analysed in this research (Tables IX, X and XI).

In the group of Saturated Fatty Acids, it was observed that the C18 acid was positively and poorly correlated with the metals Cd, Cu and Mn. The metal Zn exhibited a weak but negative correlation with C18 acid and a weak positive correlation with pentadecanoic (C15) and C16 acids. In addition, Zn also exhibited a moderate positive correlation with C14 acids (Table IX). Regarding to the Monounsaturated Fatty Acid and Polyunsaturated Fatty Acid groups, it was noted that Cd exhibited weak and negative correlations with the acids C18:4n3, C20:4n6 and C22:1n9 acids. The results highlight several relationships between Zn and fatty acids: it was the element with the highest number of correlations with these molecules. A moderate positive correlation was observed between Zn and C16:1 and ALA acid. There was also a positive but weak correlation between Zn and C18:4n3 acid. Conversely, a negative and weak correlation was observed between Zn and C18, C20:1n11, C20:4n6 and EPA acids. A weak negative correlation was found between Cu and C16:1, ALA, and C20:2n6 acids; similarly, Cu exhibited a negative yet moderate correlation with C20:4n6 (Tables X and XI).

**Table VII:** Spearman correlation matrix ( $\rho$  spearman) for metal concentrations [Cd], [Pb], [Ni], [Zn], [Cu], [Fe] and [Mn], and the index of histopathological lesions in gonads of *Paracentrotus lividus* during the study period. Significant correlations with  $p < 0.05$  are highlighted in bold (N = 96). IHPA dilat = Index of membrane dilation; IHPA reabs = Index of sex cell resorption; IHPA llp= Index of lipofuscin-like pigments accumulation; IHPA hyper = Index of hypertrophy in nutritive phagocytes; IHPA atro = Index of atrophy in nutritive phagocytes.

	IHPA dilat	IHPA reabs	IHPA llp	IHPA hyper	IHPA atro	[Cd]	[Pb]	[Ni]	[Zn]	[Cu]	[Fe]	[Mn]
IHPA	1.0											
IHPA reabs	<b>0.546</b>	1.0										
IHPA llp	<b>0.249</b>	0.179	1.0									
IHPA hyper	<b>0.283</b>	0.115	-0.002	1.0								
IHPA atro	-0.004	0.137	0.046	<b>-0.523</b>	1.0							
[Cd]	<b>0.346</b>	<b>0.406</b>	0.155	-0.074	<b>0.288</b>	1.0						
[Pb]	0.002	0.150	-0.018	0.004	-0.106	0.092	1.0					
[Ni]	-0.064	0.046	-0.022	-0.031	-0.092	0.192	0.125	1.0				
[Zn]	-0.083	-0.030	<b>-0.218</b>	-0.099	0.160	<b>0.353</b>	0.007	-0.138	1.0			
[Cu]	0.094	<b>0.259</b>	0.042	0.081	-0.024	<b>0.260</b>	0.139	<b>0.306</b>	-0.016	1.0		
[Fe]	-0.188	<b>-0.275</b>	-0.037	<b>-0.217</b>	0.077	-0.092	0.064	0.128	-0.190	0.034	1.0	
[Mn]	-0.069	0.017	0.051	<b>-0.281</b>	0.135	0.037	<b>0.224</b>	0.182	-0.129	0.146	<b>0.611</b>	1.0

**Table VIII:** Spearman correlation matrix ( $\rho$  spearman) for metal concentrations [Cd], [Pb], [Ni], [Zn], [Cu], [Fe] and [Mn], and the percentages of lipids and proteins in the gonads of *Paracentrotus lividus* during the study period. Significant correlations with  $p < 0.05$  are highlighted in bold (N = 72).

	Lipids	Proteins	[Cd]	[Pb]	[Ni]	[Zn]	[Cu]	[Fe]	[Mn]
Lipids	1.0								
Proteins	<b>0.338</b>	1.0							
[Cd]	-0.110	-0.066	1.0						
[Pb]	0.140	-0.062	0.141	1.0					
[Ni]	-0.083	<b>-0.339</b>	0.082	0.097	1.0				
[Zn]	<b>0.362</b>	<b>0.493</b>	<b>0.307</b>	0.066	-0.175	1.0			
[Cu]	-0.170	-0.068	<b>0.289</b>	<b>0.235</b>	<b>0.277</b>	0.052	1.0		
[Fe]	-0.005	-0.045	-0.030	0.118	0.181	-0.112	0.029	1.0	
[Mn]	-0.108	-0.041	0.011	<b>0.237</b>	0.100	-0.061	0.128	<b>0.628</b>	1.0

**Table IX:** Spearman correlation matrix ( $\rho$  spearman) for metal concentrations [Cd], [Pb], [Ni], [Zn], [Cu], [Fe] and [Mn], and the percentages of Saturated Fatty Acids in the gonads of *Paracentrotus lividus* during the study period. Significant correlations with  $p < 0.05$  are highlighted in bold (N = 72).

	C14	C15	C16	C18	C21	[Cd]	[Pb]	[Ni]	[Zn]	[Cu]	[Fe]	[Mn]
C14	1.0											
C15	<b>0.248</b>	1.0										
C16	<b>0.621</b>	0.050	1.0									
C18	<b>-0.773</b>	-0.019	<b>-0.441</b>	1.0								
C21	<b>0.266</b>	<b>-0.263</b>	<b>0.250</b>	<b>-0.439</b>	1.0							
[Cd]	-0.177	0.141	-0.140	<b>0.240</b>	-0.197	1.0						
[Pb]	0.018	-0.214	0.104	-0.093	0.109	0.141	1.0					
[Ni]	0.004	-0.191	0.160	0.077	-0.040	0.082	0.097	1.0				
[Zn]	<b>0.416</b>	<b>0.277</b>	<b>0.298</b>	<b>-0.333</b>	-0.055	<b>0.307</b>	0.066	-0.175	1.0			
[Cu]	-0.182	0.164	-0.094	<b>0.245</b>	-0.169	<b>0.289</b>	<b>0.235</b>	<b>0.277</b>	0.052	1.0		
[Fe]	0.108	0.066	0.188	0.038	0.142	-0.030	0.118	0.181	-0.112	0.029	1.0	
[Mn]	-0.096	-0.033	0.022	<b>0.263</b>	-0.016	0.011	<b>0.237</b>	0.100	-0.061	0.128	<b>0.628</b>	1.0

**Table X:** Spearman correlation matrix ( $\rho$  spearman) for metal concentrations [Cd], [Pb], [Ni], [Zn], [Cu], [Fe] and [Mn], and the percentages of Monounsaturated Fatty Acid in the gonads of *Paracentrotus lividus* during the study period. Significant correlations with  $p < 0.05$  are highlighted in bold (N = 72).

	C16:1	C18:1 trans	C18:1 cis	C20:1n11	C20:1n9	C22:1n9	[Cd]	[Pb]	[Ni]	[Zn]	[Cu]	[Fe]	[Mn]
<b>C16:1</b>	1.0												
<b>C18:1 trans</b>	0.139	1.0											
<b>C18:1 cis</b>	<b>0.332</b>	0.113	1.0										
<b>C20:1n11</b>	0.029	<b>-0.465</b>	<b>0.307</b>	1.0									
<b>C20:1n9</b>	<b>0.468</b>	<b>-0.354</b>	<b>0.541</b>	<b>0.534</b>	1.0								
<b>C22:1n9</b>	0.149	-0.061	<b>-0.425</b>	-0.211	<b>-0.315</b>	1.0							
<b>[Cd]</b>	-0.178	0.036	0.106	-0.196	0.079	<b>-0.330</b>	1.0						
<b>[Pb]</b>	0.117	-0.158	0.183	-0.007	0.204	0.017	0.141	1.0					
<b>[Ni]</b>	-0.084	0.169	0.078	-0.049	-0.020	0.072	0.082	0.097	1.0				
<b>[Zn]</b>	<b>0.418</b>	0.154	-0.035	<b>-0.287</b>	0.180	0.013	<b>0.307</b>	0.066	-0.175	1.0			
<b>[Cu]</b>	<b>-0.271</b>	-0.102	-0.159	-0.207	-0.100	<b>-0.441</b>	<b>0.289</b>	<b>0.235</b>	<b>0.277</b>	0.052	1.0		
<b>[Fe]</b>	0.056	0.103	0.085	-0.006	0.031	-0.068	-0.030	0.118	0.181	-0.112	0.029	1.0	
<b>[Mn]</b>	-0.083	0.070	0.050	-0.035	-0.043	-0.072	0.011	<b>0.237</b>	0.100	-0.061	0.128	<b>0.628</b>	1.0

**Table XI:** Spearman correlation matrix ( $\rho$  spearman) for metal concentrations [Cd], [Pb], [Ni], [Zn], [Cu], [Fe] and [Mn], and the percentages of Polyunsaturated Fatty Acid in the gonads of *Paracentrotus lividus* during the study period. Significant correlations with  $p < 0.05$  are highlighted in bold (N = 72).

	C18:2n6	C18:3n6	C20:2n6	C20:3n6	C20:4n6	ALA	C18:4n3	(EPA) C20:5n3	[Cd]	[Pb]	[Ni]	[Zn]	[Cu]	[Fe]	[Mn]
<b>C18:2n6</b>	1.0														
<b>C18:3n6</b>	<b>-0.476</b>	1.0													
<b>C20:2n6</b>	0.051	<b>0.249</b>	1.0												
<b>C20:3n6</b>	<b>0.456</b>	-0.089	0.119	1.0											
<b>C20:4n6</b>	<b>-0.248</b>	<b>0.384</b>	<b>0.760</b>	<b>-0.260</b>	1.0										
<b>ALA</b>	-0.109	0.215	0.059	-0.140	0.121	1.0									
<b>C18:4n3</b>	<b>0.278</b>	-0.151	-0.023	<b>0.302</b>	-0.143	<b>0.520</b>	1.0								
<b>(EPA) C20:5n3</b>	<b>-0.252</b>	<b>0.621</b>	<b>0.323</b>	<b>-0.403</b>	<b>0.678</b>	0.048	<b>-0.359</b>	1.0							
<b>[Cd]</b>	-0.128	-0.033	-0.227	-0.024	<b>-0.249</b>	-0.067	<b>-0.256</b>	<b>-0.200</b>	1.0						
<b>[Pb]</b>	-0.166	0.071	-0.203	-0.076	-0.131	0.131	0.049	0.112	0.141	1.0					
<b>[Ni]</b>	0.053	-0.058	<b>-0.255</b>	0.043	-0.182	0.001	-0.008	-0.052	0.082	0.097	1.0				
<b>[Zn]</b>	-0.072	-0.005	-0.154	0.180	<b>-0.244</b>	<b>0.437</b>	<b>0.379</b>	<b>-0.274</b>	<b>0.307</b>	0.066	-0.175	1.0			
<b>[Cu]</b>	-0.105	0.011	<b>-0.318</b>	0.140	<b>-0.441</b>	<b>-0.293</b>	<b>-0.213</b>	-0.088	<b>0.289</b>	<b>0.235</b>	<b>0.277</b>	0.052	1.0		
<b>[Fe]</b>	0.063	-0.027	-0.015	-0.011	-0.068	0.089	-0.013	0.023	-0.030	0.118	0.181	-0.112	0.029	1.0	
<b>[Mn]</b>	0.005	-0.006	0.051	0.033	-0.072	-0.126	-0.042	0.019	0.011	<b>0.237</b>	0.100	-0.061	0.128	<b>0.628</b>	1.0

## **4. DISCUSSION**

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#### 4. Discussion

As already known, sea urchins are recognized to be very sensitive to changes in environmental conditions. This sensitivity is manifested by disparities in the reproductive cycle, decline in fertility and developmental disorders (Clarke, 1993; Auernheimer & Chinchon, 1997; Savriama *et al.*, 2015). These disorders affect the growth and physiology of these echinoderms; thus, changes in biometric parameters and biological indices in this species are integrated indicators that may reflect the effects of pollution and environmental quality of a given area, especially those exposed during the development of these animals (Warnau *et al.*, 1998; Chiarelli & Roccheri, 2014). This research aimed to evaluate the impact of a fuel oil spill on the coast of Peniche using wild populations of *P. lividus* as bioindicator. Variations in the presence of trace metals in the gonads of the sea urchins were monitored for 3 months after the oil spill and the possible influence of these elements on multiple functions of these animals and, consequently, of the population was evaluated.

The monitoring of the metal contents in *P. lividus* gonads showed temporal and spatial variations according to each one of the metals analyzed. These variations may be closely associated with the availability of metals in the environment, the physiological changes on the echinoderms and the environmental disturbances related to the quality of the environment in both sampling stations (Filipuci, 2011; Choi *et al.*, 2014; Rouane-Hacene *et al.*, 2018; De Zoysa *et al.*, 2018). Trace and non-biodegradable metals which are persistent in the environment for long periods can cause serious ecotoxicological problems. In addition, some toxic metals may mimic essential metals and thus gain access to important molecular targets. To a small extent, they enter organisms, for example by ingestion of contaminated food, and are bio-persistent pollutants that accumulate at the top of the food chain (Luoma & Rainbow, 2005; Scheifler *et al.*, 2006; Chiarelli & Roccheri, 2014). A study by Richir *et al.* (2008) used *P. lividus* as a bioindicator of trace elements along the Atlantic and Mediterranean coasts, comparing the concentrations of these elements in the digestive tract and in the reproductive structures of animals. It was demonstrated that all the elements investigated in the study - among which Cd, Pb, Zn, Cu - were measurable in the gonads and in the digestive wall of *P. lividus* and a relationship was observed between the gonadosomatic index, the diameter of the carapace and the pollutant content. According to Radenac *et al.* (1997), trace metal bioaccumulation by marine organisms is also closely

associated with the reproductive cycle of the animals, being maximum before reproduction and minimum after spawning when reserves are depleted during gametogenesis.

Temporal changes in the environment may have affected the physiological variations of the parameters observed in sea urchins on the present study (Guettaf *et al.* 2000, Soualili & Guillou, 2009). Within the scope of biometric parameters, the *P. lividus* population from the Impacted Shore presented significantly higher DC and WW values (DC:  $3.45 \pm 0.33$  cm; WW:  $21.08 \pm 5.71$  g) compared to individuals collected at the Reference Shore (DC:  $3.09 \pm 0.28$  cm WW:  $15.26 \pm 4.02$  g). In addition, these values varied significantly over the three months of sampling (July, August and September) at the IS, and in September they differed statistically from the other months, showing the highest values. These patterns of spatial and temporal variability can be better understood considering in addition to aspects of the sea urchin reproductive cycle progress, environmental and ecological factors fundamental to the maintenance and distribution of these organisms and their gametogenic cycle.

The variability of biometric parameters and somatic growth of sea urchin populations are strongly influenced by factors such as local hydrodynamics (Micheli *et al.*, 2005; Siddon & Witman, 2003; Bertocci *et al.*, 2014), food availability and quality, and gonadal development (Boudouresque & Verlaque, 2001). Turon *et al.* (1995) states that in a habitat exposed to low quality food, the maximum growth and size of *P. lividus* is generally lower than in a deeper habitat where food supply is not limited. The presence of smaller individuals in a given area, therefore, may be driven by adverse conditions that these populations face in terms of high-water dynamism and intermittent food availability. Considering this idea, it is important to highlight that RS is a rocky shore clearly more exposed to the hydrodynamic impact of the waves compared to the IS, which has a bay-shaped rocky coast and a low hydrodynamic impact. Wave exposure and hydrodynamics may influence algae abundance and, therefore, the availability and quality of food for the sea urchins inhabiting these two areas. Within this context, the species richness at the RS is possibly lower due to the great disturbance suffered by the organisms, requiring physiological and morphological adaptations, directing energy for the development of efficient protection, fixation and reproduction structures to withstand environmental stress (Satyam & Thiruchitrambalam, 2018); altogether this may explain why the sea urchins at RS were significantly smaller and lighter than the animals from the IS.

Although the animals were significantly larger and heavier in the IS, the GSI values observed in this area ( $7.49 \pm 0.72\%$ ) were lower than the values recorded in the RS population ( $9.50 \pm 0.28\%$ ). The results of the present study agree with those obtained by Turon *et al.* (1995) in northeastern Spain, which detected higher somatic growth in habitats characterized by low wave exposure. As in this study, the energy invested in reproduction followed the inverse pattern, and gonad production was higher in a changing habitat subjected to strong wave action (Lozano *et al.*, 1995). This explanation may also justify the fact that the average DC of the IS population is larger than at the RS. Sea urchins inhabiting the RS zone are exposed to the action of high waves, so they may favor reproduction over somatic growth in stressful situations.

The bioaccumulated metals detected in the gonads of *P. lividus* in the present study were observed in concentrations according to the following sequence Fe > Zn > Ni > Cu > Mn > Pb > Cd. Spatial and seasonal variations of metal concentrations have been previously studied along the northwest coast of Portugal (Reis, 2013; Gadelha *et al.*, 2019). The accumulation trends of these elements are believed to be associated with the dynamics of the coast, the nature of the sediment, the geology of the region and the surrounding anthropogenic pressure. Higher concentrations of metals have generally been found near urban areas, reflecting their proximity to anthropogenic sources (Reis, 2013; Rocha *et al.*, 2018). The concentrations of metals found in this study were generally lower compared to data from other areas such as the Algerian coast - Mediterranean Sea (Soualili *et al.*, 2008).

The interaction between the factors Rocky Shores and Months had a significant effect on the mean Cd values observed in *P. lividus* gonads. The concentrations recorded in both sampling stations (IS and RS) in August and September were statistically different for the interaction. Cd concentration at IS was significantly lower ( $0.15 \pm 0.08$  mg/kg) compared to the values at RS ( $0.69 \pm 0.27$  mg/kg) in July, and in August the opposite was observed, with sea urchins from RS ( $0.19 \pm 0.08$  mg/kg) exhibiting significantly lower average concentrations than those from IS ( $0.54 \pm 0.19$  mg/kg). According to Jakimska *et al.* (2011), the presence of the metal Cd in animal tissues may indicate both short- and long-term exposure, since absorption of this element is not controlled by active homeostasis.

Complementarily, this study showed that the concentrations of Pb and Ni exhibited statistical differences between the rocky shores in July and September, being, in most cases, higher at RS in relation to the IS, except for Ni that, in September, presented higher mean concentrations at IS compared to RS. Sany *et al.* (2013) affirms that chemical properties of metals, water and sediment are associated with other environmental factors such as physical variables of the external environment (atmospheric deposition, high seawater dynamics, changes in currents, and anthropogenic pollution load shifting); changes in these factors may affect the solubility and distribution of metals in the environment and sediments which consequently affect the toxicity of the metal in marine organisms. The dissipation of metals in animal tissues depends on the duration of exposure and the concentration of the element in the immediate environment. In addition, these differences may also be associated with the reduction or increase (depending on the month) of anthropogenic activities on the coast during this period, since from July to September the visit to The Berlengas Archipelago is allowed. In July and August, especially, boat traffic is intense, decreasing again in September, when the access to the Island closes again.

In both study sites, Zn was the most abundant element in the gonads of *P. lividus*, while Cd was always the scarcest one, indicating that *P. lividus* has a greater tendency to accumulate essential metals such as Zn, Fe, Cu and Mn. Also, the metal Fe showed a weak and significant positive correlation with the GSI, suggesting that the abundant presence of this trace element may influence the increase in the GSI of the individuals. Al Najjar *et al.* (2018) investigated possible effects of the season and different tissues on the accumulation of heavy metals in the sea urchin *Diadema setosum* (Leske, 1778) collected in the Gulf of Aqaba, Red Sea. As in the present study, Zn and Fe were found in high concentrations in soft organs such as gonads. These two elements show similar accumulation tendencies in the gonads, as both metals are biologically essential and play an important role as cofactors in enzymatic processes. The high concentrations of Cu, Fe and Zn in the gonads can therefore be attributed to the high metabolic activity characteristic of this compartment.

The results of this work also demonstrated that Zn and Cd concentrations were clearly and significantly higher in the gonads of females, at both the temporal and spatial scales. According to Watling and Watling (1976), Zn is an essential element for reproduction in several marine organisms. Also, high concentrations of Zn in female gonads relative to other

tissues during the reproductive period are common in many marine invertebrates (Orren *et al.*, 1980; Ahn *et al.* 2002). Despite that, sea urchin sperm also contains considerable amounts of Zn compared to Cu and Mn, which means that spermatogenesis also requires large amounts of Zn, although the demand for spermatogenesis is lower than for oogenesis (Richir *et al.*, 2008). In male animals, including sea urchins, Zn is essential for sperm motility and for the acrosome reaction (Clapper *et al.*, 1985). Studies suggest that the primary purpose of Zn to the ovary and testis is to provide essential supplies for oogenesis and spermatogenesis (Unuma *et al.*, 2007). Regarding the results for Cd concentrations, which differed statistically between males and females, they are in agreement with the findings of Soualli *et al.* (2008) for *P. lividus* from Algiers (Belgium). These authors determined the concentrations of various metals, such as Zn and Cd, in sediments and sea urchin gonads, and found that the most numerous larval abnormalities were observed in a location near Algiers, identified as highly polluted by Pb. Levels of the other metals, including Cd and Zn, were significantly higher in female gonads than in male gonads.

Cd levels showed a significant negative correlation with gonadal weight and GSI; this means that as Cd accumulation increases, the gonadal weight and the gonadosomatic index of these sea urchins tend to decrease. Cd is a highly toxic metal whose accumulation in the gonads can have serious implications, not only for species whose gonads are commercially harvested for human consumption, but also for problems associated with non-viable gamete production in these echinoids (Järup *et al.*, 1998; Chiarelli & Roccheri, 2014). Khristoforova *et al.* (1984) conducted a study evaluating the effects of chronic exposure of the mature *Strongylocentrotus intermedius* (Agassiz, 1864) to Cd at concentrations of 5, 1, 0.5 and 0.1 mg.l<sup>-1</sup>. The results indicated the formation of anomalous sex cells and production of unviable progeny. Moreover, in *S. intermedius*, Cd concentrations of 0.5 mg.l<sup>-1</sup> resulted not only in resorption of oocytes, but also in a temporary increase in oocyte number, the latter being an adaptive response to cadmium stress. There was also a decreased percentage of fertilized eggs in the sea urchins after 10 days of exposure, suggesting a poor quality of sexual products (Khristoforova *et al.*, 1984). Warnau *et al.* (1995b) found that the gonads, along with the *P. lividus* gut, accumulated the highest levels of Cd compared to the body wall and the skeleton.

Pb and Cu correlated negatively and significantly with the individual's total weight and the DC, respectively. Several studies corroborate this record, indicating a decline in the

growth of sea urchin populations exposed to polluting metals. Pb, particularly, accumulates in the bodies of aquatic organisms and of organisms that live in the soils and is a bio-persistent pollutant that accumulates at the top of the food chain. Pb-induced toxicity to marine invertebrates varies according to species and their life stage (Guillou *et al.*, 1995; Auernheimer & Chinchon, 1997; Rouane-Hacene *et al.*, 2018; Chiarelli *et al.*, 2019)..

Despite the differences between the two sampling stations, the biometric variables evaluated in this study followed similar variation patterns in both IS and RS over time; in other words, in most cases, the lowest average values of biometric variables were recorded in August and, in contrast, the highest average values were observed in September. In addition to being influenced by all the factors mentioned in the paragraphs above, this pattern also reflects the events that occur during the process of gametogenesis, in which sea urchin gonads grow and/or increase the number and size of germ cells in two distinct periods: before gametogenesis (when nutritional reserves are stored in nutritional phagocytes) and during gametogenesis (when the number and the size of gametes are larger).

*Paracentrotus lividus* has an often-contradictory reproductive biology, following different patterns as geographical location changes and important environmental parameters such as temperature, habitat, depth and food availability change as well (Byrne, 1990; Ouréns *et al.*, 2011). This species usually has a major spawning event that occurs in spring, driven by the increase in phytoplankton density and a less intense secondary event in the fall (Guettaf *et al.*, 2000; Antoniadou & Vafidis, 2009; Ouréns *et al.*, 2011). Mature stage ovaries and testes are usually present from late winter to early spring, although mature gonads may be present throughout the year (Tenuzzo *et al.*, 2012). According to some studies, *P. lividus* may have successive spawning events in the Mediterranean and these extensions in the main annual reproductive episode are related to latitude, thermal regime, food availability and hydrodynamics (Ouréns *et al.*, 2011; Gianguzza *et al.*, 2013).

The gametogenic cycle observed in *P. lividus* specimens collected for this research followed a recognizable pattern, characterized by six gametogenic stages. The histological analyzes showed that, at both rocky shores in July and August, the sea urchins were mostly at the end of the reproductive season, with spawning and post-spawning gonads in stages V and VI. This observation agrees with the results found by Raposo (2017), which

characterized the gametogenic cycle of *P. lividus* at Abalo beach from July 2015 to June 2016. As in the present study, in the month of July, the individuals presented mainly gonads in stages V and VI and; the stage V (posture) was more incident in July and August. Similarly, Gago *et al.* (2001) evaluated reproductive and growth variables in *P. lividus* periodically from June 1999 to July 2000 for specimens collected along the Portuguese coast in Cascais. Based on the results regarding the variation in the Gonadosomatic Index, the study concluded that population spawning occurred during spring and summer, and that food availability in the area affects the size of the gonads.

Given this set of previous researches, it is important to highlight that in September 2017, at both sampling stations, *P. lividus* was mostly in initial growth (Stage I), the first phase in which the remaining gametes are reabsorbed. In addition, it is also observed - in smaller percentages – the stages IV, V and VI at IS and the stages V and VI at RS. These findings are in agreement with the observations by Raposo (2017) who reported the predominance of stage I (reabsorption), in the sea urchins collected in September/2015 in which the reabsorption of remaining gametes occurs. At this time, sea urchins are preparing to close their reproductive cycle and start a new one. The results of the present research indicate, however, a slight delay (considering the presence of stage IV in males) in the reproductive cycle of the sea urchins at IS compared to those from RS. Furthermore, the present findings also highlight the low incidence of individuals in Stage II (growth), which was detected only in August at the Reference Shore. This stage is determined by the growth in size and number of pre-vitellogenic oocytes and is therefore a fundamental moment in the gametogenic process, where the proliferating gonads contain oocytes that are still in contact with the acini wall and are surrounded by nourishing phagocytes that fill the lumen.

Some studies indicate that invertebrates exposed to certain pollutants, especially PAHs and trace metals, may have a delay in the gametogenic cycle (Gauthier-Clerc *et al.*, 2002; Siah *et al.*, 2003). Gauthier-Clerc *et al.* (2002) conducted a study on the soft-shelled clams *Mya arenaria* in a Canadian municipality well known for its multiple contaminations following a downstream linear distribution. After histological analyzes to compare the energetic state and gametogenesis of the animals collected in a reference area and in a contaminated area, it was observed that the clams in the contaminated area exhibited limited nutritional condition and late gametogenesis, and associated these discrepancies with the

dysfunction of a process vitellogenin due to exposure to contaminants. Thomas (1988) states that organic contaminants (especially PAHs) such as metals and especially Cd act alone or synergistically as endocrine disruptors in estrogen-controlled metabolism or in estrogen metabolism itself by inducing important enzymes. Parallel to these informations, Walker (2013) states that oocytes are an important nutritional source for the next vitelogenic cycle and the variability in the amount of reserves present in the ovaries in stages IV and I and the amount of material accumulated during the growth stage undoubtedly influences the gonadal condition at the beginning of the gametogenic process. Therefore, the delay in the gametogenic cycle observed in sea urchins, especially at IS, may be related to direct exposure to contaminants during the spill and to the greater incidence of lesions observed in the gonads of these animals, which may also have delayed the process.

Fuel oil compounds, including trace metals, can cause histopathological effects on the gonads of marine invertebrates such as germ cell degeneration, gonadal tissue atrophy and inflammatory reactions (Aarab *et al.* 2006; Ortiz-Zarragoitia & Cajaraville, 2006; Gonzalez-Soto *et al.*, 2019). Although several studies address this issue in other marine invertebrates (*e.g.* bivalves), only a few studies have investigated the effects of chemical pollutants on gonadal tissue of sea urchins. It is therefore very useful to investigate the effects of trace metal exposure on sea urchin gonads using histopathological changes as biomarkers.

Using a histological parameter, Vashchenko *et al.* (2001) conducted a study where they examined two Russian aquatic regions using the sea urchin *Strongylocentrotus intermedius* as a bioindicator. Histological analyzes on the gonads of the organisms of two different regions revealed large histopathological changes in the supposedly most contaminated area, such as granular and hydropic dystrophy of oocytes, reabsorption, a marked decrease in the number of gametes and accumulation of lipofuscin in the cytoplasm of accessory cells. In the present study, it was possible to identify all the five histopathological lesions previously described (section 2.4), in individuals from both sampling stations during the three months of study. Most of the histopathological indices recorded did not vary significantly between the rocky shores, presenting similar values, except for the IPHA dilatation and the IPHA atrophy (1.63 and 0.77, respectively) which in July at the Impacted Shore were significantly higher than at the Reference Shore (0.90 and 0.20, respectively). Interestingly, the gonadosomatic index of sea urchins this month was significantly lower at the Impacted Shore

compared to the Reference Shore. A histopathological study developed by Schäfer (2009) with *P. miliaris* showed that after exposure to a sublethal concentration of phenanthrene - a polycyclic aromatic hydrocarbon present in the oil - severe ovarian lesions were observed and, as noticed in the present study, the size of the gonad and the gonad index of sea urchins was significantly reduced, designating atrophy. In addition, the acinal structure was disorganized, mainly due to degenerating oocyte aggregates. This disorganization completely compromises the progress of the oogenesis process, as the migration of mature oocytes towards the final lumen is hampered. Similarly to the present study, Schäfer (2009) also recorded a significant reduction in the gonadal index in those sea urchins.

In September the IPHA LLP values were significantly higher in the Reference Shore (0.50) as opposed to the Impacted Shore (0.13). Normally, in the post-spawning phase of the gametogenic cycle or under unfavorable environmental conditions such as temperature change and presence of pollutants, the resorption processes characteristic of this stage induce the accumulation of yellow pigments in the NP cytoplasm or in different compartments of the gonad (Varaksina 1985; Syasina 1991; Vaschenko *et al.*, 2012). Schäfer (2009) states that the lipofuscin content in sea urchin gonads is closely associated with the reproductive stage of the animals, with the highest levels found in partially spawned and spawned individuals. The present work corroborates Schäfer's (2009) argument, since higher IPHA LLP in both sampling stations were recorded in July, when sea urchins were mostly in the stages V and VI of gametogenesis.

Over time, at the Impacted Shore, the IPHAs resorption, dilation and LLP varied significantly. All these indices followed the same pattern, showing higher values in July and decreasing until September. Miranda *et al.* (1999) suggested that in the final process of gametogenesis, lipofuscin that is developed and accumulated throughout the process is released from the gonads. Otherwise, larger animals that had already undergone gametogenesis in previous years would have a higher lipofuscin content than the smaller and younger ones. As lipofuscin levels decrease after spawning, it was assumed that lipofuscin is released during the recovery period. This reasoning could explain the pattern observed at the Impacted Shore not only for lipofuscin, but also for the reabsorption and dilation rates of the acinal membrane. At the Reference Shore, besides the IPHAs dilatation and resorption, IPHA hypertrophy was also significantly different over the months of study; this

last one presented a different pattern of variation than at the Impacted Shore, showing higher values in July and lower in August, rising again in September.

The only histopathological indexes with statistically significant differences between genders were IPHA hypertrophy in July at the Impacted Shore and IPHA atrophy in August at the two sampling stations. It is possible that the sex-specific differences in histopathological and biochemical parameters in *P. lividus* gonads are related to the specific function of gonadal tissue and reflect adaptive gamete mechanisms for different life spans. In this regard, it should be mentioned that in invertebrates, sperm are often more susceptible to chemical stress than eggs (Au *et al.*, 2001; Caldwell *et al.*, 2004; Fitzpatrick *et al.*, 2008).

The presence of contaminating metals in the gonads of *P. lividus* at both sampling sites probably affected the gonads and triggered the histopathological disturbances recorded in this work, which was corroborated with the correlation analyzes. As expected, since Cd is a highly toxic metal, Cd exhibited positive correlations with the IHPAs reabsorption, dilation and atrophy. Migliaccio *et al.* (2015) evaluated the effects of Cd and Mn in adults of *P. lividus* and their offspring, and concluded that both metals differentially impair the fertilization process of treated sea urchins, causing changes in reproductive status and affecting nitric oxide (NO) production in the ovaries. A detailed progeny analysis showed a high percentage of abnormal embryos, associated with an increase in endogenous NO levels and variations in transcriptional expression of various genes involved in stress response, skogenesis, detoxification and multiple drug efflux processes. Also, the metal Cu exhibited a positive correlation with IHPA reabsorption. Although Cu is an essential metal for all eukaryotic organisms, it can reach toxic levels in aquatic environments (Bryan & Langston, 1992; Stohs & Bagchi 1995; Zorita *et al.*, 2006). Sources of Cu contamination in the marine environment vary and include discharges of Cu mines as well as Cu leaks from ship-based Cu antifouling paints (Zorita *et al.*, 2006; Leon & Warnken, 2008). During exposure to Cu hydroquinone, it is suspected that reactive oxygen species may be formed, causing cytotoxicity and DNA damage (Stohs & Bagchi, 1995). On the other hand, Zn, Fe and Mn correlated negatively with IHPAs LLP, reabsorption and hypertrophy, respectively, suggesting that these essential metals play an important role in curing these lesions, since as their gonad content increases, histopathological indices decrease. Zinc, in particular, is essential for cell proliferation and differentiation, as many gene-regulating enzymes and

proteins require zinc for their function; In addition, Zn is a prerequisite for chromatin structure (Coleman, 1992; Valle & Auld, 1990). Several genes encoding these zinc proteins have been found in the sea urchin *S. purpuratus* genome, and their expression has been investigated during embryogenesis (Materna *et al.*, 2006).

It is already well understood that the biochemical aspects of the gonads are specifically influenced by the gametogenic stages of their development. However, one must also consider that the gonads are not only a reproductive organ, but also an important compartment of energy and nutritional reserves (Hughes *et al.*, 2005; Siliani *et al.*, 2016). In this study, the biochemical composition of *P. lividus* gonads showed a clear temporal pattern of variation. These nutritional variations are generally associated with the annual gonad maturation cycle, more precisely, the nutrient storage and gametogenesis processes. Typically, in temperate areas, echinoderms have a very similar reproductive pattern; in the fall, when the temperature drops, the growth of the gonad occurs, characterized by the replenishment of nutrient reserves in nutritive phagocytes, which explains the peak levels of proteins and lipids. Subsequently, phagocytes accumulate the biochemical components that will be mobilized in the developing gametes (eggs and sperm). Thus, with the onset of gametogenesis between winter and spring, nutrient content in the gonads decreases and reaches minimum levels during spawning when the gonads are depleted (Walker *et al.*, 2013).

In this study, the observed trend in lipid content and total protein content over the three months was characterized by a peak in late summer (September), during the gonad growth (Stage I). Minimum levels, in turn, were reached at the beginning of the spawning season in August when the gonads were mainly in the posture stage (stage VI). A significant variation in protein content was found over the three months. In addition, the comparison between the seasonal variation in lipid content and the gametogenic cycle suggests that lipid uptake in sea urchin gonads is favoured during the advanced stages of gametogenesis, since premature (III) and mature (IV) stages were present mainly in July and August.

In both study sites, a significant variation in the lipid content of the animals was observed over the months: the individuals collected in September presented higher values (IS:  $16.34 \pm 3.85\%$ ; RS:  $14.93 \pm 3.05\%$ ) when compared to the sea urchins collected in July (IS:  $12.49$

$\pm 4.60\%$ ; RS:  $11.95 \pm 4.12\%$ ) and August (IS:  $9.63 \pm 2.27\%$ ; RS:  $10.85 \pm 4.12\%$ ). Lipids play an important role in the structural elements of cell membranes and are generally more stable compared to proteins and carbohydrates (Marsh & Watts, 2013). According to Rocha *et al.* (2019), total lipid content throughout the year is characterized by a peak during the warm season, a minimum in the cold season, while the inflection point is observed between October and November.

For protein levels, the same distribution pattern throughout the months was observed at both sampling stations (IS and RS), although statistically significant differences were not found between the months, except between August and September at RS. The minimum level was observed in August, while in September the gonads reached the peak of protein values. According to Arafa *et al.* (2012), the maximum levels of protein content generally agree with the maximum levels of individuals' gonadosomatic index. This was also observed in the present study, with September presenting the highest GSI values at RS ( $11.09 \pm 3.07$ ); this is because there is evidence of protein requirements during the gametogenic process. Protein content is related to the reproductive cycle of the gonad, showing important levels before spawning. In general, energy is stored before gametogenesis, when food is abundant, and used later in the production of gametes with high metabolic demand.

Zn exhibited a positive correlation with both lipid and protein contents, meaning that this essential metal plays a key role in the production of these molecules. Also, Zn concentrations in the ovaries of *P. lividus* were several times higher than in the testes. This was consistent with data from other species of sea urchins and, according to Unuma *et al.* (2007), appeared to be caused by sex-specific differences in the metabolism of the main yolk protein (MYP), a transferrin superfamily protein that carries Zn, essential for gametogenesis. In the other hand, a negative correlation between Ni and protein levels in the sea urchin gonads was observed, suggesting that the higher accumulation of this metal in the gonads, the lower the protein content in these compartments. Geraci *et al.* (2004) carried out a study with heavy metals (Ni, Pb and Cd) in embryos of *P. lividus*, in which they observed that these elements are capable of triggering different responses to cellular stress, depending on the properties of the element and the duration of treatment. The study used a family of proteins as HSP70 cell markers (HSP70/72) and found that continuous fertilization treatment with toxic metals produces structurally irregular late embryos, which then degenerate.

Some marine animals such as sea urchins, characterized by indirect development and planktonic larvae, provide larval development through nutrients supplied within the egg. The size, growth and survival of early juveniles are strongly driven by the quality of these nutrients accumulated and stored by the larvae (Vařtilingon *et al.*, 2001; Pechenik, 2006; Pernet *et al.*, 2006). Maternal supply of nutrients, including essential fatty acids, is therefore an important aspect of normal embryo development and plays an important role in offspring performance.

The characterization and quantification of fatty acids (FA) in *P. lividus* gonads performed in this work agrees with other biochemical studies conducted with the species (Martinez-Pita *et al.*, 2010; Carboni, 2013; Rocha *et al.*, 2019). As often happens, polyunsaturated fatty acids (PUFA) were the dominant class of FA, followed by saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). Higher percentages of these FAs were found in the ovaries compared to the testes. It is generally recognized that FA compositions from sea urchin gonads reflect dietary intake, although reproductive status may alter the relative abundance of FA in *P. lividus* (Hughes *et al.*, 2005; Martinez-Pita *et al.*, 2010). In addition, environmental factors such as water temperature can also modulate FA composition in marine organisms (Boudouresque & Verlaque, 2001; Martinez-Pita *et al.*, 2010).

PUFAs such as docosahexaenoic acid (DHA, 22:6n3), eicosapentaenoic acid (EPA, 20:5n3) and arachidonic acid (ARA, 20:4n6) are identified as essential components of all cells' membranes and of tissues and play important roles in physiology, growth and ontogenesis (Van der Merwe *et al.*, 2012). In addition, PUFAs also play important roles in the inflammatory response and, consequently, in metabolic disorders (Montes *et al.*, 2013). Sanina & Kostetsky (2002) state that there is an increase in PUFAs when temperature values decrease to maintain optimal membrane fluidity. This observation agrees with the results obtained for PUFAs in the present study, considering that the highest values found for this FA class were registered in September, when summer ends, and the temperature starts to decrease. Additionally, there are studies demonstrating the association of this FA class with the regulation of cortisol production by modulating corticotropin-stimulated inter-renal cells in fish species, suggesting a relationship with stress responses (Ganga *et al.*, 2006).

Notwithstanding the above regarding PUFA's, the EPA values found in the sea urchin gonads in July were significantly lower at the Impacted Shore (14.61%) compared to the Reference Shore (19.10%). This result may indicate some deficit regarding the synthesis of this essential compound for sea urchin gonadal development. According to Mai *et al.* (1996), the proportions of EPA are a reflection of the presence of macroalgae in the diet of sea urchins, since this fatty acid is found in macroalgae species such as *Laminaria digitata*; that is, high levels of EPA in the gonads may reflect preferential algae intake by IS urchins, since EPA can be found in concentrations of up to 50% of the total FA content in some macroalgae species (Rocha *et al.* , 2019).

In an overview, the Impacted Shore had significantly higher values of C15, C18, C18:1 trans and C18:2n6 compared to the Reference Shore. Barberá *et al.* (2011) found in their study that fatty acid composition varied among sampling sites and suggested that this composition reflected the availability of food resources at the locations where sea urchin populations were collected, but also possible dietary adaptations. According to Mao *et al.* (2015) a relatively calmer and lakeside environment provides favorable conditions for algae growth and, as discussed earlier, the Impacted Shore is characterized by low hydrodynamic impact and, following this reasoning, is an area with a greater abundance of algae. In addition, gonadosomatic index values in this site were also lower as in the study by Barberá *et al.* (2011). Still in this reasoning, Cook *et al.*, (2007) points out that the gonadal development also is a determining factor in targeting fatty acid profile. This may justify the higher levels of these fatty acids at IS.

In August, statistically significant differences were not identified between the percentages of fatty acids found in the gonads of individuals collected in the two sampling stations. However, in September, the FAs C18:4n3, C20:4n6, C20:2n6 and ALA were statistically more representative at the Impacted Shore compared to the Reference Shore, while C16 values were statistically higher at the Reference Shore. According to Rezanka & Sigler (2009), C16 is an important component of the lipid fraction in the gonads of some sea urchins and is especially abundant in algae and sediment; even so, it cannot be considered a valuable trophic marker as it is present in abundance in many organisms. Similarly to the present study, Barberá *et al.* (2011) found that C16 was present in smaller proportions in the

gonads of *Spatangus purpureus* collected on bare sand bottoms and suggested that this result may be related to the low availability of algae in this site. Palmitic acid C16 is synthesized in plant chloroplasts by synthesis of malonyl CoA, and usually biosynthesizes C18:2n6 and C18:3n3 in the first instance and subsequently to C20:4n6, C20:5n3 and C22:6n3 (these last two were not identified in the present study). These FAs are extremely important for sea urchin tissues and have structural functions even when undetected in the diet. Sea urchins are unable to synthesize any of these fatty acids, and, therefore, it is suggested that these compounds are synthesized by sea urchins from lower fatty acid precursors (Takagi *et al.*, 1980; Cook *et al.*, 2000; Liyana-Pathirana *et al.*, 2002).

A recent study by Rocha *et al.* (2019) evaluated the effects of sex, season and gametogenic cycle on the FA composition of male and female *P. lividus* gonads in wild populations on the Portuguese coast and observed that the FA composition in the gonads clearly oscillated during the months. All individuals generally presented PUFA, MUFA and SFA concentrations in November, when the gonads are more nutrient filled, since they were in one of the growth stages (stage II). Although in the present research only the summer months of 2017 were studied, it is possible to make comparisons considering the gametogenic stages. The C14, C16, C16:1, ALA, C18:4n3, C20:4n6 and C22:1n9 FAs identified in the present study showed, as in the study by Rocha *et al.* (2019), higher values in September, when most individuals were in the stage I of the gametogenic cycle, which is characterized by a growth stage. On the other hand, there are studies that associate the differences found on a temporal scale with dietary lipid intake and not necessarily with the gametogenic stage (Carboni, 2013). However, not all fatty acids that significantly varied between months in the present study showed higher values in September, such as C16, C18 and C18:1 trans. As mentioned earlier in this research, diet plays an important role in determining the nutritional composition of the gonads. Pereira *et al.* (2019) conducted a study with the macroalgae *Laminaria ochroleuca*, ingested by *P. lividus*, on the north coast of Portugal. The results showed a seasonal pattern of growth and reproduction for these algae, showing interestingly that *L. ochroleuca* accumulated more nutrients during its growth period (winter), which agreed with the increase of the quantified nutrient concentration in the gonads of sea urchins, including FAs. Although in the present study analysis for identification and quantification of FAs in algae species consumed by *P. lividus* in the sampling areas were not performed and, therefore, animal fatty acid correlations cannot be

adequately established, it is likely that the diet of different algae has influenced the FA composition of sea urchins.

The percentages of some of the fatty acids identified in this study varied significantly between the sexes of the individuals. In the Impacted Shore, the FA C14, C18:1 trans and C20:3n6 exhibited statistically higher percentages in females compared to males; however, the FA C15 and C20:4n6 exhibited significantly higher values in male sea urchins. At the Reference Shore, the FA values of C14, C16, C16:1, ALA, C18:4n3 and C20:1n9 were statistically higher in females compared to male sea urchins; only the percentages of C18 were significantly higher in males. The results found in this study agree with the results of Hughes *et al.* (2005), which analyzed changes in the FA profile of *Psammechinus miliaris* gonads over gametogenic cycles, observing significant differences in fatty acids between males and females before spawning. The authors suggested that this fact is probably associated with the presence of high levels of gametes with different stage IV fatty acid compositions immediately before spawning. In the same way, Martínez-Pita *et al.* (2010), on the south coast of Spain, developed a study with the species of sea urchins *P. lividus* and *Arbacia lixula*, where one of the objectives was to analyze the fatty acids of the gonads of males and females. As in the present research, the results showed that both species presented higher percentages of FA C14, C16 and C18:4n3 in the gonads of females as compared to males and, on the other hand, it was observed higher levels of FA C18 in males compared to females. These gender differences probably indicate different needs of males and females during gametogenesis, although the presence of a large number of gametes in mature gonads may also influence FA composition.

The possibility of the presence of toxic metals found in the gonads of sea urchins - confirmed in this research - modulating their fatty acid profile should not be ruled out. Contact with toxic substances and exposure to adverse environmental conditions may affect the nutritional quality of gametes and, consequently, the reproductive success of marine species. There are studies that corroborate this statement, indicating that sublethal exposure to trace metals such as Cu, Zn and Cd, in the laboratory, led to poor embryonic and larval development in sea urchins such as *Strongylocentrotus intermedius* (Khristoforova *et al.*, 1984; Durkina, 1994). The accumulating trace metals can cause physiological stress in FA at different trophic levels in the food chain and, ultimately, in humans.

In this study, the Spearman correlation analyzes performed showed that the metals Cd, Cu, Ni, Zn and Mn had an effect, sometimes negative, sometimes positive, on some of the identified fatty acids. Cd correlated negatively and significantly with the FAs C18:4n3, C20:4n6 and C22:1n9. In addition, there was also a significant negative correlation of Cu with C16:1, ALA, C20:2n6 and C20: 4n6, Ni with C20:2n6 and, lastly, Zn with the FAs C18, C20:1n11, C20:4n6 and EPA. This basically means that the presence of these metals decreases the content of those FA's in the sea urchin gonads. In aquatic animals, metal ions such as Cd, Cu and Zn accumulate in cells, predominantly in the cytosol (Cain *et al.*, 1992; Wallace *et al.*, 2003). Some studies indicate that, depending on the concentration, Cd ions decrease the enzymatic activity in animals tissues; for example, Sarosiek *et al.* (2009) developed a study whose objective was to determine the effects of copper, zinc, cadmium and mercury ions (100, 10 and 1mg/l) on the activity of some carp sperm enzymes. This study showed that acid phosphatase activity was relatively insensitive to zinc ions, but its activity was effectively inhibited by copper, mercury and cadmium ions. Effects observed on enzyme reaction rates may affect tricarboxylic acid cycle and fatty acid synthesis.

In a recent study, Mahboob *et al.* (2019) determined the effects of water contamination on the FA profile of the periphyton, zoobenthos, and 3 species of carps, captured from three different sites classified as highly polluted, less polluted and unpolluted from the Indus River (Pakistan). The study observed that the pollution was a determining factor in the FA composition in the different freshwater animals analyzed. In fact, some important fatty acids were absent in the organisms from the polluted sites. The presence of 6 and 9 omega-series FAs was detected in the muscle samples of fish caught in polluted places compared to sites without pollution and the total proportions of PUFA and SFA were reduced in the periphyton and zoobenthos as the pollution of the trace metals site increased compared to the unpolluted site. All trace metals showed significant negative correlations with the total FAs in the periphyton, zoobenthos and fish samples. This fact suggests that the increase of pollution in the water may have caused trophic transfers to periphyton, zoobenthos and fish. Fatty acids in fish muscles were affected by the level of contamination due to changes in the food chain associated with increased exposure to adverse environmental conditions. It was inferred that abiotic factors and chemical pollutants induced trophic transfer in food and, finally, the loss of essential FAs in fish meat (Mahboob *et al.*, 2019). It is therefore possible that the presence

of trace metals in the present study disturbed the biosynthesis and composition of the FAs identified in sea urchin gonads. In view of this scenario, the risk of some spread of these contaminants in the food chains should also be considered, namely because *P. lividus* is a species captured as shellfish for human consumption.

In the present study significant positive correlations between Zn and the FAs C14, C15, C16, C16:1, ALA and C18:4n3, and between Cu and Mn metals with FA C18 were also identified. These trace metals are considered sources of essential nutrients for sea urchins in appropriate concentrations, but at high levels, they may cause inhibitory or toxic effects (Lawrence, 2001). Pereira *et al.* (2018) studied the profile of essential and nonessential elements in three species of edible hares: *Aplysia depilans* Gmelin, *Aplysia fasciata* Poiret and *Aplysia punctata* Cuvier. Mn, Fe, Zn, Cu and Se were identified by those authors as the main essential elements and a correlation was found between the levels of these elements and the FA desaturation-elongation indices of those species. Also, Cd and Se seemed to promote the desaturation-elongation process involved in the production of C20:4n6c and, on the other hand, Ni, Cr and Fe may potentiate the conversion of C18:3n3c to C20:5n3c. In addition, Ni and Cu appear to decrease the desaturation index  $\Delta 9$  (Pereira *et al.*, 2018).

## **5. CONCLUSIONS**

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## 5. Conclusion

This study allowed to conclude that the accumulated metal concentrations in the gonads of *Paracentrotus lividus* sea urchins collected at the two studied sites on the coast of Peniche varied according to seasonal biotic fluctuations in the sea urchins (biometric, biochemical parameters and reproductive cycle), environmental factors (local hydrodynamics, food supply) and also anthropic actions related to the expansion of urban areas to the coastlines, which increase the levels of pollutants in the marine environment.

The comparison of biometric variables between the two rocky shores revealed that the sea urchins from IS, the epicenter of the spill, were significantly larger and heavier than those from RS. On the other hand, individuals collected at IS had significantly lower GSI values than individuals collected at RS. These findings reinforce the idea that these patterns of spatial and temporal variability are strongly associated with a series of factors such as aspects of the progress of the reproductive cycle of sea urchins, the local hydrodynamics of the habitat, availability and quality of food and development of the gonad. The study also points out the negative and weak correlations found between the toxic metals Cd, Pb and Ni accumulated in gonads of sea urchins with the biometric variables GW, WW and GSI, indicating that the presence of these metals adversely affects these biometric variables.

Additionally, this study allows to confirm the relationship between histological lesions in female and male gonads of sea urchins and metal bioaccumulation, especially at the IS. The results allowed to discriminate two toxic metals (Cd and Pb) in sufficiently high concentrations in sea urchin gonads from IS and RS to explain the appearance of certain histological lesions in the observed gonads. The intensity of the biological impact at that location, however, does not exclude the possibility that other pollutants may be involved. It should also be considered, associated with the latest findings, that the fact of the GSI of sea urchins in July had been significantly lower at IS compared to the RS, corroborates the idea that there is a negative relationship between the rate of histopathological lesions and gonadal development.

Metal contamination with Cd, as expected since it is a highly toxic metal, accentuates the IHPAs reabsorption, dilation and atrophy. Likewise, Cu contamination appears to increase

IHPA reabsorption. On the other hand, the essential metals Zn, Fe and Mn correlated negatively with the IHPAs LLP, reabsorption and hypertrophy, respectively, what demonstrates that these essential metals play an important role in curing these lesions, since as their gonad content increases, histopathological indices decrease.

The present investigation also allowed to conclude that the biochemical parameters (lipids, proteins and fatty acids) may differ according to the concentrations of trace metals found and had a significant variation over the months in the two sampling stations (IS and RS). Zn plays a fundamental role in the production of these molecules, evidenced by the positive correlations found between this metal and the lipid and protein contents. This study also highlights the fact that the concentrations of Zn in the ovaries of *P. lividus* were several times higher than in the testicles. On the other hand, a negative correlation was observed between the levels of Ni and proteins in the gonads, suggesting that the greater the accumulation of this metal in the gonads, the lower the protein content in these compartments.

According to the present study, *Paracentrotus lividus* affected by contaminants may experience a delay in the reproductive cycle and have lower GSI values. In the month of September, a time when sea urchins generally prepare to end a gametogenic cycle and start a new one, almost the entire population was in the "immature" stages (Stage I) at both sampling stations; however, this restart was not identical between the 2 sampling stations, since the sea urchins from IS presented a delay in this function; this late gametogenesis may be related to direct exposure to contaminants during the spill and to the higher incidence of lesions observed in the gonads of these animals, which may also have delayed the process.

The use of bioindicators in marine ecosystems represents an adequate methodology for environmental monitoring work, because, in general, it can offer results with high specificity of responses, which makes interpretation more efficient. *Paracentrotus lividus* is an excellent bioindicator, responding proportionally to the degree of disturbance, which most likely reflects the general response of the population. The trophic position of *P. lividus* as a sea urchin is one of the relevant characteristics regarding its degree of importance as a biological indicator, considering that the lower the trophic level of the organism and the more it serves as food for other beings of higher levels of the food chain, the greater the

relevance of the organism as a bioindicator because, in view of the contamination in that organism, it is assumed that the entire food chain might be contaminated. Within this context, and in view of the results observed in this study, strong and direct evidence of *P. lividus* contamination by the oil spill that occurred in July 2017 were not observed, although slight effects were detected in the reproductive function. Therefore, it can be assumed that this accident did not produce strong negative impacts in the ecosystem in question, since potential detrimental effects of this accident were successfully attenuated with the immediate control measures taken by the company involved and the responsible environmental agencies.

Within the scope of environmental monitoring investigations, the present study brings together a set of data and results that may serve, in the future, as a reference for ecotoxicological studies using *Paracentrotus lividus* as an environmental indicator on the coast of Portugal.

## **6. FUTURE PERSPECTIVES**

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## 6. Future Perspectives

The use of bioindicators such as *Paracentrotus lividus* in environmental assessment works enables knowledge about the level at which the pollutant interacts with the organism, and at what level it is more susceptible to its action. The results of researches like this are essential to design effective strategies to understand the problems and recover the biodiversity of aquatic ecosystems. For the future, it is important to think about establishing integrated inspection and control programs for environmental contamination of the marine environment through studies using bioindicators; this strategy will certainly provide a more accurate knowledge of the environmental quality of certain areas that may suffer from possible contamination. Investigations using biological indicators will allow the assessment and characterization of the environmental health status of marine ecosystems, producing reliable data that will allow the implementation of appropriate measures for their protection and/or recovery, in addition to allowing the assessment of the risks that such contaminations confer to public health and the biodiversity of these ecosystems.

Keeping this in mind, future studies can be improved by increasing the number of contaminants analysed and extending the analysis of metals to other sea urchin storage organs, such as the intestine, to determine whether metals are also accumulated there. In addition, future researches can be developed with the purpose of analysing the sea urchins studied in the present work again, in the years after the year of the naphtha spill, in order to identify, over time, the appearance of differences in the analysed parameters. Depending on the extent of the impact on the ecosystem, the damage to the biological community can be intense, taking several years to fully recover. Future analyzes would allow to measure the state of recovery of the ecosystem and also to monitor possible adaptations and changes in the organisms over time.

Since all species are interconnected in an ecosystem and depending on the physical environment to live, the disturbances that occur in a species or an ecological compartment will reflect in the entire trophic web, causing much greater damage, in the entire ecosystem. In this sense, it would also be interesting to carry out analyzes on other organisms associated with the feeding of sea urchins, namely seaweed, in order to verify the existence of any correlations between the trophic levels.

Also, for a more comprehensive analysis, the development of embryological analyzes can be considered, studying the sensitivity of sea urchins in different stages of development to trace metals and other pollutants from contamination by naphtha. Parallel to this, it is also possible to complement the research by performing analysis of contamination by metals in environmental samples, namely, water and sediment samples, in order to analyze the various physiological responses of *Paracentrotus lividus* to the presence of metals and their bioaccumulation. Finally, future studies could monitor the accumulation of metals in the tissues of these organisms under controlled conditions in the laboratory to better understand the effects that these contaminants cause in these animals, and analyzes with sea urchins and oxidative stress biomarkers can also be performed.

The information reported in this study confirms that *P. lividus* has the characteristics of a good indicator species and can faithfully reflect the health of a given environment. There is an urgent need for more research and appropriate scientific environmental strategies to encourage studies of this type and use the results for the management of harmful polluting sources.

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## Appendices

**Appendix I:** Two-way ANOVA and Post-hoc tests results for the biometric characterization of gonads of *Paracentrotus lividus* from 2 different sampling stations in the coast of Peniche, from July to September of 2017, following an oil spill event. Only the variables that presented significant results were represented ( $p$ -value  $< 0.05$ ). Oil spill Impacted Shore (IS) and Reference Shore (RS). df – degrees of freedom; MS – Mean Square.

<b>Two-way ANOVA</b>					
<b>Factor</b>	<b>Dependent variable</b>	<b>df</b>	<b>MS</b>	<b>F-statistic</b>	<b><math>\rho</math>-value</b>
<b>Rocky Shores</b>	Carapace Diameter	1	5.769	66.008	0.000
	Gonadosomatic Index	1	182.582	19.932	0.000
<b>Months</b>	Carapace Diameter	1	0.834	9.545	0.000
	Gonadosomatic Index	1	8.079	9.288	0.000
	Gonadal Weight	1	1.185	10.826	0.000
<b>Rocky Shores*Months</b>	Total Wet Weight	2	0.032	3.223	0.042
<b>Post-hoc tests</b>					
<b>Dependent variable</b>	<b>Test</b>	<b>Condition</b>		<b><math>\rho</math>-value</b>	
Carapace Diameter	<i>Tukey HSD</i>	Comparison:			
		August and July			0.016
		August and September			0.000
Gonadal Weight		August and September			0.000
		July and September			0.000
Gonadosomatic Index		July and September			0.000
	<i>Bonferroni test</i>				
Total Wet Weight		August and July			0.003
		August and September			0.000
		July and September			0.028

**Appendix II:** Kruskal-Wallis and post-hoc tests results for the concentrations of Cd, Pb, Cu, Zn, Ni, Fe and Mn in gonads of *Paracentrotus lividus* collected in the sampling sites at Peniche during the study period. Only the variables that presented significant results were represented ( $p$ -value  $< 0.05$ ). It was considered the effects of the different months (July, August and September) as factor. The following symbols stand for: df - degrees of freedom; MS - Mean Square.

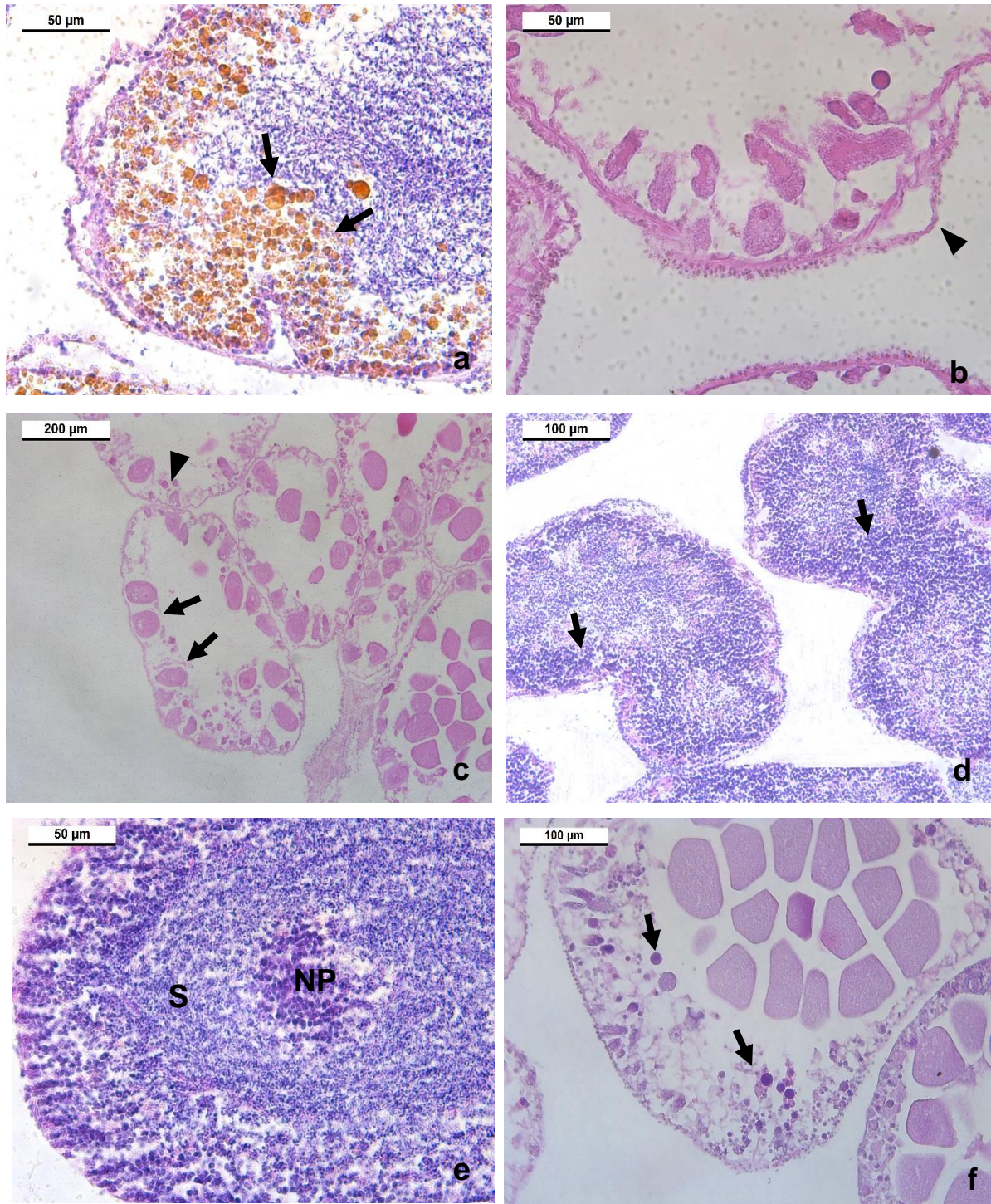
<b>Kruskal-Wallis test</b>				
	<b>Dependent variable</b>	<b>df</b>	<b>H de Kruskal-Wallis</b>	<b><math>\rho</math>-value</b>
<b>Impacted Shore</b>	Cd	2	10.510	0.005
	Cu	2	13.535	0.001
<b>Reference Shore</b>	Cd	2	10.745	0.005
	Pb	2	8.314	0.016
	Ni	2	14.724	0.001
	Cu	2	18.959	0.000
	Fe	2	12.310	0.002
	Mn	2	6.497	0.039
<b>Pairwise Method Comparisons</b>				
	<b>Dependent variable</b>	<b>Condition</b>		<b><math>\rho</math>-value</b>
<b>Impacted Shore</b>	Cd	Comparison: August and September		0.000
	Cu	July and September		0.018
		August and September		0.000
<b>Reference Shore</b>	Cd	July and September		0.002
		August and September		0.017
	Pb	July and August		0.026
		August and September		0.007
	Ni	July and September		0.000
		August and September		0.015
	Cu	July and August		0.000
		July and September		0.000

**Appendix II (continued):**

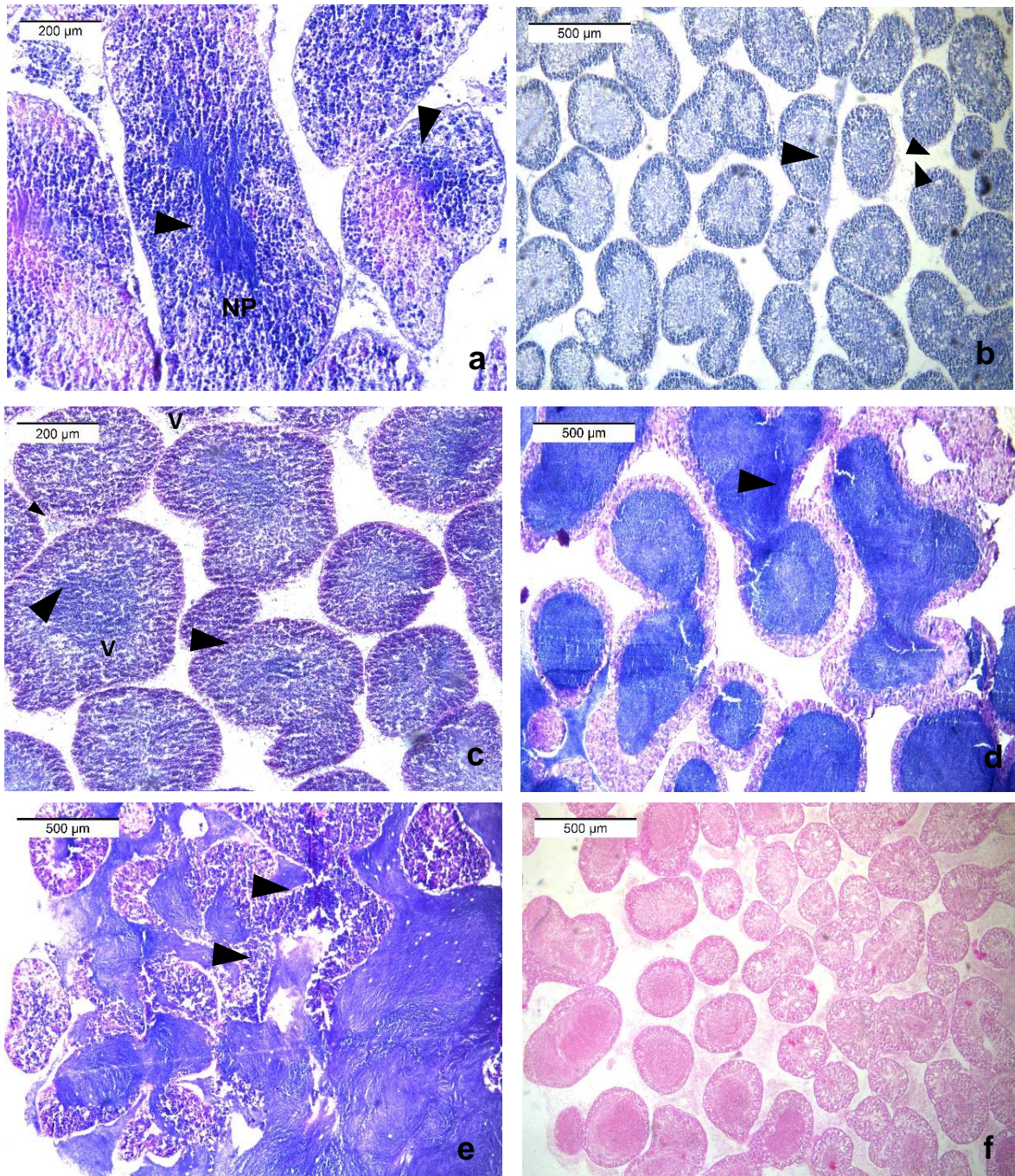
<b>Pairwise Method Comparisons</b>			
	<b>Dependent variable</b>	<b>Condition</b>	<b>ρ-value</b>
<b>Reference Shore</b>	Fe	July and August	0.004
		July and September	0.002
	Mn	July and August	0.015

**Appendix III:** Kruskal-Wallis and post-hoc tests results for the percentages of lipids and proteins in gonads of *Paracentrotus lividus* collected in the sampling sites at Peniche (Portugal) during the study period. Only the variables that presented significant results were represented ( $\rho$ -value < 0.05). It was considered the effects of the different months (July, August and September) as factor. The following symbols stand for: df - degrees of freedom; MS - Mean Square.

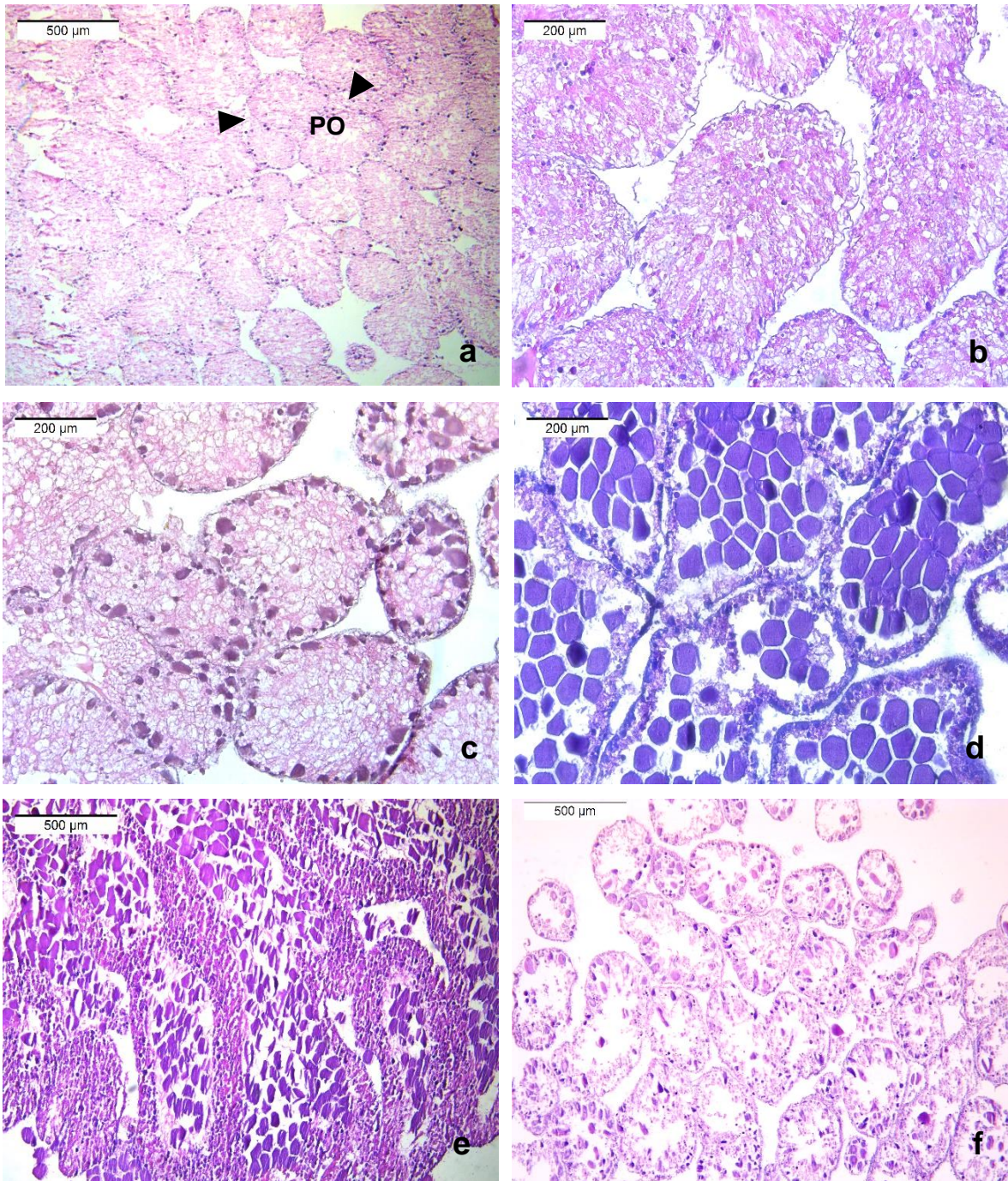
<b>Kruskal-Wallis test</b>				
	<b>Dependent variable</b>	<b>df</b>	<b>H de Kruskal-Wallis</b>	<b>ρ-value</b>
<b>Impacted Shore</b>	Lipids	2	14.974	0.001
<b>Reference Shore</b>	Lipids	2	6.695	0.035
	Proteins	2	8.263	0.016
<b>Pairwise Method Comparisons</b>				
	<b>Dependent variable</b>		<b>Condition</b>	<b>ρ-value</b>
<b>Impacted Shore</b>	Lipids		Comparison:	
			August and September	0.000
			July and September	0.014
<b>Reference Shore</b>	Lipids		August and September	0.004
	Proteins		August and September	0.011



**Appendix IV:** Gonadal lesions on *Paracentrotus lividus* from the two collection areas in 2017. **a.** Male with globular inclusions of LLP (arrows); dilations of the ascinal wall (arrow heads); **b.** Female with dilated membrane; **c.** Female with atretic oocytes (arrows) and hypertrophy of NPs (arrow heads); **d.** Male with hypertrophy of NPs (arrow); **e.** Male with large aggregations of NPs in the center; **f.** Female with hypertrophy of NPs (arrows); NP–nutritional phagocytes; LLP–Lipofuscin-likepigments.



**Appendix V:** Male gametogenic cycle of *Paracentrotus lividus*. a) Stage I: a high amount of nutritive phagocytes (NP) is observed; (b) Stage II: absorption of nutrients supplied by nutritive phagocytes (NP) and growth of pre-yolk gametes; NPs are found mainly along the ascinal wall of the testis; (c) Stage III: partial posture of the testicles with nutritive phagocytes in the center; V: empty spaces left by released sperm; (d) Stage IV: mature testicles filled with mature sperm ready for release; (e) Stage V: testicles at the beginning of the posture with spaces left by the released sperm (V); (f) Stage VI: reorganization of the internal structure of the testicles.



**Appendix VI:** Female gametogenic cycle of *Paracentrotus lividus*. a) Stage I: pre-vitelline oocytes are observed in the ascinal wall; b) Stage II: oocyte and ovarian growth; c) Stage III: ovary: premature with oocytes in all stages of development; d) Stage IV: ovary full of mature eggs and a very small amount of nutritive phagocytes; e) Stage V: ovary with loose eggs. shortage of nutritive material and emergence of empty spaces. indicate the beginning of laying; f) Stage VI: the ovary loses its internal structure leading to disorganization.