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Ecotoxicological risk assessment of deep-sea mining in the Clarion-Clipperton Zone

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

2023

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Master’s in Marine and Coastal Systems

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2023

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Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

I declare to be the author of this work, which is original and unpublished. Authors and works consulted are duly cited in the text and are included in the list of references.

(Leandro Teixeira Marinho)

Abstract

Deep-sea mining has the potential to become the next great extractive industry on a global scale, focusing on metals exploitation. The objective of this work is to assess the ecotoxicological effects of deep-sea mining plumes and to help elaborate a risk assessment model to evaluate the environmental risks of deep-sea mining. The work involved the analysis of deep-sea Actiniaria spp. and sediment samples collected from the MANGAN21 cruise to the Clarion-Clipperton Zone in the Pacific Ocean. During this cruise, a nodule collector trial generated a plume of sediments with potential negative ecotoxicological impacts that deposited over the deep-sea fauna. The effects of deep-sea sediment (10 mg/L and 50 mg/L) were evaluated in the marine mussel *Mytilus galloprovincialis* after a 14-day exposure assay in the laboratory. An array of biomarkers (SOD, CAT, GPx, GST, LPO, AChE, and DNA damage) and metals (As, Cd, Co, Cr, Cu, Mn, Ni, Pb, V, and Zn) were analyzed on mussel's gills, from the sediment exposure assay, and on deep-sea Actiniaria specimens collected from control and plume exposed locations. Additionally, metals and REEY concentrations were assessed in waters from the mussel assay. Results show that the single anemone specimen exposed to the plume appears to have induced SOD and LPO activity in response to the high concentrations of Cr, Co, Ni, Cu, Cd, Pb, and Mn in its tissues. In the case of the mussels, AChE and DNA damage were significantly altered after 14 days exposure to 50mg/L of deep-sea sediments while Mn, and Cr were accumulated in the gills from the same mussels. While no apparent signs of oxidative stress and damage were observed, this research demonstrates that deep-sea sediments from the Clarion-Clipperton Zone trigger genetic and neurological toxicity in shallow-water mussels. This robustly supports the idea that ecotoxicological harm can manifest even at low concentrations of these sediments. These findings can contribute to the development of a comprehensive, multi-disciplinary Weight of Evidence (WOE) approach for assessing the environmental risks associated with deep-sea polymetallic nodule mining in the Clarion-Clipperton Zone.

Keywords: Deep-sea mining, Ecotoxicology, Biomarkers, *Mytilus galloprovincialis*, Actiniaria

Resumo

A mineração no mar profundo tem o potencial para se tornar a próxima grande indústria extrativa à escala global, com foco na exploração de certos metais. Durante dez anos (1972-1982), a pesquisa sobre mineração no mar profundo, principalmente para extração de nódulos polimetálicos na Zona Clarion-Clipperton (CCZ), atravessou uma "era de ouro" (Glasby, 2002). Mais recentemente, vários fatores contribuíram para o elevado interesse na mineração no mar profundo. Em primeiro lugar, os preços dos metais têm aumentado desde o início deste século (Cronan, 2022). Em segundo lugar, em 2022, as Nações Unidas (ONU) estimaram que a população mundial pode aumentar para 8,5 mil milhões até 2030 e quase até 10 mil milhões de pesssoas até 2050 (ONU, 2022a), o que significa que serão necessários mais materiais para alcançar os Objetivos de Desenvolvimento Sustentável definidos até 2030 (ONU, 2022b), nomeadamente matérias-primas críticas, designadas pela Comissão Europeia, que são essenciais para o desenvolvimento da indústria de energias renováveis emergentes (Comissão Europeia, 2023) e de baterias elétricas (Martins et al., 2021; Hein et al., 2020). Terceiro, a ISA (Autoridade Internacional dos Fundos Marinhos), uma organização das Nações Unidas responsável por regulamentar e controlar todas as atividades relacionadas com minerais em áreas internacionais do fundo marinho, emitiu licenças para certas entidades explorarem apenas em locais de mineração selecionados (ex. Zona de Clarion-Clipperton). Atualmente, existem 21 empreiteiros aprovados pela ISA com direitos de exploração em 29 áreas do mar profundo distribuídas por diferentes partes dos oceanos Pacífico, Índico e Atlântico, das quais 17 estão localizadas na CCZ (ISA, 2023). Todo este entusiasmo em torno dos nódulos polimetálicos deve-se ao seu potencial valor económico, uma vez que na sua composição podem ser encontrados vários dos elementos necessários para um futuro próspero da humanidade. Para além do manganês (Mn), outros metais como Fe, Ni, Cu, Co, Mo, Ti, Li, Zn, Y, W, REEs entre outros, compõem os nódulos polimetálicos encontrados no mar profundo (Wang, 2009; Hein et al., 2013; Wegorzewski & Kuhn, 2014; Kuhn et al., 2017; Sharma, 2022). No entanto, a mineração no mar profundo pode acarretar vários riscos ambientais que apresentam um elevado nível de incerteza devido à falta de informações essenciais sobre os ecossistemas marinhos do mar profundo. Os impactos podem incluir alterações diretas na estrutura do fundo do mar (devido à remoção da camada superficial bentónica para extrair nódulos), perturbações nos sedimentos e criação de plumas (pelo contacto dos equipamentos de mineração com

o leito marinho), contaminação dos ecossistemas profundos em torno dos locais de mineração (associada à pluma de sedimentos que pode conter altas concentrações de metais), poluição luminosa e sonora (do navio de superfície, do veículo de mineração e do sistema de bombas de elevação) e descargas de águas processadas (profundidade de descarga ainda não estabelecida) que podem alterar a temperatura da água e introduzir produtos químicos (potencialmente mais concentrados pelo processamento do minério no navio de superfície) ou mesmo espécies invasoras (pela potencial libertação de águas de lastro adicionais) (Hein & Koschinsky, 2014; Visbeck & Gelpke, 2014; Hauton et al., 2017; van Doorn et al., 2022).

O objetivo deste trabalho consiste então em avaliar os efeitos ecotoxicológicos das plumas geradas pela mineração no mar profundo e contribuir, com dados, para a elaboração de um modelo de avaliação de risco para avaliar os riscos ambientais da mineração no mar profundo. O trabalho envolveu a análise de indivíduos da ordem Actiniaria (anémonas) de águas profundas e de amostras de sedimentos recolhidas durante a expedição MANGAN21 à Zona de Clarion-Clipperton no Oceano Pacífico. Durante esta expedição, um protótipo de coletor de nódulos (Patania II) realizou um percurso que gerou uma pluma de sedimentos, com impactos ecotoxicológicos potencialmente negativos, que se depositaram no fundo e consequentemente sobre os organismos daquela região. Os efeitos dos sedimentos do mar profundo também foram avaliados no mexilhão *Mytilus galloprovincialis* após uma exposição a duas concentrações (10 mg/L e 50 mg/L) durante 14 dias, em laboratório. Foram analisados uma série de biomarcadores (SOD, CAT, GPx, GST, LPO, AChE e danos no DNA) e metais (As, Cd, Co, Cr, Cu, Mn, Ni, Pb, V e Zn) nas brânquias do mexilhão, e em espécimes de Actiniaria do mar profundo recolhidos em locais de controlo e exposição à pluma. Além disso, foram avaliadas as concentrações de metais (As, Cd, Co, Cu, Mn, Ni, Pb, V e Zn) e REEY nas águas dos aquários do ensaio com mexilhão. Os resultados mostram que o único espécime de anémona que foi exposto à pluma aparenta ter atividades de SOD e LPO induzidas, em resposta às elevadas concentrações de Mn, Co, Ni, Cu, Cd e Pb medidas nos seus tecidos. No caso do ensaio com mexilhões, os resultados mostram que os sedimentos do mar profundo, mesmo com uma concentração de apenas 50 mg/L, libertaram significativamente vários metais e REEYs em solução nas águas dos aquários. No entanto, apenas a atividade da AChE e o dano no DNA foram significativamente alterados após 14 dias de exposição a 50 mg/L de sedimentos em

resposta ao Mn e Cr, que foram significativamente acumulados nas brânquias dos mesmos mexilhões. Embora não tenham sido observados sinais evidentes de stress e dano oxidativo, esta pesquisa demonstra que os sedimentos em águas profundas da Zona de Clarion-Clipperton desencadeiam ações de toxicidade genética e neurológica em mexilhões de ecossistemas costeiros. Isto solidifica a ideia de que danos ecotoxicológicos podem ocorrer mesmo com baixas concentrações destes sedimentos. No entanto, a falta de estudos abrangentes realizados em escalas realistas, tanto espaciais como temporais, deixa-nos incertos quanto à extensão, duração e consequências reais dos efeitos provocados pela mineração no mar profundo. Além disso, ao avaliar o risco ecotoxicológico associado à mineração no mar profundo, é essencial reunir dados de várias fontes e integrá-los para obter uma compreensão mais abrangente dos potenciais riscos envolvidos (ISA, 2022). O modelo "Weight of Evidence" (WOE) é uma ferramenta promissora utilizada na avaliação de risco ambiental para caracterizar o risco associado à exposição a sedimentos, e é aplicada precisamente quando uma abordagem abrangente e multidisciplinar é necessária para chegar a uma conclusão (Burton et al., 2002; Piva et al., 2011; MERAG, 2016; Mestre et al., 2017; Suter et al., 2017a; Suter et al., 2017b; van Doorn et al., 2022). Assim, o modelo WOE tem-se mostrado uma abordagem útil na avaliação de risco ambiental de ambientes marinhos, especificamente na ressuspensão de depósitos de minas que acumulam no fundo do mar (Mestre et al., 2017), e deverá ser aplicado à mineração no mar profundo de acordo com a ISA (2022). Estes resultados contribuem assim para o desenvolvimento de um modelo WOE para avaliar os riscos ambientais associados à mineração de nódulos polimetálicos no mar profundo na Zona de Clarion-Clipperton.

Palavras-chave: Exploração mineira no mar profundo, Ecotoxicologia, Biomarcadores, *Mytilus galloprovincialis,* Actiniaria.

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Abbreviation Index

As- Arsenic

CCZ or CCFZ- Clarion-Clipperton (Fracture) Zone

Cd- Cadmium

Co- Cobalt

Cu- Copper

Cr-Chromium

DSM- Deep-sea mining

EU- European Union

ISA- International Seabed Authority

Mn-Manganese

Ni- Nickel

NM- Nautical miles

Pb- Lead

RREY- Rare Earth Elements and Yttrium

UN- United Nations

UNCLOS- United Nations Convention on the Law of the Sea

U.S.- United States of America

V- Vanadium

WOE- weight of Evidence

Zn- Zinc

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I. Objectives

II. General Objective

The goal of this study is to assess the ecotoxicological effects of deep-sea mining plumes and contribute to elaborate a risk assessment model to evaluate the environmental risks of mining deep-sea polymetallic nodules.

III. Specific objectives

To determine metal concentrations (Ar, Cd, Pb, Co, Cu, Cr, Mn, Ni, V and Zn), enzyme activities (CAT, SOD, GST) and assess LPO levels in Actiniaria individuals retrieved from control and plume exposed sites of the GSR and BGR nodule exploration areas in the CCZ.

To determine metal concentrations (Ar, Cd, Pb, Co, Cu, Cr, Mn, Ni, V and Zn), enzyme activities (CAT, SOD, GPx, GST, AChE) and to quantify LPO in the gills of mussels, *Mytilus galloprovincialis* exposed to deep-sea sediments from the CCZ for 14 days.

1. Introduction

The first observation of deep-sea minerals occurred during the HMS Challenger expedition (1872-1876) where manganese oxide “black bodies” were identified. But it was only after John L. Mero’s book in 1965, “The Mineral Resources of the Sea” that a real economic and political interest for polymetallic nodules was revealed (Mero, 1965; Sparenberg, 2019; Cronan, 2022; Sharma, 2022). By this time, the world was facing “great pressures” for metal demand. In one hand humanity was still recovering from the massive use of raw materials (such as metals) during the WWII and rapid growth afterwards (Scharf, 2013; Cronan, 2022), on the other hand, the Cold War was accelerating the development in oceanography and marine engineering due to investments in military science (Oreskes, 2003, Sparenberg, 2019). In 1965 Mero depicted polymetallic nodules as an endless resource of Mn, Co, Ni and Cu that was allegedly growing at a faster rate than world consumption at that time would predict (a process that today is known to take millions of years), something that sparked global interest in deep-sea minerals and its exploration (Mero, 1965; UN, 1967; Glasby, 2002; Sparenberg, 2019; Cronan, 2022; Sharma, 2022).

Nowadays, the deep-sea mining industry and associated technology are well developed (Kang et al., 2021; Cheng et al., 2023) mainly due to the urgent demand of metals and REEs for electric and electronic uses occurring in the 21st century (Zhang et al., 2017; Watari, et al., 2021; IEA, 2021). Nevertheless, to avoid the same mistakes of overexploitation of resources and environmental pollution committed in the past in other industries (ie: fishing and oil and gas exploitation), holistic assessments of possible environmental impacts must be taken prior to exploitation of these mineral deposits (Kwan, et al., 2023).

1.1. Historic Contextualization of DSM

During the 20th century, prompted by the desire of several states to gain control over seabed resources, a worldwide political effort took place to regulate the areas beyond national jurisdiction. This had long been necessary since the sea was still largely governed by an ancient doctrine proposed in 1609 by Hugo Grotius, *Mare Liberum* (“The Free Sea”), in which the sea and its resources could not become anyone's property and should be shared by all (Grotius & Welwod, 1609; Buzan, 1976; Tanaka, 2012). By the end of the 17th century the Grotian doctrine was commonly accepted for the High Seas, but for coastal States, a maritime territory belt adjacent to its coasts, the Territorial Sea, was customarily claimed and limited by the distance at which a cannon could shoot, around 3 nautical miles (NM) from the coast, in which states had exclusive jurisdiction or sovereignty over the territory and its resources (Buzan, 1976; Anderson, 1982; Tanaka, 2012; Scharf, 2013). The seabed passed almost unnoticed through these changes in the law of the sea, being referenced only when few exceptions on legislations for exploitation of a state living resources beyond its territorial sea were recognized (Buzan, 1976). In fact, it was only from the middle of the 19th century, with scientific and technology developments like, the first deep-sea surveys (like the Challenger expedition), the laying of telegraph cables and building of the first oil derricks and piers off the California coast that the seabed and subsoil received recognition as potential sources of new resources, yet it was still legislated by the Grotian doctrine (Buzan, 1976; Anderson, 1982; Ermida, 2019).

During the 20th century several political conflicts and consequent technological development led states to make legal claims over the seabed and subsoil beyond its territorial sea. The most relevant claim, that practically launched the development of the law of the sea, occurred in September of 1945, when the U.S. President Harry Truman issued a proclamation with respect to the natural resources of the subsoil and seabed of the “appurtenant” continental shelf of the U.S. (Truman Proclamation) placing the area and such resources under U.S. legislation and control but keeping the right to free navigation above the continental shelf (Truman, 1945), which was soon delimited by the 200 meter isobath (UN, 1967; Buzan, 1976; Anderson, 1982; Hoagland et al., 2001). This document changed the perspective on the seabed, as the continental shelf adjacent to a coastal state became an extension of its territory, and soon several other coastal states started to follow and asserting rights over vast areas of the seabed (the largest ones were up to 200 NM), some even claiming the water column above the continental shelf as territorial sea (Buzan, 1976; Anderson, 1982; Scharf, 2013), something that increased divergences in the oldest norms applied at sea, thus creating the need for a uniform codified international law of the sea.

In 1958, the first United Nations Conference on the Law of the Sea (UNCLOS I) took place in Geneva, with 86 states participating, which culminated in four separate Conventions that codified the law of the sea:

- The Convention on the High Seas (1958), that came into force in 1962;

- The Convention on the Territorial Sea and the Contiguous Zone (1958), that came into force in 1964;
- The Convention on the Continental Shelf (1958), that came into force in 1964;
- The Convention on Fishing and Conservation of the Living Resources of the High Seas (1958), that came into force in 1966.

These documents not only, vaguely, defined and delimited the maritime zones of each state but also regulated the sovereign rights and duties of each state in each zone and provided regulations to avoid overexploitation of the living resources of the sea. Unfortunately, the participating states of UNCLOS I failed to reach a consensus on specific limits, regarding the Continental Shelf and the Contiguous Zone, making the conventions relating to these zones virtually free to the interpretation of each state (Buzan, 1976; Anderson, 1982; Tanaka, 2012). In addition, they also failed to consider the possibility that the seabed could be exploited, for military or commercial purposes, beyond a depth of 200 meters, due to the fast-developing status of ocean technology (UN, 1967; Buzan, 1976). A second conference (UNCLOS II) to define the limits of the territorial sea and the jurisdiction of fishing areas of the states occurred in Geneva 1960, but it was not able to solve any of these two matters and did not produce a convention (Buzan, 1976; Tanaka, 2012).

Deep-sea minerals did not receive much attention until this time because, although the presence of polymetallic nodules was recognized, their demand was especially scarce due to: the technical difficulties of operating in an economically viable way in the deep sea, and due to the overwhelming evolution of the use of the oceans, both in the increased capture of fish, the increase in maritime transport and the expansion of the oil industry to deeper marine regions (Buzan, 1976). In other words, the marine mineral resources that were indeed extracted, came from regions closer to the coast and from shallower waters, having little influence on the jurisdictional expansion of coastal states over the seabed. Starting in the mid- to late 60's, with Mero's publication in 1965 and the speech of the of Malta's ambassador, Arvid Pardo, to the UN General Assembly in 1967, deep-sea technology saw much research and development. In his speech, Pardo showed high concerns over the intensive use of the oceans and proposed the adoption of a well-defined international "régime" (the yet to come UNCLOS, 1982) to legislate the law of the sea (UN, 1967). In this treaty Pardo called for the incorporation of fundamental principles, adopted until today, that is, "*the seabed and ocean floor… beyond the limits of national* *jurisdiction*" (hereinafter referred to "the Area") is a "*common heritage of the mankind*" and "*should be reserved for peaceful purposes*" and for the benefit of mankind as a whole and, such areas should "*not be subject to national appropriation in any manner…*" (UN, 1967). Scientific research occurring in the Area should be unrestrictedly allowed and its results made "*available to all*" (UN, 1967). The exploration and exploitation of resources from the Area should be taken in the "*interests of mankind*", taking in special consideration "*the needs of poor countries*", and should not cause serious harm to the marine environment (UN, 1967). Pardo added that an international agency (International Seabed Authority, ISA) should be established to ensure the principles and provisions of the treaty in any activity occurring in the Area.

For ten years (1972-1982) research for deep-sea mining, mainly for polymetallic nodules on the Clarion-Clipperton-Zone (CCZ) went through a "golden age" (Glasby, 2002). Between Germany and France 68 cruises were sent in search for nodules in the CCZ and the Soviet Union took about 100 cruises to map nodule distribution in all deep-sea ocean basins worldwide (Glasby, 2002 and references therein). Furthermore, companies from Belgium, Canada, Germany, and Japan, under the US guidance, formed several international consortia focused on assessing the feasibility of nodule mining (Kang & Liu, 2021) and, in 1978, OMI concluded the first successful deep-sea polymetallic nodule mining trial in the CCZ (Glasby, 2002; Sparenberg, 2019; Kang & Liu 2021). In 1982, 117 states signed UNCLOS on the day it opened for signature, and it was finally adopted (UNCLOS, 1982). However, states showed reluctance in ratifying the convention mainly due to concerns over the deep-sea mining regime for The Area, set in Part XI on UNCLOS. In fact, UNCLOS only entered into force in 1994 along with the Implementing Agreement (1994), that modified numerous provisions of Part XI of UNCLOS. Currently UNCLOS has 168 Parties (167 member states and the EU). Facing the lengthy provisions of UNCLOS, that would channel research efforts to improve mining technology (Kang & Liu, 2021) and to first assess environmental aspects of deep-sea nodule mining (Sparenberg 2019; Glasby, 2022) mining companies considered nodule mining unviable (Glasby, 2002). So, the commercial interest in mining the deep sea declined between the mid-1980s and the beginning of the twenty-first century also due to fluctuations in metal prices and the increase of environmental awareness (Glasby, 2002, Cronan, 2022).

More recently, several factors have contributed to the renewed interest in deep-sea mining. First, metal prices have been increasing since the start of this century (Cronan, 2022). Second, in 2022 the United Nations (UN) estimated that the world population may increase to 8,5 thousand million until 2030 and almost up to 10 thousand million until 2050 (UN, 2022a), meaning that more materials will be necessary to reach the Sustainable Development Goals defined to 2030 (UN, 2022b), specifically critical raw materials, appointed by the European Commission, that are essential for the development of emerging renewable energy industry (European Commission, 2023) and electric batteries (Martins et al., 2021; Hein et al., 2020). And third, the ISA, an organization under the UN to regulate and control all mineral-related activities in international seabed areas, has issued licenses to contractors to explore only on selected mine sites (i.e., Clarion-Clipperton Zone). Currently there are 21 Contractors approved by the ISA with exploration rights over 29 deep-sea areas distributed over different parts of the Pacific, Indian and Atlantic Oceans, 17 of which are located in the CCZ (ISA, 2023).

1.2. Deep-sea minerals

There are three main deep-sea minerals with commercial interest, (cobalt-rich) ferromanganese crusts, (hydrothermal) polymetallic massive sulphide deposits, and (manganese) polymetallic nodules, all forming at depths ranging from 1000 to 6000m (Sharma, 2022). Ferromanganese crusts are accumulations of iron (Fe) and manganese (Mn) oxides that precipitate from cold seawater onto hard substrate rocks at a rate of a few millimeters per millions of years. Manganese crusts are mainly found on flanks of seamounts and other deep-sea outcrops (e.g., guyots), that were kept free of sediments for millions of years due to strong enough deep-sea currents that didn’t allow sedimentation. Besides iron and manganese, these crusts have several other metals and elements in its chemical constitution like Co, Ni, Cu, Mo, W, REEs (rare earth elements) among others (Halbach et al., 2017). Massive sulphide deposits form when seawater seeps through seabed cracks near a sub-seafloor heat source, where leaching of metals from adjacent rocks results in a hot fluid (up to 400 ºC), enriched in dissolved metals and sulfur (Cherkashov, 2017). Mineral accumulations form chimney-like structures (hydrothermal vents) when deposited, after being precipitated from the hot fluid to the surrounding cold waters (Cherkashov, 2017). Seafloor sulphide deposits are therefore widely distributed along Mid-Ocean Ridges and Island Arcs, where magmatic and tectonic activity promote metal leaching and high seabed porosity, respectively. This deep-sea mineral has high concentrations of Cu, Zn, Pb, Au and Ag, and lower amounts of Co, As, REEs, among others, however its composition can vary considerably (Cherkashov, 2017; Sharma, 2022).

1.2.1. Polymetallic nodules

Polymetallic nodules are approximately spherical layered accumulations of iron and manganese oxides around a nucleus (hard substrates, like a shark tooth or any rock piece) loosely lying on abyssal plains in about 4000-6000 m depth in all major oceans (Kuhn et al., 2017). Each layer has distinguished chemical and mineralogical compositions depending on two different growth processes: hydrogenetic growth and diagenetic growth. The first occurs as a result of element precipitation from oxygen-rich seawater while the second occur as a result of element precipitation from suboxic pore water (Wegorzewski & Kuhn, 2014). Usually, manganese nodules are mixtures of both types of layers (Kuhn et al., 2017). Even though the main factor controlling chemical and mineralogical composition of manganese nodules is the chemical condition of the surrounding environment (Kuhn et al., 2017), there are studies reporting influence of other factors on nodule growth like biogenic processes driven by microorganisms/bacteria that creates several "micronodules" that later assemble to large nodules (Wang et al., 2009), or that biological productivity, sedimentation rate and tectonic plate movements controlled nodule abundance and its grading during its formation periods (von Stackelberg et al., 1991). All this enthusiasm around polymetallic nodules is due to its economic value, being that several of the necessary elements for the planned prosperous future for mankind can be found on their composition. Apart from manganese (Mn), other metals like Fe, Ni, Cu, Co, Mo, Ti, Li, Zn, Y, W, REEs and a few more make the composition of polymetallic nodules found on the deep sea (Wang, 2009; Hein et al., 2013; Wegorzewski & Kuhn, 2014; Kuhn et al., 2017; Sharma, 2022).

1.3. Clarion-Clipperton Zone and its rich metal seafloor

Polymetallic nodule fields (Fig.1) are spread worldwide but only a few areas are of economic interest. These are the Central Indian Ocean Basin (CIOB), the Peru Basin, the Cook Islands and the Clarion-Clipperton (Fracture) Zone (CCZ) (Kuhn et al., 2017). The CCZ is an area of the seafloor with about 4 million km^2, water depths between 4000 and 6000 m and is located in the Eastern Central Pacific Ocean between Hawaii and Mexico (ISA, 2010; Hein et al., 2013; Lodge et al., 2014; Kuhn et al., 2017). The CCZ gets its name from its North and South boundaries, the Clarion Fracture Zone, and Clipperton Fracture Zone respectively. Of all the areas represented in Fig.1, the CCZ is the region with the largest and richest occurrence of Mn nodule fields (Hein et al., 2013; ISA, 2010), therefore, it is also the area of greatest economic interest with 17 exploration contracts (Fig.2) approved by the ISA so far (ISA, 2023).

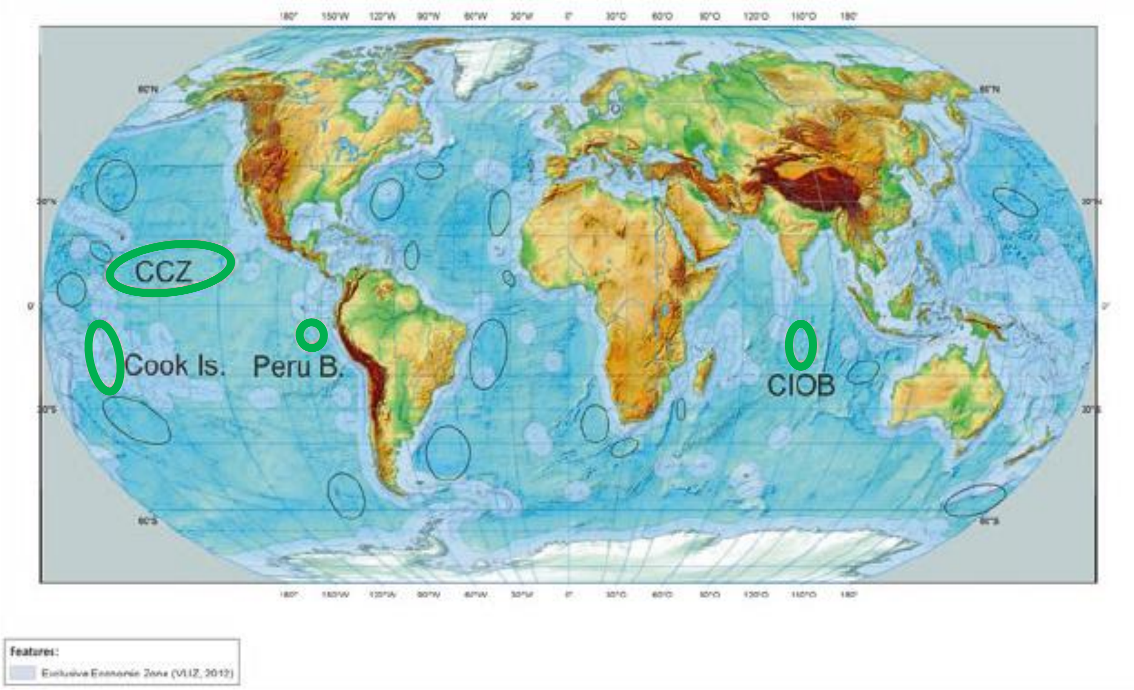

Fig.1.1- Polymetallic nodule fields in worldwide abyssal plains. Areas marked in green are economically important regions. CCZ- Clarion-Clipperton Zone, Cook Is.- Cook Islands, Peru B.- Peru Basin and CIOB- Central Indian Ocean Basin. (adapted from: Khun et al., 2017)

According to the ISA (2019), for polymetallic nodules, the entitled exploration area allocated to each contractor is 75,000 km² and a mechanism called “reserved areas” (Fig.1.2 yellow areas) ensures that developing countries will be able to access deep-sea mineral resources in the future. Nine “Areas of Particular Environmental Interest” (APEIs) with 160,000 km² each, were also designated in the CCZ and are protected from any future exploitation (ISA, 2012). So far, several studies have been carried out to understand the total and local abundance and distribution of polymetallic nodules in the CCZ with abundances up to 30 kg/m² and an average of 15 kg/m² (Skornyakova & Murdmaa, 1992; ISA, 2010; SPC, 2013; Mewes et al., 2014; Peukert et al., 2018). There are also reports of rare rich areas reaching 75 kg/m² (Visbeck & Gelpke, 2014). Currently a conservative “standard” value of total abundance of polymetallic nodules in the CCZ is 21,100 million tons (ISA, 2010) which amounts to 6000 million tons (aprox.) of manganese, more than the total amount found on land reserves (Visbeck & Gelpke, 2014).

Due to great amounts of metals being found on the deep-seafloor, deep-sea mining is becoming more and more realistic. So, comparisons with land terrestrial reserves were made to understand its relative worth (Hein et al., 2013; Visbeck & Gelpke, 2014).

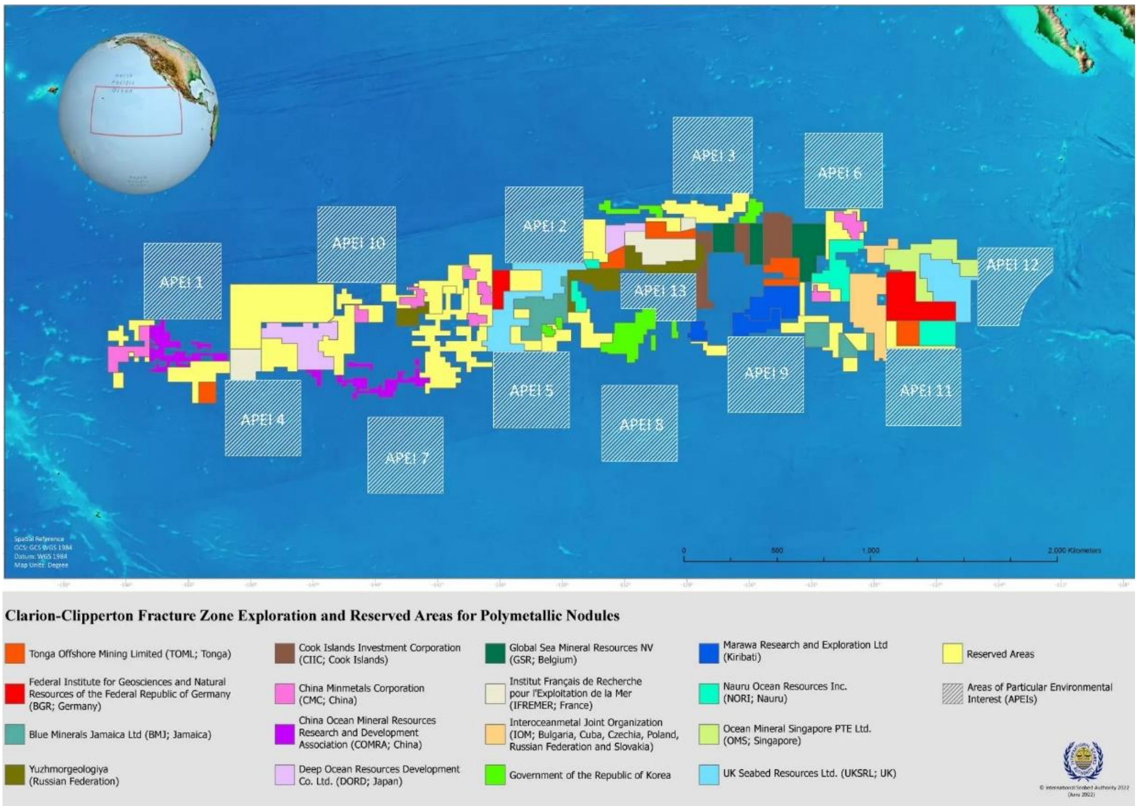

Fig.1.2- Polymetallic nodule exploration areas on the CCZ and respective contractors. (Source: ISA, 2023. Link: https://www.isa.org.jm/maps/clarion-clipperton-fracture-zone/

The comparison of total amounts of metals between the CCZ and global land reserves are shown in Table 1. Nodules in the CCZ have 10,000 times more Tl, 10 times more Mn, 4 times more Te, 3.4 times more Ni, 5.8 times more Co, and 4 times more Y than land reserves. These numbers may show significant amounts of metals in deep-sea deposits however, it should be noted that not all nodules nor land-metals would be recovered, and that only economically recoverable land reserves are represented, meaning that more ore exists on land (Hein et al., 2013), however it may become more and more intrusive for the environment and local or indigenous communities to try to mine on land.

Table 1.1-Comparison between metal tonnages ($\times 10^6$ tons) on the CCZ and on global economically recoverable terrestrial land reserves. Red values show metals with higher abundance in the CCZ. (Adapted from: Visbeck & Gelpke, 2014)

Metal content of manganese nodule occurences in millions of tons		
Elements	Clarion-Clipperton Zone (CCZ)	Global reserves on land (economically recoverable reserves)
Manganese (Mn)	5,992	630
Copper (Cu)	226	690
Titanium (Ti)	67	414
Rare earth oxides	15	110
Nickel (Ni)	274	80
Vanadium (V)	9.4	14
Molybdenum (Mo)	12	10
Lithium (Li)	2.8	13
Cobalt (Co)	44	7.5
Tungsten (W)	1.3	3.1
Niobium (Nb)	0.46	3
Arsenic (As)	1.4	1
Thorium (Th)	0.32	1.2
Bismuth (Bi)	0.18	0.3
Yttrium (Y)	2	0.5
Platinum group metals	0.003	0.07
Tellurium (Te)	0.08	0.02
Thallium (Tl)	4.2	0.0004

1.4. Risks of deep-sea mining

As it is, the CCZ is not richer in every metal, however, according to some authors the prospect of deep-sea mining relies more on reducing industry costs (economic, societal, and environmental) than amounts of product extracted. Bear in mind that, allegedly, deep-sea mining may have fewer impacts when compared with land-based mining (Hein et al. 2013). For example, transportation routes and infrastructures (roads and building complexes) would be replaced by ore transport systems (bottom to surface) and ships, that move freely on surface and should allow for high-grade selective mining. There is also an advantage of extracting several metals at the same time against land mines that usually extract only one or two metals. Deep-sea mining would have reduced societal impacts as it would not remove territory of native human populations that are many times affected by the necessity of reaching more inland to find land minerals. Further, no personnel would be in harm since nobody would be at the deep ocean mine. Still, given the lack of knowledge concerning environmental and societal impacts of deep-sea mining it is not possible yet to adequately assess the costs and benefits in comparison to land-based mining.

1.4.1. Environmental risks of DSM

Deep-sea mining may entail environmental, economic, societal, and legal risks (van Doorn et al., 2022). Environmental ones may have a higher order of uncertainty due to lack of essential information on deep-sea ecosystems. Impacts could include direct changes on the structure of the seafloor (by removal of the benthic surface layer to mine nodules), sediment disturbances and plume creation (by passage/contact of mining gear on/with the seabed), contamination of deep ecosystems around the mine sites (associated with the plume of sediments that may have high concentration of metals), light and noise pollution (from the surface vessel and the mining vehicle and from the lifting pump system) and processed water discharges (depth of discharge still not assessed) that may change water temperature and input chemicals (potentially more concentrated by ore processing at the surface vessel) or even introduce invasive species (by potential release of additional ballast waters) (Hein & Koschinsky, 2014; Visbeck & Gelpke, 2014; Hauton et al., 2017; van Doorn et al., 2022). To better understand and measure environmental impacts, small-scale mining equipment tests and mining simulation experiments have been carried out like the Deep Ocean Mining Environmental Study (DOMES) (Morgan et al., 1999), the Japan Deep-Sea Impact Experiment (JET) (Kaneko et al., 1996, 1997), the Interoceanmental Joint Organization- Benthic Impact Experiment (IOM-BIE) (Kotlinski & Tkatchenko, 1997) and the DISturbance-reCOLonization experiment (DISCOL) (Schriever et al. 1997). Such studies focused mainly on impacts to benthic communities made by mining vehicles and sediment plumes. JET results indicate significant impacts to benthic communities in areas of re-sedimentation (associated with plume). Also, DISCOL showed significant changes in the benthic habitat within tracks created by the device representing the mining system. Studies made 26 years later, on several regions where mining tests occurred showed that the tracks created by mining vehicles remain visible, biological recovery can be severely difficult depending on taxa and overall losses of heterogeneity in disturbed areas (Miljutin et al., 2011; Simon-Lledó, 2019). Since these experiments are only small-scale, when compared to full-fledged long-term DSM operations, little biological, geomorphological and baseline data has been collected over the extensive area of the deep sea (Amon et al., 2022).

1.5. Biomarkers of Ecotoxicological Risk

Biomarkers, in an environmental context, are biochemical, cellular or tissue alterations in an organism, that can be assessed through laboratory techniques, and that reflect changes due to exposure to environmental contaminants, physical-chemical alterations, the presence of microorganisms, among others, (Shugart et al., 1992).

Reactive oxygen species (ROS) are constantly produced in cellular pathways of aerobic metabolism (Regoli & Giuliani, 2014). The most common ROS found in cells are the singlet oxygen ($^1O_2$), the superoxide anion ($O_2•^-$), the hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO•). However, these can readily react to form other molecules with different reactivity and potential to cause damage to proteins, lipids, and DNA in cells (Stohs & Bagchi, 1995; Regoli & Winston, 1999; Lumb A., 2017). Under normal conditions the antioxidant system of marine organisms, an apparel of low molecular weight scavengers and antioxidant enzymes that interact in a complex network, blocks the unhealthy effects of ROS (Regoli & Giuliani, 2014). However, it is recognized that ROS production can be highly induced by a wide variety of stressing situations and that the antioxidant systems reacts accordingly to defend or recover from cellular damage, not always being sufficient to prevent deleterious conditions in organisms. So most effects of excessive ROS in organisms are harmful and imbalances between ROS and antioxidants are linked to several diseases (Lumb, 2017). A very detailed description of the antioxidant system of marine organisms can be found in Regoli & Giuliani (2014), so to understand the connection to this work, only a few selected pathways of ROS will be detailed.

The first stage in ROS production and activation begins with the spontaneous reduction of molecular oxygen (O_2) to $O_2•^-$, by receiving a free electron or by reacting with certain enzymes (Nordberg & Arnér, 2001). The superoxide anion is relatively stable for a radical and does not cross lipidic membranes (Nordberg and Arnér, 2001; Lumb, 2017) however it can oxidize small molecules and react with other enzymes to catalyze the production of highly potent ROS (Fridovich, 1999). Superoxide dismutases (SODs) are antioxidant enzymes responsible for metabolizing $O_2•^-$ to H_2O_2 (reaction 1).

$$2\,O_2•^- + 2\,H^+ \rightarrow H_2O_2 + O_2 \qquad \text{(reaction 1)}$$

Two metal-containing SOD isoforms, Cu/Zn-SOD and Mn-SOD can be found in the cytosol and within the mitochondria, respectively, of eukaryotic cells (Nordberg & Arnér, 2001; Regoli & Giuliani, 2014). SODs catalyze reaction 1 with unparallel efficiency being limited only by the rate of diffusion (Fridovich I., 1999; Nordberg & Arnér, 2001). Regardless of being an antioxidant enzyme, SOD is a source of hydrogen peroxide, which although not a free radical has the ability to cross cell membranes playing as an intermediate in the formation of more reactive ROS (Nordberg & Arnér, 2001), so Catalases (CAT) and glutathione peroxidases (GPx), antioxidant enzymes reductors of H_2O_2, must harmonize its activity with SOD. Catalases catalyze the dismutation of two molecules of H_2O_2 to one molecule of O_2 and two molecules of water (H_2O) (reaction 2) and take part in detoxifying other substrates, like phenols and alcohols, through H_2O_2

$$2\ H_2O_2 \rightarrow O_2 + H_2O \qquad \text{(reaction 2)}$$

reduction (Nordberg & Arnér, 2001; Regoli & Giuliani, 2014), and GPx catalyzes the reduction of H_2O_2 to H_2O, using reduced glutathione (GSH) as an electron donor. GPx are also known to reduce other peroxides to alcohols, like lipid hydroperoxides, generated during oxidation of cell membranes (lipid peroxidation) (Ayala, et al., 2014; Regoli & Giuliani, 2014). As a result, CAT and GPx are highly important in preventing ROS damage in aerobic systems, since the conversion of H_2O_2 to HO• can occur naturally via the Fenton/Haber Weiss reactions, and that the HO• is assessed has the most dangerous ROS (Zhengwei., 2005; Ayala, et al., 2014; Lumb, 2017). The generation of hydroxyl radicals is given by the reaction between H_2O_2 and a cation of a transition metal (usually Fe^{2+} or Cu^{+}) leading to the production of hydroxide ion (OH^{-}, not a ROS) and the hydroxyl radical (reaction 3), this is also known as the Fenton reaction (Halliwell & Gutteridge, 2007).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + HO\bullet \qquad \text{(reaction 3)}$$

A complementary reaction occurs, in which the ferric ion (Fe^{3+}) reacts with a superoxide anion and is reduced to ferrous ion (Fe^{2+}) (reaction 4), which in turn will promote more Fenton reactions.

$$O2\bullet^{-} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \qquad \text{(reaction 4)}$$

Both reactions, 3 and 4, are called the Haber-Weiss reaction that ends up working like a redox cycle of Fe ions that catalyzes the production of very dangerous ROS (Ayala et al., 2014; Regoli & Giuliani, 2014). This system explains the most important mechanism for ROS generation by trace metals and, besides Fe and Cu, other metal ions like several Cr cations, V^{5+}, Co^{2+} and Ni^{2+}, can catalyze Fenton-like reactions (Regoli & Giuliani, 2014).

The hydroxyl radical poses the biggest threat to cells as the most reactive species of oxygen that indiscriminately damages the cellular biomolecules closest to its site of generation such as lipids, proteins, and DNA (Zhengwei, 2005; Valavanidis et al., 2006; Ayala et al., 2014).

Lipid peroxidation (LPO) is a process in which chain-like reactions occur to oxidize lipids, particularly affecting polyunsaturated fatty acids (PUFAs) that are widely present in the phospholipids of cell membranes (Zhengwei, 2005). Such process can be divided in three phases: initiation, propagation, and termination. During initiation, free radicals (like HO•) remove hydrogen (H) from lipids yielding free radicals of lipids (L•). Propagation follows with the rapid reaction of O_2 with L• to produce lipid peroxyl radicals (LOO•), which in turn will remove H from other lipids creating lipid hydroperoxides (LOOH) and new L• resulting in a chain-reaction process. The decomposition of lipid hydroperoxides produces more lipid radicals and harmful aldehydes and hydrocarbons (Zhengwei, 2005; Ayala et al., 2014; Regoli & Giuliani, 2014). Under normal antioxidant balance, termination occurs when L• or LOO• receive an H, from lipid-soluble (low molecular weight) radical scavengers, like Vitamin E (or α-tocopherol), to stop the propagation of radicals. This reaction produces Vitamin E radicals which usually can´t initiate LPO and can be reconverted into radical scavengers again, therefore preventing irreversible LPO damage.

Besides LPO (ROS damage in lipids), ROS can cause damage in nucleic acids, like DNA that contains several potential metal-binding sites (Kazakov & Hecht, 2011). Damage to DNA is presented by formation of DNA adducts, modifications in nitrogenous bases, and single or double strand breaks (Halliwell & Aruoma, 1991; Almeida et al., 2003; Regoli & Giuliani, 2014; Lumb, 2017).

There are other toxic compounds that enter cellular media that cannot be dealt by antioxidant enzymes (SOD, CAT, GPx), like xenobiotic compounds (PAH, PCB). In this scenario bio transformation enzymes, like glutathione *S*-transferases (GST), act through a xenobiotic biotransformation system that conjugates the toxic into less toxic products, until excretion is possible. This system can be described in 3 phases in which the first consists of reactions between phase I enzymes and hydrophobic compounds, making them more polar. The more common essayed enzymes of phase I are the cytochrome P450 and flavinamonoxigenases (MFO). Phase II follows with conjugation enzymes, like GST, that will catalyze the combination of cellular substrates with the products from phase I, that will increase its solubility, so that excretion, by the multiple resistance drug proteins (MRP) can easily occur in cell membranes (phase III) (Regoli & Giuliani, 2014).

Besides both systems mentioned above, there are enzymes, like acetylcholinesterase (AChE), that are more related to neurotoxic compounds and act in neuronal synapses and neuromuscular junctions. The role of AchE is to hydrolyze the neurotransmitter acetylcholine (ACh) in acetil Coenzyme A and in choline, preventing continuous muscular contraction and nerve firings, which is essential for normal functioning of sensory and neuromuscular systems (Payne et al., 1996; Cajaraville et al., 2000). Certain compounds, mainly carbamates and organophosphates that are commonly found in commercial pesticides, inhibit AchE activity, leading to accumulation of ACh and consequent enhanced stimulation of muscle and nerve fibers, eventually resulting in paralysis and death (Colović et al., 2013).

It is important to understand that in vivo conditions do not follow such “linear” approaches like the ones explained above. All the defense mechanisms work as one, or at least at the same time, constantly. Besides, there are many more reactions and interactions occurring between ROS, enzymes, scavengers, auxiliary enzymes that work as cofactors, and other compounds or products that may be found in cells of marine organisms (Regoli & Giuliani, 2014) that were not mentioned here due to its complexity. Therefore only the pathways and systems relevant for this work were presented.

1.6. The mussel as a test organism

Mussels are sessile, filter-feeding organisms, with a wide geographical distribution, found in highly dense and stable populations and are easy to collect and work in laboratory conditions. Therefore the mussel *Mytilus galloprovincialis* is widely recognized as a great bioindicator species (Viarengo & Canesi, 1991) and there is extensive background literature about physiological responses under several stressing conditions (Cravo et al., 2009; Serafim et al., 2011; Gonzalez-Rey & Bebianno 2013; Gomes et al., 2012; Trombini et al., 2016; Pinheiro et al., 2019; Gonçalves et al., 2022). Here, we assessed a battery of biomarkers in mussels *Mytilus galloprovincialis* exposed to deep-sea sediments to help understand the potential ecotoxicological risk of DSM.

2. Methods

2.1. Study area and sampling

The MANGAN21 expedition occurred onboard of the Norwegian ship “ISLAND PRIDE” and took place in the Global Sea Mineral Resources, GSR (Belgian-sponsored), and the Federal Institute for Geosciences and Natural Resources, BGR (German-sponsored), contract areas of the Clarion-Clipperton Zone (Fig.3), from April to mid-May 2021.

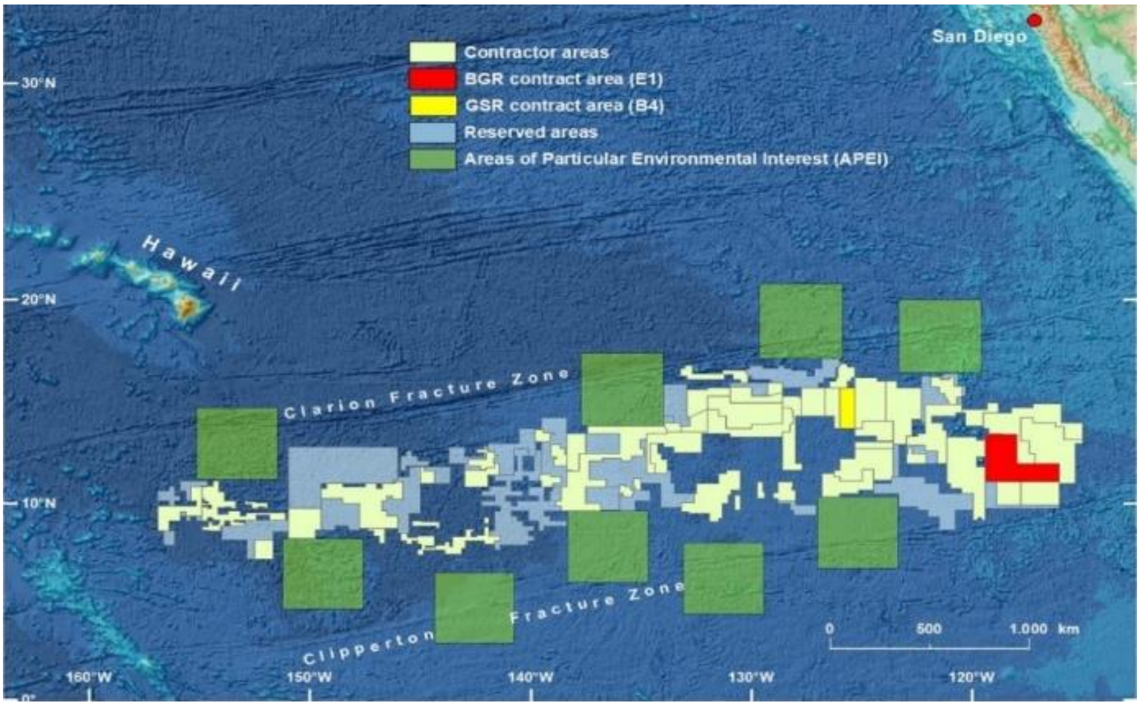

Fig.2.1- CCZ with highlight of the BGR (red) and GSR (yellow) contract areas. (Source: BGR; Link:https://www.bgr.bund.de/EN/Themen/MarineRohstoffforschung/MiningImpact-Logbuch/Bilder/2021-05-14_Logistik1_g_en.jpg?__blob=normal&v=2)

Global Sea Mineral Resources has developed a pre-prototype nodule-collecting robot named Patania II (Fig.4) that was successfully operated at commercial driving speeds and nodule pick-up rates. With a collector width of 4 m-wide, the total mined surface area was estimated to 30,899 m² for the GSR trial and 23,600 m² for the BGR trial.

Fig.2.2- Pre-prototype nodule collector, Patania II, used to simulate a small-scale mining activity on the BGR and GSR contract areas of the CCZ. Source: © GSR

Sediment samples were collected at depths greater than 4,000 m, in both contract areas (4 samples per area), using a multi-corer (MUC) capable of removing 30 cm long cores of the top sediment from the seafloor. All cores were handled in a cold environment (4 ºC) to keep the *in situ* temperatures and preserve chemical equilibria in the sediments. Seven biological samples (Actiniaria sp.) were collected using a ROV manipulator arm, 5 from the BGR trial and 2 from GSR trial. Only one of the samples (GSR) was collected 1 week after the Patania II trial, the rest were collected before the trial. Sediment and nodules were preserved at 4 ºC, with the first 1 cm top layer stored apart (also at 4 ºC) for each core, and Actiniaria samples were preserved at -80 ºC until laboratory analysis at the Centre for Marine and Environmental Research (CIMA) at University of Algarve, Portugal.

2.2. Sediment exposure assay on shallow-water mussels

On June 2022, mussels *M. galloprovincialis* (6.09±0.36 cm length, 3.34±0.25 cm width, 2.42±0.20 cm height) were hand-picked from the Ria Formosa Lagoon, Portugal (37◦00′30.6″N 7◦59′39.6″W) during low tide and were transported to the laboratory alive, where they were scrap-cleaned and divided into 18 mussels per tank, each with 7 L of natural seawater (T: 18.78 ± 0.40 ºC; S: 34.89 ± 0.46 ‰, pH: 7.94 ± 0.11, O_2: 98.06 ±

5.06 %) and were acclimated for 5 days with a 12h/12h light/dark cycle and kept under constant aeration. After acclimatation, a mixture of dry sediments from 1-10 cm seabed cores from the GSR contract Area was added in two different concentrations (plus control), 10 mg/L (C10) and 50 mg/L (C50) in a 4 tank per treatment design (except for T0 with a duplicate design). The water was replaced every other day and contamination restored for each renewal. The mussels were not fed, and no deaths occurred during the exposure.

2.3. Sampling sediment, water, and mussels

A set of dried deep-sea sediment triplicates were stored in microtubes for trace metals analysis. Water samples were taken, from each treatment, at 4 different moments (T0, T2, T8, and T14) using pre-decontaminated (at HCL 20%) 50 ml syringes and tubes, and 0.2 µm cellulose acetate syringe filters. First, to rinse the filter, 50 ml of milli-Q water was filtered, following 20-30 ml of natural seawater, then water from each treatment was filtered to a 50 mL digiTUBE. Each treatment, per time, was sampled once for dissolved metals. Water samples for dissolved metals analysis were added 50 µL of HCl (34%) and stored at 4 ºC. Blanks taken, at each sampling moment, consisted of 50 ml Milli-Q filtered water (after rinsing with Milli-Q and natural seawater) and were stored, for dissolved metals, as described above. Mussel sampling also occurred in several moments (T0, T7 and T14 days), where they were prepared for different analyses. For antioxidant enzymes activity (SOD, CAT and GPx), biotransformation enzyme activity (GST), lipid peroxidation (LPO), neurotoxicity enzyme activity (AchE) and for evaluation of trace metals accumulation in mussels, the gills of the exposed mussels were dissected and were immediately frozen in liquid nitrogen (N_2) and stored at -80 ºC until further analysis. Additionally, haemolymph of six mussels collected after 0, 7 and 14 days, exposed and unexposed to deep-sea sediments, was extracted from the posterior adductor muscle with a sterile hypodermic syringe and processed immediately after.

2.4. Trace elements analyses

The concentrations of trace metals in mussel tissues and anemones, and in waters were prepared and analysed at the Portuguese Institute of Sea and Atmosphere (IPMA, Lisbon) and at the Federal Institute for Geosciences and Natural Resources (BGR, Hannover), respectively.

2.4.1. Trace elements in mussels and anemones

Tissues were first freeze-dried, grounded, and homogenized. Then, samples were digested in a microwave (CEM, MARS 5), with nitric acid (HNO_3, distilled, 65% v/v), at 160 ºC, for 30 min following the procedure described by Brito et al. (2020). The labware was previously decontaminated with HNO_3 (20%). Digested samples were analysed by quadrupole ICP-MS (Perkin-Elmer NexION 2000C) equipped with a cyclonic spray chamber, a concentric Meinhard nebulizer and a dual detector (Figueiredo et al., 2020). The 75As, 111Cd, 208Pb, 59Co, 63Cu, 55Mn, 60Ni, 51V and 66Zn were the quantified isotopes, as they present minimum isobaric and polyatomic interferences under routine conditions (137Ba++/137Ba and 140Ce16O/140Ce ≈ 0.010). The equipment was set up by ensuring low variability of counts (RSD <2%). The isotope 115In was used as an online internal standard. Calibration curves (7 points) were used in different dynamic ranges depending on the metal concentration in samples. For most of the samples, concentration intervals of calibration curves were 0.44–39.8 µg/L for As, 0.052–4.0 µg/L for Cd, 0.057–4.0 µg/L for Pb, 0.12-9.9 µg/L for Co, 0.50-9.9 µg/L for Cu, 0.23-19.9 µg/L for Mn, 0.10-9.9 µg/L for Ni, 0.23-19.9 µg/L for V and 7.9-404 µg/L for Zn. Three procedural blanks were included within each batch of 10 samples and accounted for less than 1% of the total concentrations determined in the samples. The accuracy of the analytic method was also evaluated through the evaluation of international certified reference materials (BCR 668 and DORM-4). The percentage recovery was 90 ± 7.4%. Metal concentrations are shown in microgram per gram of tissue dry weight (µg/g, dw). The detection limits were 0.14 µg/L for As, 0.009 µg/L for Cd, 0.014 µg/L for Pb, 0.031 µg/L for Co, 0.040 µg/L for Cu, 0.46 µg/L for Mn, 0.067 µg/L for Ni, 0.064 µg/L for V and 0.69 µg/L for Zn.

2.4.2. Trace elements in waters

Concentration of trace elements in tank waters were directly determined with a triple quadrupole ICP-MS (Thermo iCAP), using an automated offline sample preconcentration method (SeaFAST, Elemental Scientific Inc.) equipped with an APEX Q (Elemental Scientific Inc.) sample introduction system for increased sensitivities. Sample concentrations were quantified by external standard calibration and validated with the certified reference materials (CRM; National Research Council of Canada (NRC)) NASS-7 and the SLRS-6.

2.5. Condition Index

To evaluate the physiological status of control and exposed mussels to 10 mg/L and 50 mg/L of deep-sea sediment, the soft tissues and shells of mussels were weighted, and the Condition Index (CI) calculated as the percentage (%) of the ratio between the drained weight of the soft tissues (g) and the shell weight (g) (Simpson & Batley, 2016). A total of 42 mussels were evaluated, 6 per treatment and time of exposure.

2.6. Homogenization of tissues for antioxidant enzyme activities

Tissues of mussels and anemones were defrosted, weighted, and immersed in 5 ml of 20 mM Tris-Sucrose buffer (0.5 M sucrose, 0.075 M KCl, 1 mM DTT, 1 mM EDTA, pH 7.6) using 15 ml falcon tubes as container. The samples were then homogenized in a VWR® Star-Beater, until no fragments of tissue were observed. To obtain the cytosolic fraction samples were centrifuged first for 15 minutes at 500g and 4ºC and second for 45 minutes at 12,000g and 4ºC, saving the supernatant and discarding the pellet in both centrifugations. The final supernatants were aliquoted into microtubes for the different enzyme activity analyses (Total Protein, SOD, CAT, GPx and GST) and stored at -80ºC.

2.7. Total Protein

The methodology described below was used to calculate the total protein concentrations in all the enzymes analysed: SOD, CAT, GPx, GST, AchE and LPO.

The determination of total protein concentration was assessed in the cytosolic fraction according to the colorimetric Bradford method (Bradford, 1976), using Bovine Serum Albumin (BSA) as standard (1 mg/ml). The Bradford method assumes the binding of the dye Comassie Blue G-250 to proteins (Bradforf, 1976). During this assay the dye is converted, from its cationic form (red) to its anionic form (blue), by binding to protein (Sedmak & Grossberg, 1977). Aliquots of 5 µL BSA standard or samples were pipetted in each microplate well with 200 µL of diluted Bradforf reagent (1:5) and incubated for 20 min at room temperature. The absorbance of the blue protein-dye form was detected at 595 nm using a microplate reader (Infinite M200 Pro, Tecan®). To determine Total Protein concentrations in samples, a standard curve (with BSA) was obtained, and the following formula (1) was applied. Results are expressed in mg/g.

$$(1)\ \ Total\ Protein\ (mg/g) = \ [TP](mg/ml) * \frac{Volume\ Tris\ (ml)}{W\ tissue\ (g)}$$

2.8. Superoxide dismutase (SOD) activity

SOD activity was measured according to the method described by McCord & Fridovich (1969). In brief, samples were mixed in glass cuvettes (Vtotal=3 ml) with a phosphate buffer (50mM, pH 7.8) with EDTA (0.1mM), hypoxanthine (1.5mM), cytochrome c oxidized (0.15mM) and xanthine oxidase (56 mU/ml) and read at 550 nm, during 1 minute, using a UV-Vis spectrophotometer (Jasco V-650 controlled with Jasco’s Spectra Manager™ software). Blanks had neither sample nor xanthine oxidase (XO). To test for XO viability only, samples were not added. SOD activity is expressed in Units (U), meaning that 1 U of activity represents the necessary amount of sample to inhibit 50% of the cytochrome c reduction reaction caused by the anion superoxide ($^{\cdot}O_2^{-}$), which is generated by the xanthine/hypoxanthine system. The determination of SOD was based on the increase in absorbance generated by the production of reduced cytochrome c. Results were calculated with formula (2) and are expressed as U/mg protein.

$$(2)\ \ SOD\ Activity\ (U/mg\ prot) = \frac{\frac{\%I}{50(M/cm)} * \frac{Vtotal}{Vsample} * 1.4 * 1cm}{Protein\ (mg/ml)}$$

2.9. Catalase (CAT) Activity

Quantification of CAT activity was based on the method described in Greenwald (1985) by measuring the consumption of hydrogen peroxide (H_2O_2) at 240 nm. Samples were mixed in a quartz cuvette (Vtotal=3 ml) with phosphate buffer (KH_2PO_4 80 mM, K_2HPO_4 80 mM, pH 7.5) and H_2O_2, while blanks only had the phosphate buffer. Readings were performed with a UV-Vis spectrophotometer (Jasco V-650 controlled with Jasco’s Spectra Manager) that detects the consumption of H_2O_2 over one minute by measuring the decline in absorbance (dAbs/min). Results were calculated with formula (3) an’ expressed as mmol/min/mg of protein.

$$(3)\ \ CAT\ Activity\ (mmol/min/mg\ prot) = \frac{\frac{dAbs/min}{40} * \frac{Vtotal}{Vsample}}{Protein\ (mg/ml)}$$

2.10. Glutathione Peroxidase (GPx) Activity

GPx is an antioxidant enzyme family that is induced by peroxides (ROOH), like H_2O_2, and uses reduced glutathione (GSH) as co-factor. GPx activity was determined in a microplate reader with an adapted method from McFarland et al. (1999). A daily assay mixture – DAM (3 mm GSH, 0.25 Mm NADPH, 0.67 U/ml GR) was prepared in 20 ml GPx phosphate buffer (50mM potassium phosphate, pH 7.6, 0.1 mM EDTA, 0.15 mM NaN_3). While keeping the microplate over ice, 20 µl of blank (Tris-Sucrose buffer from chapter 2.5) and 40 µl of samples were added to microplate wells in duplicate. Afterwards, 200 µl of DAM solution was pipetted in each well (out of the ice) and incubated at 28ºC for 2 minutes. Next, 50 µl of substrate (1.00 mM Cumene hydroperoxide) were added to each well and finally, read with a microplate reader (Infinite M200 Pro, Tecan®). at 340 nm for 5 minutes (readings each 30 seconds) at 28ºC. GPx activity was assessed through the decrease in absorbance of nicotinamide adenine dinucleotide phosphate (NAPDPH) at 340 nm (Ɛ 340 (NADPH) = 6.22 mM^{-1} cm^{-1}), that is used as cofactor in the regeneration of GSH. GPx activity is expressed as µmol NADPH/minute/mg protein and calculated according to formula (4):

$$(4)\quad GPx\ Activity\ (\mu mol/min/mg\ prot) = \frac{\left(\frac{\Delta Abs_{sample} - \Delta Abs_{blank}}{5}\right) * Vtotal\ * 1000}{6.22 * 0.7485 * Vsample * Protein(mg/ml)}$$

In which Vtotal is 0.29 ml and the lightpath is 0.8085 cm.

2.11. Glutathione S-Transferase (GST) Activity

GST activity was determined according to the method described by Habig and Jakoby (1981) but adapted to a 96-well microplate assay (McFarland et al. 1999). A daily assay solution – DAM, was prepared with 0.5 ml of CDNB (60mM in ethanol) and 20 ml of phosphate buffer (0.2 M, pH 7.9). The mixture in each well of the microplate was composed of 25 µl of homogenization buffer (from chapter 2.5), as blank, or 25 µl of samples, in triplicates, and 200 µl of a mixture, prepared before the analysis, with 5 ml of DAM and 125 µl of GSH (10mM). Absorbance was measured at 30 second intervals at 340 nm for 3 minutes, with a microplate reader (Infinite M200 Pro, Tecan®). GST activity was assessed through the change in absorbance, that CDNB (Ɛ 340nm CDNB= 9.6 mM) suffers when it is conjugated with GSH, and is expressed as µmol of CDNB conjugate formed/minute/mg protein and calculated with formula (5):

$$(5)\quad GST\ Activity\ (\mu mol/min/mg\ prot) = \frac{\left(\frac{\Delta Abs_{sample} - \Delta Abs_{blank}}{3}\right) * Vtotal * DF}{9.6 * 0.6135 * Vsample * Protein(mg/ml)}$$

In which, 0.6135 is the lightpath and DF is the dilution factor.

2.12. Acetylcholinesterase (AChE) Activity

For AChE activity analysis, tissues were weighted and then homogenized, in 5 ml of Tris-HCl buffer (100 mM, pH 8.0) and 50 µl of Triton 0.1% (10 µl/ml of Tris-HCl), with a VWR® Beater, Mixer Mill. Homogenates were then centrifuged at 12,000 g, over 30 minutes at 4ºC, and the supernatant was divided in two aliquots: one for determination of Total Protein (Bradford, 1976) and the other for the determination of AChE activity. AChE activity was determined according to the method of Ellman et al. (1961), adapted to a microplate reader by Matozzo et al. (2005), by measuring the change in absorbance given by the reaction between acetylthiocholine (ATC) and dithiobisnitrobenzoate (DTNB). To fill the microplate, 50 µl of Tris-HCl buffer (0.1M, pH 8.0), for blanks, or 50 µl of samples, in triplicates, were added in each well and incubated with 200 µl of DTNB (0.75 mM) over 5 minutes. Afterwards, 50 µl of ATC (3 mM) was pipetted to each well and incubated for 10 minutes. Absorbance was measured at 30 second intervals at 405 nm for 5 minutes, with a microplate reader (Infinite M200 Pro, Tecan®). Results are expressed as nmol of ATC/minute/mg protein and were calculated using formula (6):

$$6)\quad AChE\ Activity\ (nmol/min/mg\ prot) = \frac{\left(\frac{\Delta Abs_{sample} - \Delta Abs_{blank}}{5}\right) * Vtotal * 1000}{13.6 * 0.8385 * Vsample * Protein(mg/ml)}$$

In which, DTNB molar coefficient extinction at 405 nm is 13.6 mM/cm and the lightpath is 0.8385 cm.

2.13. Lipid Peroxidation (LPO)

LPO was assessed by measuring the concentration of malondialdehyde (MDA), a terminal product of the peroxidation of the lipids of the membranes caused by reactive oxygen species (ROS). Tissues were homogenized at 4ºC in a Tris-HCl buffer (0.02 M; 5 mL per g of tissue, pH 8.6) with butylated hydroxytoluene (BHT, 10 µL/mL). The homogenate was separated in soluble and insoluble fractions by centrifugation (30 000 g, 45 min, 4ºC) and the supernatant was used to measure total protein content (Bradford, 1976) and LPO levels. Total protein concentration of the cytosolic fraction was measured by the Bradford method (Bradford, 1976). For determination of LPO, the method proposed by Erdelmeier et al. (1998) was followed, with a maximal absorbance at 586 nm, and using malondialdehyde bis (dimethyl acetal) as standard. Three solutions were prepared daily, Reagent 1 (R1- 0.01M 1-methyl-2-phenylindole in acetonitrile), a dilution of R1 (18 ml of R1 in 6 ml of methanol) and a solution of methanosulfonic acid (R2- 15.4 M). an MDA solution (20 mM in Tris-HCl pH 8.6) was prepared for determination of a standard curve. Standard solutions of MDA and samples (200 µl) were well mixed with R1 (650 µl), then well mixed again with R2 (150 µl) and were then incubated at 45 ºC for 60 minutes in a water bath. After, only samples were centrifuged at 15,000g for 10 minutes at 4ºC. In each well of the microplate 4 replicates of 150 µl of MDA standard or samples were pipetted and read with a microplate reader (Infinite M200 Pro, Tecan®) at 586 nm. MDA concentrations are expressed as nmol/mg protein and were calculated according to formula (7):

$$7)\quad MDA\ (\mathrm{nm}ol/mg\ prot) = \frac{\dfrac{\dfrac{Abs - b}{a}\,(\mathrm{\mu mol/l}) * Vtris\ (ml)}{weight\ tissue\ (g)}}{Total\ proteing\ (mg/g)}$$

2.14. DNA damage Assessment

To assess the damage to DNA the comet assay was selected, following the procedures described in Singh et al. (1988) and Gomes et al. (2013). Haemolymph of six mussels collected after 0, 7 and 14 days, exposed and unexposed to deep-sea sediments, was extracted from the posterior adductor muscle with a sterile hypodermic syringe. A sub-sample (100 µl) from each experimental condition was used to assess cell viability by staining with trypan blue and measuring the percentage of live cells by randomly counting 100 cells. For the comet assay, microscope slides were firstly coated with 0.65% normal melting point agarose (NMA) in Tris-acetate EDTA. Haemolymph samples were centrifuged at 3000 rpm for 3 minutes (4 ºC) and the pellets were suspended in 0.65% low melting point agarose in Kenny's salt solution and casted on microscope slides. Slides (with cells) were then immersed in a lysis buffer (2.5 m NaOH, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% dimethylsulfoxide, 1% Sarcosil, pH 10, 4 ºC) for 1h. Afterwards, slides were placed in an electrophoresis chamber with buffer (300 mM NaOH, 1mM EDTA, pH 13, 4 ºC) and let rest for 15 minutes to allow DNA unwinding. After electrophoresis ran for 5 minutes at 25 V and 300 mA, slides were immersed in a neutralizing solution (0.4 mM Tris, ph 7.5) for 15 minutes, rinsed again in bi-distilled water for another 15 minutes and left to dry at room temperature. Finally, 4,6-diamidino-2-phenylindole (DAPI, 1 µg/ml) was used to dye the slides, and the pictures (50 cells per slide) were taken using an optical fluorescence microscope (Axiovert S100) coupled to a camera (Moticam 1080), and the % of DNA tail in each cell was calculated. Scoring analysis was performed using Imaging Software Komet 7.1 (Kinetic Imaging Ltd). Comet results are expressed as DNA tail % (mean ± sd).

2.15. Statistical analysis

All data gathered from biomarkers and metal concentrations was, separately, tested for normality (Shapiro-Wilk test), followed by one-way ANOVA and a pairwise post-hoc Tukey's multiple comparison tests, to identify significant differences ($p < 0.05$). All statistical analysis were done in R software (Version 4.3.0) with the tool Rcmdr.

3. Results

3.1. Metals in deep-sea anemones

For the measurements of metal concentrations in deep-sea anemones it was only possible to show the mean (μ) ± SD (σ) for the individuals from the BGR contract area that were not subjected to the Patania II trial. As only 1 individual per condition were retrieved, from the GSR area, one from 1 week after the trial with Patania II occurred and one before the trial, no significant differences can be ascertained, and a visual description is presented here (Fig. 3.1).

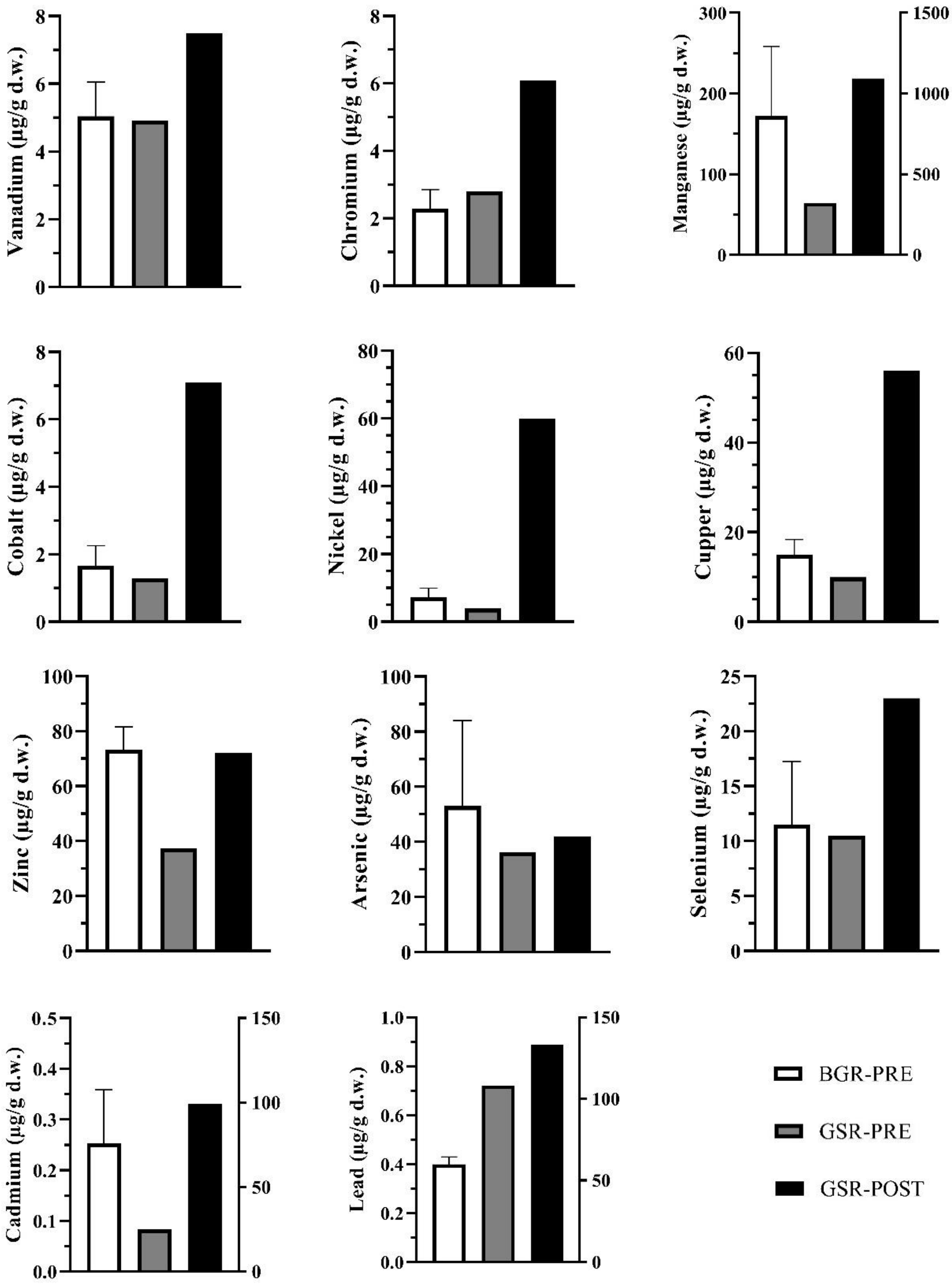

Fig.3.1-Metal concentrations in deep-sea anemones collected from the deep-sea contract areas GSR and BGR. It was not possible to calculate any error for the individuals from GSR-POST and GSR-Pre (n=1, respectively). Manganese, Cadmium and Lead have a right Y-axis for GSR-POST concentrations, and a left one for BGR-PRE and GSR-PRE, to better visualize the concentrations.

Every metal, except Zinc and Arsenic, show contrasting concentrations in the GSR-Post individual, ranging from 1.49 times (Vanadium) to 393 times (Cadmium) higher, than the concentrations found in the BGR-Pre group (Table 3.1, ratio1). Besides Cadmium, Lead was also around 300 times more concentrated in the individual exposed to the plume generated by Patania II, hence the log10 scale in the “y” axis of both graphs in Figure 3.1. Overall, but without Cd and Pb, metals concentrations were almost 3.5 times higher in the affected individual when comparing to the group BGR-Pre. On the other hand, the GSR-Pre individual and the BGR-Pre group show more similar values (Table 3.1, ratio 2), with an average ratio for all metals of 0.82, ranging from 0.332 (Cd) to 1.80 (Pb).

Table 3.1- Metals concentrations in deep-sea anemones whole tissue (ug/g d.w.). a: n=4; b: n=3.

	V	Cr	Co	Ni	Cu	Zn	As	Se	Cd	Pb	Mn	n
BGR-PRE (µ±σ)	5.05 ±1.01	2.32 ±0.571	1.65 ±0.571^a	7.26 ±2.62^a	14.9 ±3.51^a	73.2 ±8.44^a	39.4 ±4.30^a	11.5 ±5.86	0.251 ±0.105^a	0.400 ±0.0306^b	172 ±86.2^a	5
GSR-PRE	4.93	2.80	1.30	3.93	9.98	37.5	36.2	10.5	0.0833	0.721	64.4	1
GSR-POST	7.51	6.09	7.13	60.0	56.2	72.4	42.0	22.8	98.6	133.3	1092	1
Ratio1	1.49	2.63	4.32	8.26	3.79	0.990	1.07	1.99	393	333	6.35	-
Ratio2	0.98	1.21	0.787	0.541	0.671	0.512	0.919	0.916	0.332	1.80	0.374	-

3.2. Biomarkers in deep-sea anemones

SOD, CAT, GST and LPO were measured on anemone whole tissues and the sample size (n) was only of 1 individual for GSR-PRE and GSR-POST and 5 individuals for BGR-PRE (Table.3.2), so no statistical tests between areas or treatments were feasible. Nevertheless, as such data might be relevant for future deep-sea studies, it is reported in this work.

Table.3.2- Biomarkers of cellular oxidative stress in anemones retrieved from the deep-sea contract area GSR and BGR. GSR values do not show standard deviation due to having only one individual available for the analysis for each treatment (pre and post Patania II).

Area/Treatment	SOD (U/mg prot)	CAT (µmol/min/ mg prot)	GST (µmol/min/ mg prot)	LPO (nmol/mg prot)	n
GSR-PRE	83.635	9.949	0.03422	1.2534	1
GSR-POST	311.12	6.3814	0.001478	3.33706	1
BGR-PRE (mean ± SD)	83.67±19.45	8.0938±2.41	0.0696±0.05448	2.0164±0.2261	5

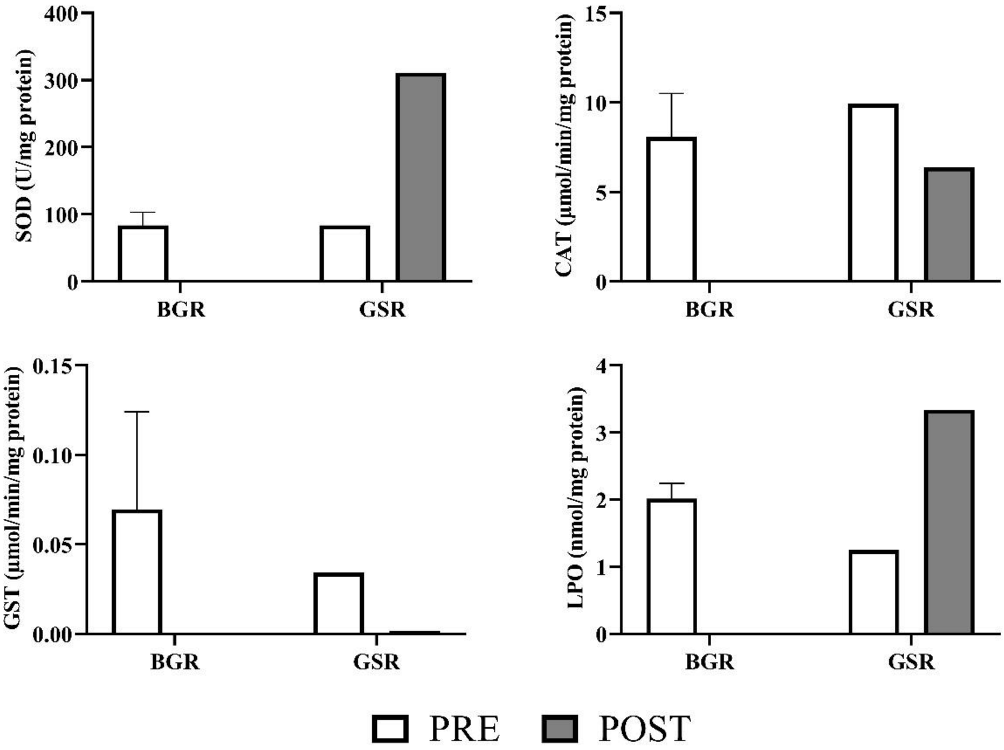

Fig. 3.2- Antioxidant enzymes (SOD and CAT), GST, and LPO activities in deep-sea anemone collected from the contract areas GSR and BGR. GSR readings do not have error bars since only one individual was analysed for each treatment in this area.

The individual from GSR-Post showed almost 4 times more activity of SOD when compared to individuals from pre-Patania treatment, CAT activity showed similar activity in all samples, and GST showed high variance in individuals from the group BGR-Pre and an accentuated lower activity for the individual exposed to the plume (GSR-Post) (Fig. 3.2). LPO concentrations were 1.5 and 2 times higher in GSR-Post individual when compared to BGR-Pre and GSR-Pre, respectively (Fig. 3.2).

3.3. Metals in water from mussel exposure experiment

Since each tank was sampled once per each sampling day per each treatment, it was not possible to run statistical tests having Time of Exposure and Treatment as factors at the same time (n=1), so one had to be ignored (by averaging observations over one factor) according to the testing hypothesis. Observations were then averaged over the levels of Time of Exposure (n=4), to test whether metals were (or were not) more concentrated in tanks with higher concentrations of sediments. From the 13 analysed metals, only 4, Cobalt, Copper, Manganese and Gallium showed statistically significant differences ($p<$

0.05). The tanks exposed to 50 mg/L of deep-sea sediment showed greater concentrations of these 4 metals when compared to the control groups (Fig 3.3).

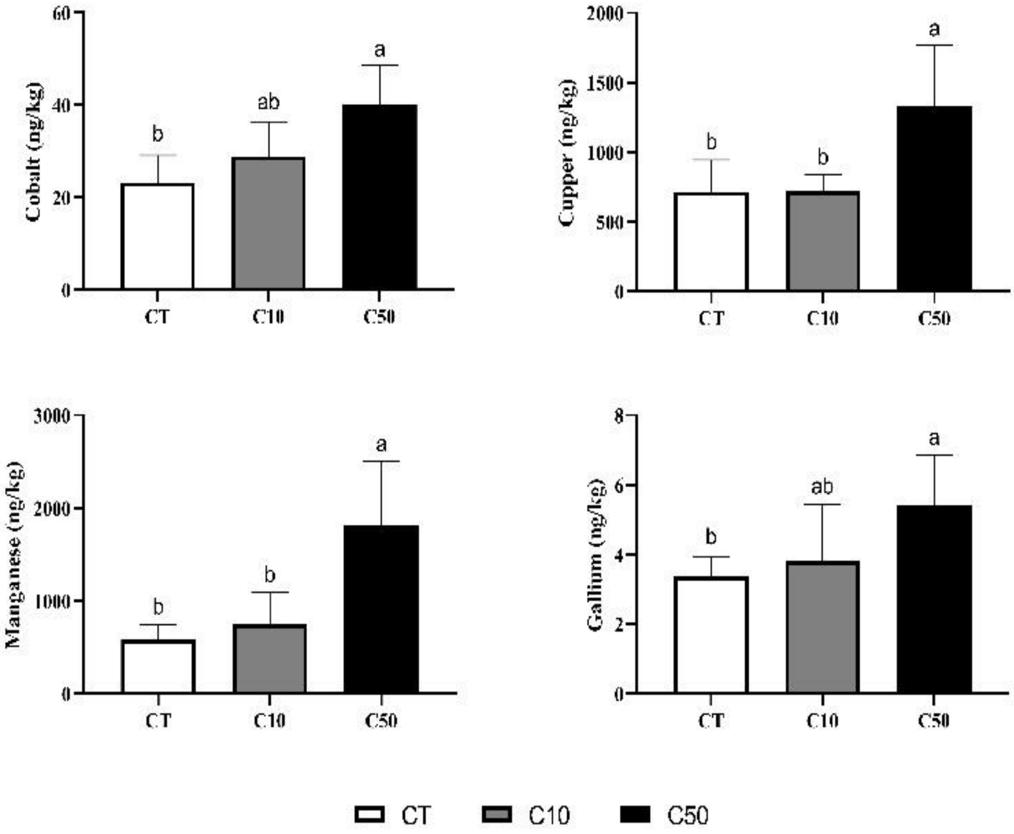

Fig.3.3- Metal concentrations (Co, Cu, Mn, and Ga) in tank waters unexposed (CT) and exposed to two concentrations of deep-sea sediments (10, and 50 mg/L). Different letters indicate a significant difference among treatments (ANOVA; $p < 0.05$).

Furthermore, the tanks exposed to 10 mg/L showed no significantly different concentrations when compared to the control tanks ($p > 0.05$). Tanks exposed to 50 mg/L showed higher concentrations of Copper and Manganese when compared with tanks exposed to 10 mg/L while Cobalt and Gallium showed no significant differences for the same comparison (Fig. 3.3). The remaining results of heavy metal concentrations in tank waters can be found in Annex 1.

3.4. REEY concentrations in waters

From the 14 Rare Earth Elements and Yttrium (REEY) that were analysed, only 7, Eu, Yb, Dy, Y, Ho, Er and Lu, showed statistically significant results, as seen in Figure 3.4.

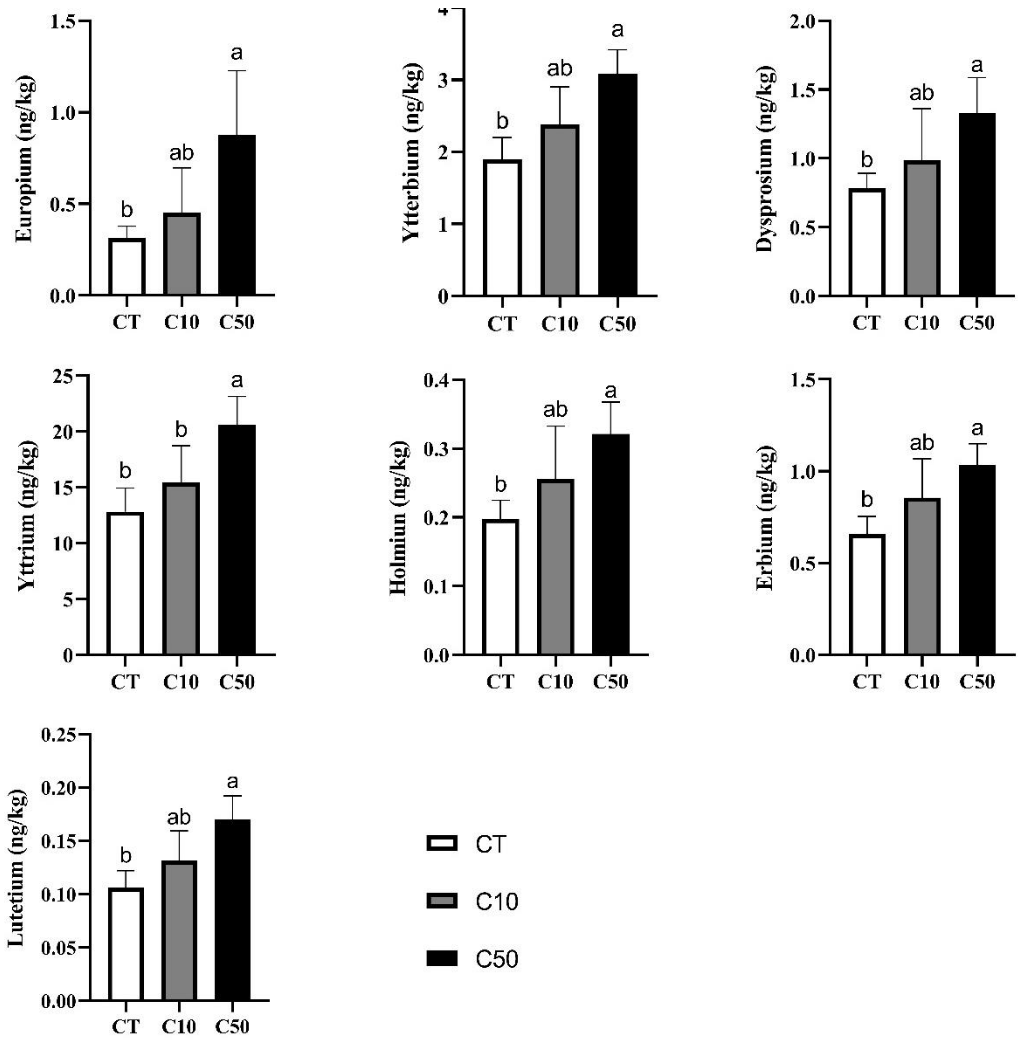

Fig.3.4- REEY concentrations in tank waters (ng/kg) unexposed (CT) and exposed to two concentrations of deep-sea sediments (10, and 50 mg/L). Different letters indicate a significant difference among treatments (ANOVA; $p < 0.05$).

For these elements, control tanks had significantly lower concentrations than tanks exposed to 50 mg/L ($p< 0.05$). Tanks exposed to 10 mg/L didn´t show any significant difference when compared with control groups ($p> 0.05$). Moreover, tanks exposed to 10 mg/L showed no statistically significant differences amongst tanks exposed to 50 mg/L, except for Yttrium that showed statistically lower concentrations in the 10 mg/L treatment tanks. The graphs for the remaining REEs concentrations are Annex 2.

3.5. Metals in mussel gills

From the 10 metals that were measured in the mussels gills, only chromium and manganese, showed statistically significant differences (Fig. 3.5).

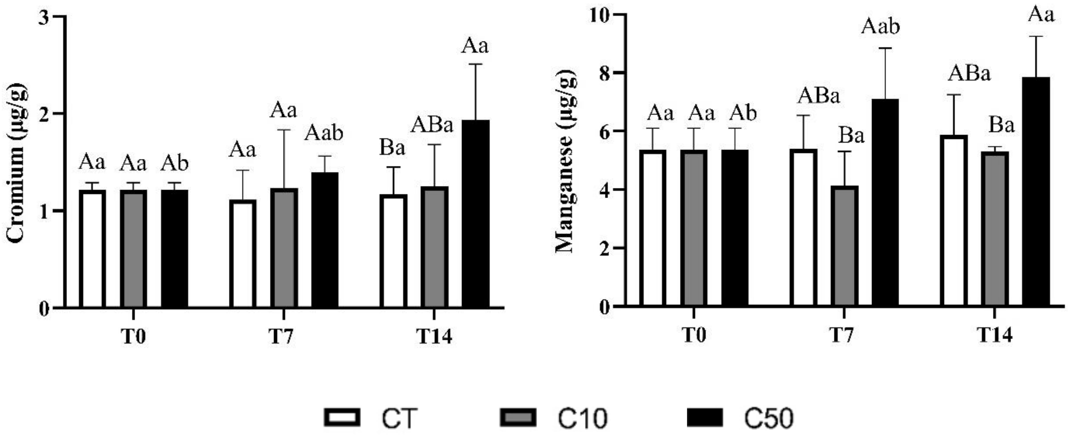

Fig.3.5- Metal concentrations (Mn, and Cr) in gills of *Mytilus galloprovincialis*, unexposed (CT) and exposed to two concentrations of deep-sea sediments (10, and 50 mg/L) for 14 days. Different capital letters indicate a significant difference among treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment among different times (ANOVA; $p < 0.05$).

After 7 days and 14 days of exposure, manganese showed higher concentrations in mussels exposed to 50 mg/L of deep-sea sediment when compared with the ones exposed to 10 mg/L ($p< 0.05$) and no differences were obtained when compared to the control groups of each correspondent exposure time ($p> 0.05$). Also, manganese had significantly higher concentrations in mussels exposed to 50 mg/L for 14 days, when compared to initial conditions ($p< 0.05$), but no significant differences were reported when compared to mussels exposed for 7 days to the same concentration of deep-sea sediment. Mussels showed statistically significant differences in Chromium concentrations between the groups of mussels sampled after 14 days, with mussels exposed to 50 mg/L having higher concentrations than the control group ($p< 0.05$) only. Also, Chromium had significantly higher concentrations in mussels exposed to 50 mg/L after 14 days of exposure, when compared to initial conditions ($p< 0.05$), and no differences were found when compared to mussels exposed for 7 days ($p> 0.05$). The figures for the remaining metal concentrations measured in the gills of *Mytilus galloprovincialis* are in Annex 3.

3.6. Biomarkers in mussel gills

Given the experimental design of the bioassay, biomarkers results are presented here following firstly comparisons between groups not exposed to deep-sea sediment (control), followed by comparisons between different treatments for the same exposure time and finally comparison overtime for the same treatment.

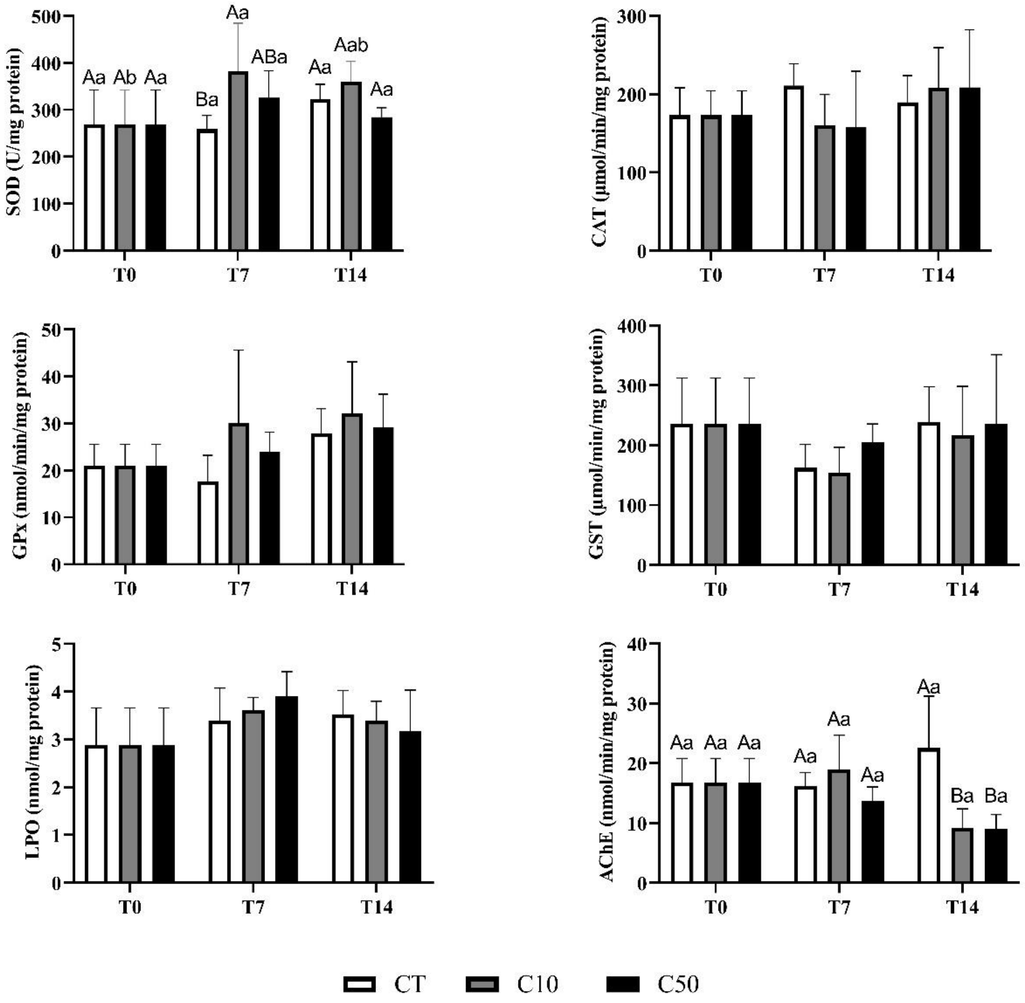

Fig.3.6- Biomarkers of oxidative stress (SOD, CAT, and GPx), GST, LPO, and AChE activity in gills of Mytilus galloprovincialis, unexposed (CT) and exposed to two concentrations of deep-sea sediments (10, and 50 mg/L) for 14 days. Different capital letters indicate a significant difference among treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment among different times (ANOVA; $p < 0.05$).

Overall, there were no significant differences in the control groups of all biomarkers ($p > 0.05$) (Fig. 3.6). Concerning comparisons between exposed groups for the same exposure time, SOD activity was only significantly higher for mussels exposed to 10 mg/L of sediment after 7 days of exposure, compared to control ($p < 0.05$) (Fig. 3.6). Similarly, SOD activity showed to be significantly higher after 7 days of exposure, compared to mussel's initial conditions ($p < 0.05$). AChE activity was significantly lower in the mussels exposed to 10 and 50 mg/L of sediment after 14 days, compared to controls (Fig. 3.6). Both groups, 10 and 50 mg/L, showed similar AChE activity (Fig. 3.6).

There were no statistically significant differences amongst control groups, nor between groups for the same exposure time nor overtime for the remaining biomarkers ($p>0.05$) (Fig. 3.6).

3.7. DNA damage

The DNA damage, expressed as % DNA tail, in the hemocytes for the different treatments and times, of control and exposed mussels to the deep-sea sediments, is displayed in figure 3.7.

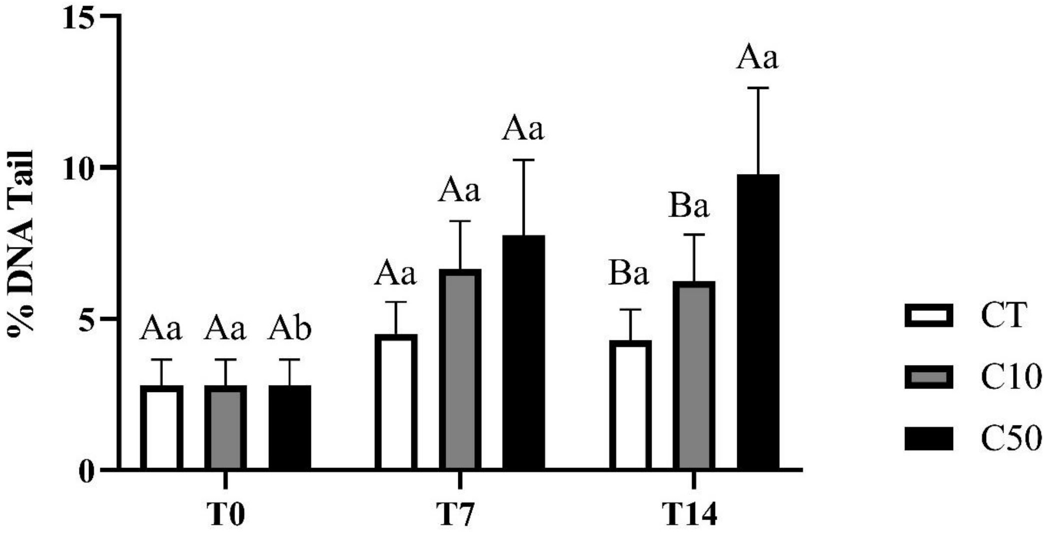

Fig.3.7- DNA damage in hemocytes of *Mytilus galloprovincialis*, unexposed (CT) and exposed to two concentrations of deep-sea sediments (10, and 50 mg/L) for 14 days. Different capital letters indicate a significant difference among treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment among different times (ANOVA; $p < 0.05$).

There were no statistically significant differences among control groups ($p > 0.05$), neither between groups exposed to deep-sea sediment after 7 days of exposure ($p > 0.05$). The mussels collected after 14 days of exposure that were exposed to 50 mg/L of sediment showed significantly higher values than the control group and the group of mussels exposed to 10 mg/L ($p < 0.05$). The latter were not significantly different from each other ($p > 0.05$). The same group (T14, C50) was also significantly higher than the mussels from initial conditions ($p < 0.05$). The mussels that were exposed to 50 mg/L, after 7 days, showed significantly higher values than initial conditions ($p < 0.05$), but were not different from the control groups of its respective time of exposure ($p > 0.05$).

4. Discussion

The deep sea is still a distant world for us, raising many difficulties as we try to understand it. The depths one might want to study are practically unreachable for the “ordinary” scientist and that’s why it’s important to share small efforts like this with the rest of the scientific community to help determine the chemical parameters and their natural ranges/backgrounds associated with such environment and its fauna, as well as assessing possible toxic effects due to human activities (like deep-sea mining). Bearing in mind, that only one of the anemones grabbed from the deep sea was subjected to the sediment plume produced by the nodule collector prototype (Patania II), it could still be worth to discuss whether such trial showed any apparent impact on this single individual. Fortunately, with replicates from areas that were not affected by the plume, it is possible to help understand what the normal, or at least expected, conditions might be for the anemones that inhabit the areas close to the mining zones. Comparing it with the exposed individual might shed a light on whether the plume had an impact in metal bioaccumulation and selected enzymes activity. From figure 3.1 it is noticeable that metals, except Zn and As, seem to be in higher concentrations in the individual exposed to the plume when compared to anemones that were not exposed to it. The same comparison between the GSR-Pre individual and the BGR-Pre group (Table 3.1) can help in understanding how different both areas might be under normal circumstances and if the values from GSR-Post could be seen as anomalous. It seems that metal concentrations in non-exposed anemones of both areas look somewhat related, meaning that were found in the same order of magnitude or were at least closer (in the case of Cd and Mn) than when comparing with the individual from GSR-Post. It is notorious that Mn, Co, Ni, Cu, Cd and Pb are the metals with greater differences between the affected individual and the BGR-Pre group and the GSR-Pre individual, which could be somewhat expected since the polymetallic nodules found at the abyssal plains of the CCZ are mainly composed of such metals (Hein & Koschinsky, 2014). Besides, Lin et al. (2022) has concluded that the sediments from a western contract area of the CCZ are naturally richer in metals found in nodules, like Mn, Co, Cu and Ni, compared to several coastal sediments. So, the differences found between anemone treatments might be related with a direct effect of the sedimentation of the plume on the anemone that could have led to the bioaccumulation of several metals in its tissues. This is supported by studies made on a shallow-water anemone, *Aiptasia pallida* (Brock & Bielmyer, 2013) and a deep-sea anemone from a hydrothermal vent field *Paraphelliactis pabista* (Escobar-Chicho et al., 2019), that show the metal bioaccumulation capacity of Actiniaria species when exposed to metal mixtures or naturally metal enriched vent fluids. The concentrations of metals, except Zn and Co, found in the control (Pre-Patania II) anemones from this study are in the same order of magnitude than the anemone studied in Escobar-Chico et al. (2019). Furthermore, Mn, Zn and As were found at higher concentrations in both studies, for the same comparison between controls, although Mn showed higher concentration than Zn in this study, and the opposite was noted in the anemone from the vent system, and As was the third highest concentration in both studies. According to Escobar-Chicho et al., the anemone *P. pabista,* showed no visible sign of stress or malformations while exhibiting high concentrations of trace metals. The same could apply to the Actiniaria spp. studied in this work, since both species live in deep-sea ecosystems enriched in metals (although with different compositions and proportions).

The possible physiological effects of exposure to a metal-enriched natural environment, as well as those of exposure to the sediment plume were evaluated. The activity of antioxidant enzymes and LPO showed visually interesting values. The first antioxidant enzyme that neutralizes ROS species in cells, SOD, shows a considerably higher activity in the anemone collected one week after exposure to the plume, however CAT, an enzyme expected to work alongside SOD to fight ROS, does not show an obvious difference in activity between treatments, while LPO levels appear to be higher in the exposed individual. There is more than one way to explain such results but, due to low number of replicates (GSR-Post n=1; GSR-Pre n=1; BRG-Pre n=5), no significant statistical evidence can be presented, however a logical and supported discussion might clarify the possible ecotoxicological effects of sediment plumes from nodule fields in deep-sea fauna. The simplest way to understand what might have happened in the anemone exposed to plume is, after exposure, SOD activity was activated as expected due to high production of ROS, but could not keep up with ROS production, what possibly lead to the cellular damage indicated by LPO levels. It could also be, that LPO was somewhat held, and CAT was masked by GPx activity (not analysed), an enzyme that catalyzes the reduction of the same product as CAT (H_2O_2) and might reduce LPO levels by dealing with some of its sub-products (Regoli and Giuliani, 2014). Another factor that might explain the apparent absence of an expected increase in CAT activity, is the fact that certain metals catalyze Haber -Weiss and Fenton reactions (Halliwell and Gutteridge,

2007) that convert H_2O_2 to $HO^{\cdot}$, a highly reactive initiator of LPO. Since the exposed anemone had high concentrations of several metals in its tissues it is likely that such reactions might have occurred, also reasoning in favor of the higher LPO levels found. Finally, Brock & Bielmyer (2013), have reported a decrease in CAT activity in the shallow-water anemone, *A. pallida*, after 3-day exposure to mixtures of metals (Cu, Zn, Cd and Ni) at 3 different concentrations (10µg/L, 50 µg/L and 100 µg/L of each metal) and a normalization back to control activity after 7 days of exposure at 10µg/L and 50 µg/L of the metal mixtures. The only deep-sea anemone from the GSR area, that was collected 7 days after the plume was produced, appears to be in the same ranges for that time, following that a possible inhibition of CAT activity previously to collection might have occurred.

Regarding the experiment of exposure of mussels *Mytilus galloprovincialis* to deep-sea sediment over the period of 14 days, results generally translate an uncommon physiological response, nevertheless, it is important to notice that environmental alterations were found among the different tanks. While the antioxidant system, GST, and LPO of mussels exposed to deep-sea sediments showed no alterations compared to control conditions, AChE and DNA damage, biomarkers of neurotoxicity and genotoxicity respectively, were significantly influenced. AChE activity was inhibited in both groups of mussels exposed to both concentrations of deep-sea sediments after 14 days of exposure. Damage to DNA of hemocytes seems to show an increasing trend with increasing concentrations of deep-sea sediment, being that after 14 days of exposure to the highest concentration, mussels had significantly more DNA damage overall. It seems that it was only after 14 days that mussels, mainly the ones exposed to 50 mg/L of sediment, show signs of oxidative damage to DNA and neurotoxicity.

It has been assessed that metals associated with nodules have been accumulating in the seabed over the last millions of years, and those metals might also get enriched in the surrounding waters of nodule plains during mining activities, however information about potential toxic metals being released during DSM is scarce (Kwan et al., 2023). The enrichment of certain metals (Co, Mn, Cu, and several REEY) in the water tanks treated with 50 mg/L of sediment (Fig. 3.3 and Fig. 3.4) indicates that the metal composition of the deep-sea sediments used for the exposure assay resembles the composition of polymetallic nodules. Manganese, as expected, and Chromium, that was not measured in waters, were the only metals to be in significantly higher concentrations in the gills of mussels of those same tanks (Fig. 3.5), indicating a possible accumulation of Mn and Cr after 14 days.

Interestingly, studies that assessed the environmental quality of potential “contamination hotspots” (Cravo et al. 2009) and the environmental hazard of plumes from marine mine tailings (Mestre et al. 2017), presented lower concentrations of Cr in whole tissue and gills respectively, of *Mytilus galloprovincialis*, when compared to the amounts of Cr found in the gills of mussels exposed to 50 mg/L of deep-sea sediment for 14 days (this study). Based on this, focus will be given in Cr and Mn toxicity, to try to explain the damage found in mussels. Polymetallic nodules are mainly made of manganese oxide mineral layers, like phyllomanganates, that have the potential to sorb metals (Kuhn et al., 2017). In fact, Dai et al. (2009), confirmed that Cr(III) is easily oxidized to Cr(VI) in the presence of birnessite, a phyllomanganate, with the concomitant reduction of the manganese oxides. In turn, Cr(VI), which exists as chromate (CrO_4) in the environment, can pass through cell membranes using sulfate transporters due to its structural similarity with sulfate (SO_4) and reach the nucleus of cells (DesMarias & Costa, 2019; Peana et al. 2021). In the cell, Cr(VI) is highly toxic. Its reduction to Cr(V), Cr(IV), and Cr(III) produces ROS that cause oxidative damage, changes epigenetic gene expression, and cause direct DNA damage (Shi et al., 1994; DesMarias & Costa, 2019; Peana et al., 2021). Furthermore, several studies have reported the effects of Cr(VI) exposures at different ranges (ng/L, µg/L and mg/L), different time scales (4h, 4 days and 7 days) and with chronic and acute toxicity assays (Emmanouil et al., 2006, 2007; Barmo et at., 2011; Franzellitti et al., 2012; Mu et al., 2023). Succinctly, acute exposures to 10.4 µg/g and 0.104µg/g of Cr(VI) show increased DNA damage in gills of *Mytilus galloprovincialis* at 4h after exposure (Emmanouil et al., 2006, 2007). It is worth to mention that DNA damage reported by the comet assay can be related to repair of oxidative DNA damage under the lowest concentrations mentioned (Emmanouil et al., 2006). An exposure in the range of 10’s ng/L of Cr(VI) for 1 week reported significant accumulation the in digestive gland of *Mytilus galloprovincialis* and altered expression of mRNA encoding genes related metal resistance and antioxidant processes among others (Franzellitti et al., 2012). Two studies, Emmanouil et al. (2007) and Barmo et al. (2011), exposed the mussel *Mytilus galloprovincialis* to Cr(VI), in the total range of 0.1 to 200 µg/L, for 7 and 4 days, respectively. There were no significant alterations of CAT and GST activity from 0.1 to 100 µg/L nor in LPO in all concentrations tested, but Cr(VI) exposure was related with metal accumulation in the digestive gland of mussels, affected lipid metabolism and gene expression. Exposure to higher concentrations of Cr(VI), in the range of mg/L, have showed CAT inhibition, induced GR activity and MDA content (LPO product) as well as GST increased expression in the clam *Geloina erosa* (Mu et al., 2023). The Cr concentration found in the gills of affected mussels (≈2 µg/g) corresponds to a contamination of around 50 ng/L, according to the results of Franzellitti et al (2012). The biomarkers response from this work are in accordance with the results of the studies mentioned above for low concentrations of Cr(VI), in which oxidative stress does not appear to occur but cause DNA damage.

Manganese toxicity has been widely studied in rats and humans due to its relationship with neurotoxicity, and has been showed to affect the cholinergic system, either by induction or inhibition of AChE activity, depending on the methodology of exposure, however it is not completely understood how Mn acts on this system (Santos et al., 2012; Peres et al., 2016). In echinoderms, it has been shown that exposure to 12 mg/L of Mn causes motoric disorder and Ache induced activity (Sköld et al., 2015). Studies in other marine organisms have also showed inhibition on AChE activity in the presence of mixtures of metals found in contaminated locations and in petrochemical sites contaminated with several hydrocarbons and metals (Gaitonde et al., 2006; Maisano et al., 2017). A recent study also denotes the importance of Cr(VI) and its neurotoxicity once it reaches the human brain, as well as an inhibitory effect on AChE activity (Jr. Wise et al., 2022). Overall, based on literature, and considering that the concentrations of deep-sea sediment used in this work were very low, the biomarkers responses are somewhat in agreement with other studies of similar exposures.

As seen by the mussel assay, small concentrations of deep-sea sediment (50 mg/L) are enough to cause damage in cells. This is not remotely close to a full-scale deep-sea mining operation, let alone several operations at a commercially viable extraction rate. Recent modeling of deep-sea mining plumes, at an industrial scale, in the CCZ accounted for a discharge rate of 280 kg/s and predicted that 50% of the sediments would settle within 10 days and within several kilometers of the source region (Gillard et al., 2019). Such great amounts of sediments in suspension are probably going to release great amounts of metals, which, according to the results found in this study, might became toxic in a long-time exposure, even to deep-sea fauna, however the lack of comprehensive studies conducted at realistic magnitudes in both spatial and temporal dimensions leaves us uncertain about the extent, duration, and outcomes of the effects brought about by deep-sea mining. Moreover, when assessing ecotoxicological risk associated with deep-sea mining plumes, it is essential to gather data from multiple sources and integrate all to provide a more comprehensive understanding of the potential hazards involved (ISA, 2022).

Weight of evidence (WOE) is a promising tool used in environmental risk assessment, to characterize sediment hazard and it is applied when different types of science-based evidence (Lines of Evidence- LOEs) are needed to draw a conclusion (Burton et al., 2002; Piva et al., 2011; MERAG, 2016; Mestre et al., 2017; Suter et al., 2017a; Suter et al., 2017b; van Doorn et al., 2022). Including multi-disciplinary LOEs allows for a final integration of the results which facilitates decision-makers to take a balanced and informed decision based on potential risks caused by any specific activity. Several general WOE frameworks have been developed to infer qualitative and quantitative data, and there isn’t a standardized way of building a WOE, so each author must adapt the model to its study (Burton et al., 2002; Chapman, 2007; MERAG, 2016; Suter et al., 2017a, 2017b). Traditionally, assessment of environmental risks and impacts requires previous knowledge of how ecosystems and organisms respond to anthropogenic disturbances (Glasson et al,.2012). However, little biological and geomorphological documentation of the deep sea represent a major issue for risk assessment plans (Kaikkonen et al., 2018). A few studies have used WOE approaches to assess environmental risks of sediments in shallow marine environments (Benedetti et al., 2012; Benedetti et al, 2014; Mestre et al., 2017, Regoli et al, 2019; Manfra et al., 2021) and fewer or even none have applied it in risk assessment of deep-sea sediments (no literature was found). Nevertheless, WOE has proved to be a useful approach in environmental risk assessment of marine environments, specifically in resuspension of marine mine tailing deposits (Mestre et al., 2017), and should be applied to deep-sea mining according to the ISA, (2022). The present work gathered fundamental data to feed the WOE model, but still more baseline data is needed to adequately use this tool in deep-sea environments. Indeed, waters remaining from ore dewatering at surface must be discharged back into the ocean, and will probably be enriched in many metals, so ecotoxicological assessments must also be applied to such waters, or simulations of those conditions applied in laboratory ecotoxicological bioassays.

5. Conclusions

In the single deep-sea anemone exposed in situ to the sediment plume, several metals seem to have accumulated in its tissues and these seem to have induced oxidative stress and oxidative damage, when compared to control locations. In the experimental trial where *M. galloprovincialis* were exposed to 10 and 50mg/L of deep-sea sediment some metals were more accumulated, and genotoxicity and neurotoxicity were induced in the highest concentration after 14 days. The organisms analysed in this work were exposed to small concentrations, well below from the expected full scale of deep-sea mining operations at a commercially viable extraction rate, and there is strong evidence that ecotoxicological harm is induced. This work has further contributed with data on bioaccumulation of metals and biomarkers that may help in the elaboration of a multi-disciplinary WOE to evaluate the environmental risks of mining deep-sea polymetallic nodules in the Clarion-Clipperton Zone.

6. References

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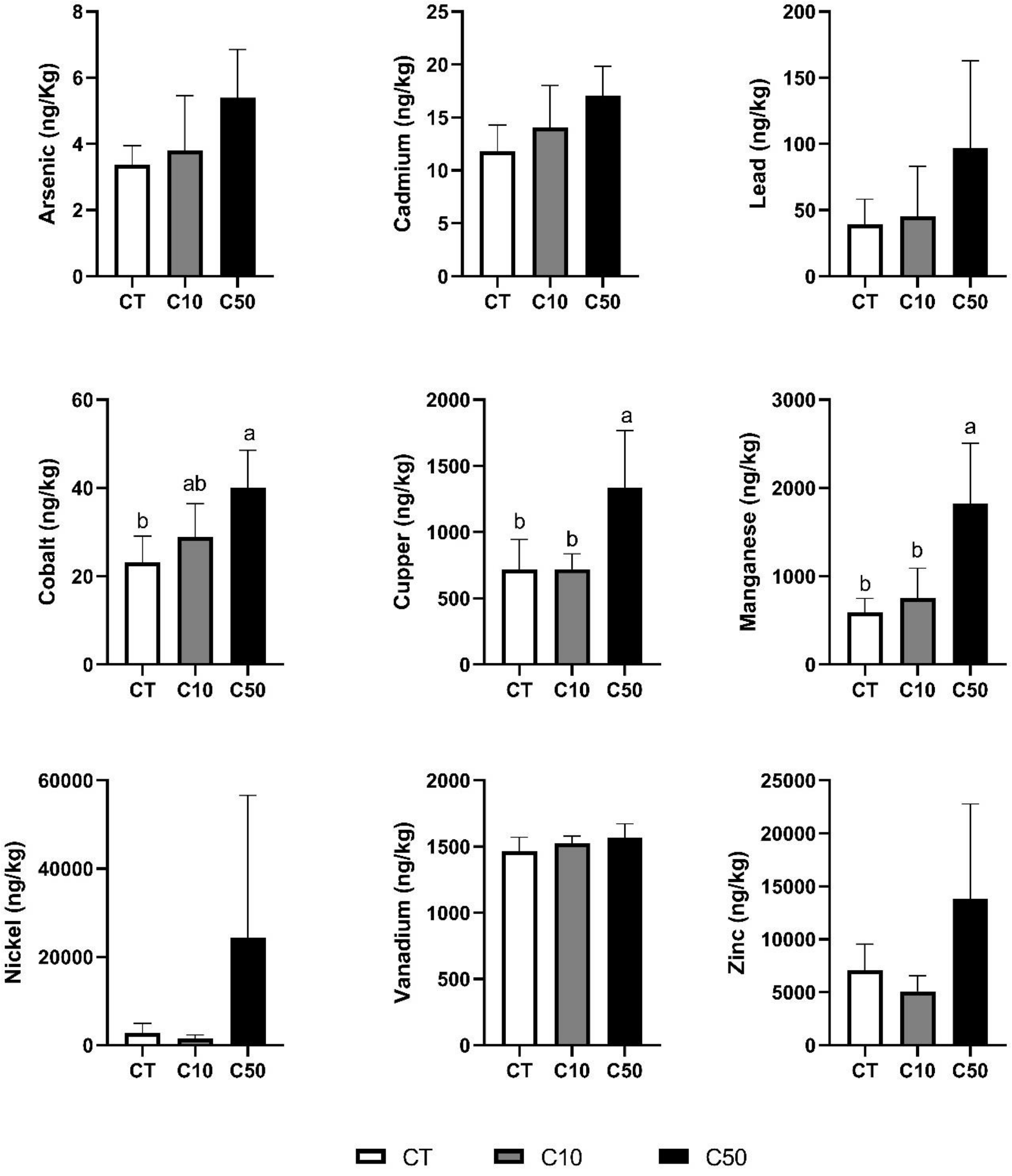
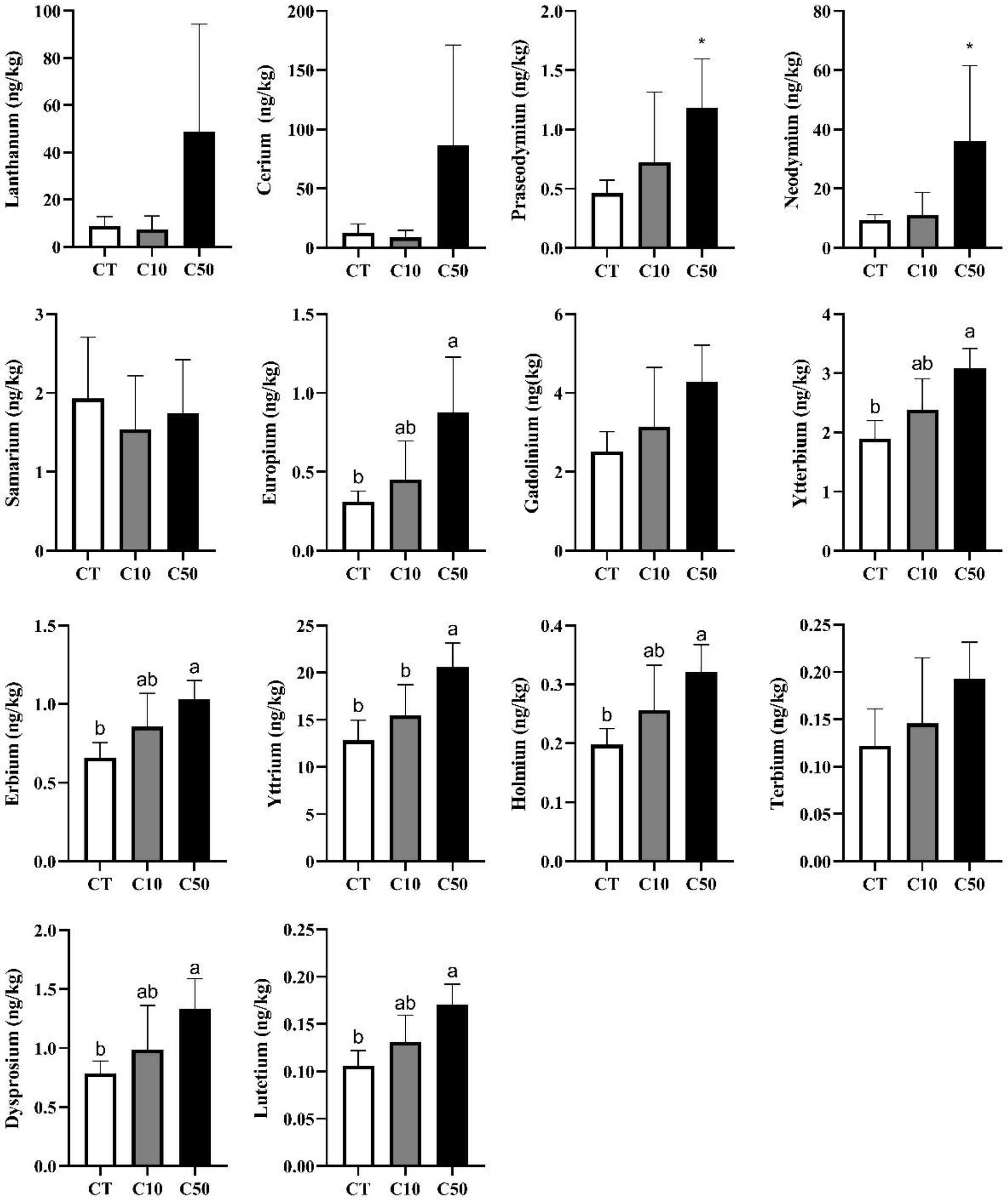
ANNEXES

ANNEX 1



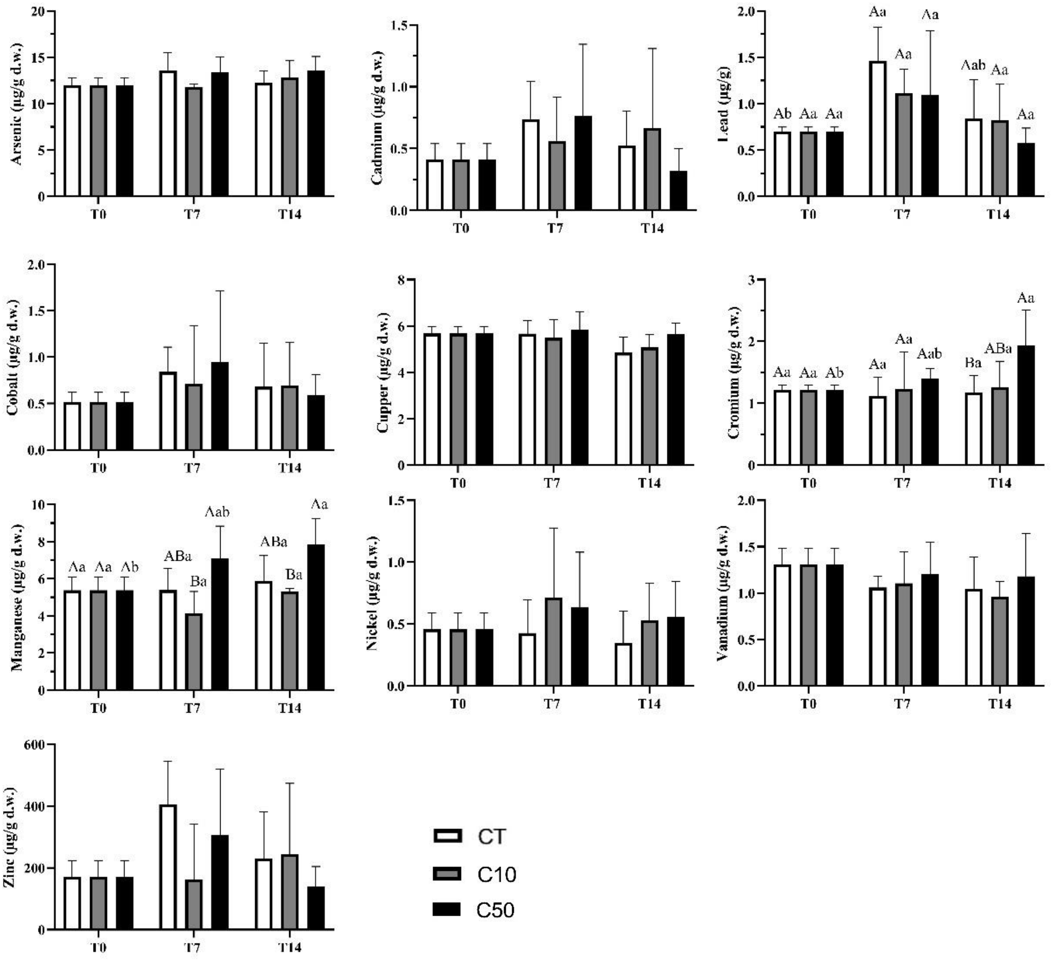
Annex 1. Metal concentrations in tank waters, unexposed (CT) and exposed to two concentrations of deep-sea sediments (10, and 50 mg/L). Different capital letters indicate a significant difference among treatments within the same treatment averaged over the levels of Time.

ANNEX 2



Annex 2. REEY concentrations in tank waters, unexposed (CT) and exposed to two concentrations of deep-sea sediments (10, and 50 mg/L). Different capital letters indicate a significant difference among treatments within the same treatment averaged over the levels of Time.

ANNEX 3



Annex 3. Metal concentrations in gills of *Mytilus galloprovincialis*, unexposed (CT) and exposed to two concentrations of deep-sea sediments (10, and 50 mg/L) for 14 days. Different capital letters indicate a significant difference among treatments within the same treatment averaged over the levels of Time.