Science of the Total Environment 951 (2024) 175628

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Review Article

Potential biomarkers of metal toxicity in deep-sea invertebrates – A critical review of the omics data

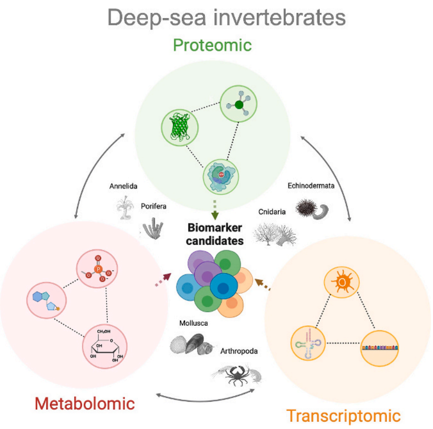
Cármen S.V. Sousa[a], Jin Sun[b], Nélia C. Mestre[a,*]

[a] Centre for Marine and Environmental Research (CIMA), Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
[b] Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao 266003, China

HIGHLIGHTS

- Fifty omics studies on metal-related exposure in deep-sea invertebrates reviewed
- 41 biomarkers candidates prevailed in different omics approaches and various species.
- 28 potential biomarkers identified from the few studies with metal challenges
- Metal toxicity induced changes in immune system, metabolism and oxidative stress.
- Validated biomarkers can be helpful to implement future disturbance threshold levels.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Susanne Brander

Keywords:
Mineral exploitation
Deep-sea organisms
Omics
Ecotoxicology
Biomarkers

ABSTRACT

Deep-sea mining (DSM) activities are expected to release potentially toxic metal mixtures through the generation of sediment plumes to the marine environment. This may disrupt the normal functioning of biological mechanisms, adversely affecting deep-sea invertebrate organisms. It is thus essential to understand the ecotoxicological effects from these toxic elements in deep-sea organisms and the omics approaches applied to ecotoxicology are seen as promising tools. Here, we provide an overview of the principal biological modifications identified in deep-sea invertebrates when exposed to metals and critically evaluate the current knowledge and discuss which potential biomarkers may be useful after metal exposure. Most of the 50 omics studies on deep-sea invertebrates revised are comparative transcriptomes ($n = 41$). Forty-three potential biomarker candidates are highlighted from immune system, 46 from cellular metabolism and 29 from oxidative stress. The processes mostly affected by metal toxicity in deep-sea invertebrates are related to innate immune defense; sulfur, chitin, and catabolic metabolism; antioxidation; and detoxification. We acknowledge the current limitations and future perspectives for their uses and emphasize the need to invest in further ecotoxicological studies using the omics approaches.

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* Corresponding author.
E-mail address: ncmestre@ualg.pt (N.C. Mestre).

https://doi.org/10.1016/j.scitotenv.2024.175628
Received 5 April 2024; Received in revised form 30 July 2024; Accepted 16 August 2024

1. Introduction

The deep sea (>200 m depth) is widely regarded as the largest ecosystem that covers approximately between 50 and 65 % (360 million km^2) of the Earth surface (Danovaro et al., 2014; Taylor and Roterman, 2017). This unique ecosystem is characterized by its harsh conditions such as the extremely high-hydrostatic pressure, cold temperature, low dissolved oxygen content, and no sunlight (Miller et al., 2021). In addition, the availability of food can be critical and sometimes limiting except for chemosynthetic ecosystems (Vecchione et al., 2023). The deep-ocean floor is mostly abyssal plain at depths between 3000 and 6000 m but comprises other features such as canyons, seamounts, mid-ocean ridges and trenches, underwater volcanoes, or hydrothermal vents (Miller et al., 2018).

The deep sea provides a range of important ecosystem functions largely driven by chemosynthetic derived energy (instead of photosynthesis), such as global biogeochemical cycling, carbon sequestration and vast energetic and mineral reserves (Thurber et al., 2014). Chemosynthetic ecosystems from the deep sea (e.g. hydrothermal vents and cold methane seeps) share common characteristics, for instance the presence of reduced chemical compounds which composition varies among regions and geological settings (e.g. sulfide, methane, hydrogen, radionuclides and mineral-rich particles), local hypoxia or anoxia, increased microbial primary production and transmission of chemical and heat energy to the water column via plumes (Levin et al., 2016). On the other hand, high temperatures (exceeding 350 °C) are observed at hydrothermal vents when compared to cold seeps where temperature varies between 2 and 3 °C (Levin et al., 2016). Some of these ecosystems have already been damaged by human activities (e.g. fisheries, contaminants, oil and gas extraction) or face planned activities that may adversely affect them, such as those related to the extraction of mineral resources (Andrews et al., 2021). Deep-sea mining (DSM) is defined by removing extensive accumulations of minerals from the deep ocean. Four types of resources have been studied so far (Kleiv and Thornhill, 2022; Levin et al., 2019) (Fig. 1):

(1) Polymetallic or ferromanganese nodules that can be found on the sediment-covered abyssal plains at depths of 3500–6500 m and predominantly composed by manganese (Mn) among other metals (e.g. cobalt, copper, nickel). One example is the abyssal seafloor Clarion-Clipperton Zone (CCZ) located in the western Pacific Ocean that is largely known due to their nodules richness (Hein et al., 2013, 2014);

(2) Seafloor massive sulfides deposits that are present in hydrothermal vents (active and inactive) on the mid-ocean ridges and volcanoes at depths of 1000–5000 m. These deposits are naturally enriched by metal sulfides, iron, zinc, copper, lead, but also gold, silver, arsenic, cobalt, molybdenum, platinum, and Rare Earth Elements (REEs). Mid-Atlantic Ridge North between the equator and the Azores Triple Junction is well-known of having several active sites with high-temperature hydrothermal vents (e.g. Snake Pit and TAG) (Beaulieu et al., 2013; Boschen et al., 2013; Cherkashov, 2017);

(3) Cobalt-rich ferromanganese crusts at depths of 400–7000 m are present on seamounts. These crusts are mainly composed by cobalt, iron, and Mn among others, such as nickel, copper, platinum, and REE as well. The crust deposits are spread worldwide mainly in the Atlantic and Pacific regions (Hein et al., 2013, 2014);

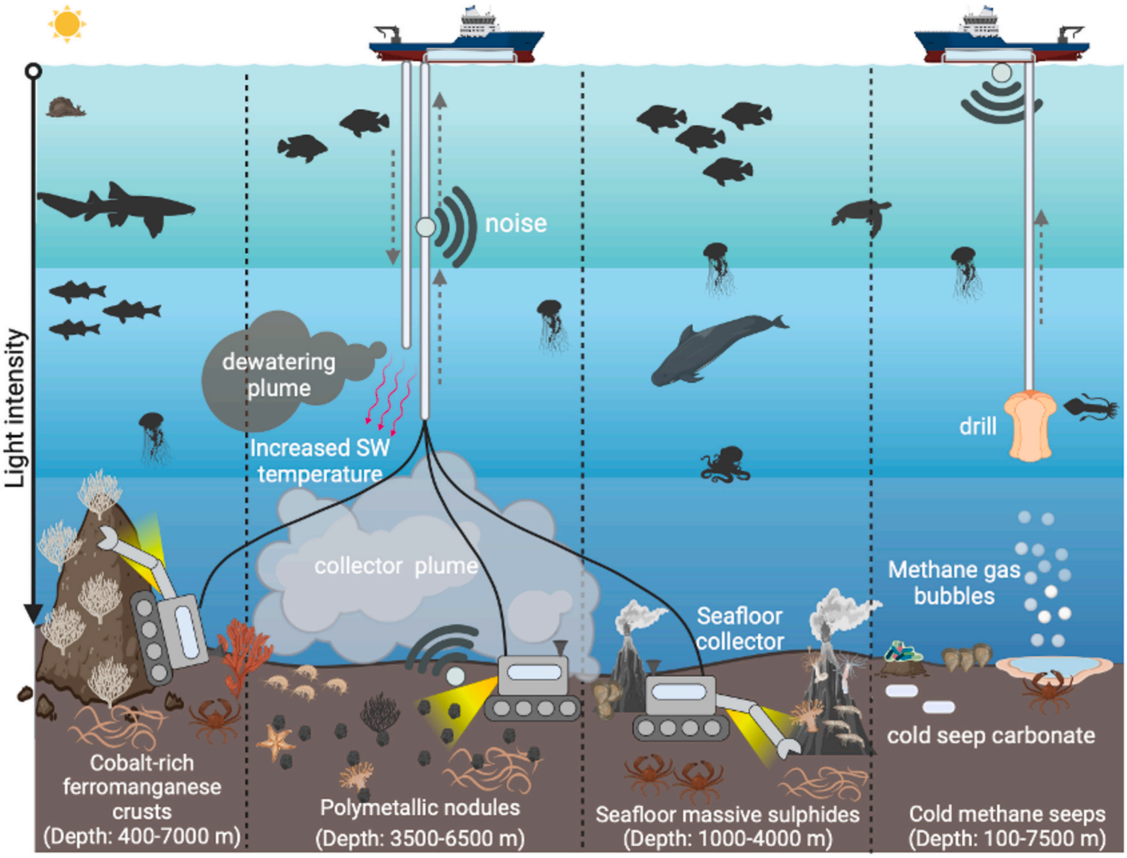

Fig. 1. Deep-sea ecosystems under deep-sea mining scenario. Cobalt-rich ferromanganese crusts, polymetallic nodules and seafloor massive sulfides, located at different depths (dashed black line).
This schematic illustration is not performed at scale and it was created with BioRender.com.

(4) Gas hydrates are primarily constituted of methane at depths of 500–7500 m. These ice-like crystals are present along continental margins and permafrost regions, whose stability is dependent of higher pressure and low temperature, salinity, and gas composition (Kvenvolden, 1999; Ranjith et al., 2017);

DSM is considered as an important strategic tool for the future of the world economy, especially for green technology (e.g. electric vehicles), which main goals are to protect the environment, repair the damage performed by humans in the past and preserve the natural resources by using alternative fuels (Kang et al., 2019). However, DSM raises so many issues and valid concerns as it will harvest non-renewable resources from the deep ocean and cause many disturbances to the environment, such as light, vibration and noise. The necessary machineries working on the deep seafloor will remove the seafloor habitat and associated organisms, with potential to disrupt food webs and ecosystem health, as well as modify the substrate, disrupting the biogeochemical processes, with implication to carbon cycling (Hallgren and Hansson, 2021; Hauton et al., 2017; Miller et al., 2018; Simon-Lledó et al., 2019). In addition, the generation and release of sediment plumes and metals, potential sub-lethal impacts of chronic exposure, rises of seawater temperature by metal washing, and other cumulative effects should be considered (Hallgren and Hansson, 2021; Hauton et al., 2017; Miller et al., 2018; Simon-Lledó et al., 2019). Consequently, these may cause irreversible damage to these unique ecosystems. Meanwhile, a consensus exists between the narrative that is in favor and against DSM regarding to the need of more environmental impact studies to obtain more knowledge for proper regulation of the activities (Hallgren and Hansson, 2021). The time sensitivity of this discussion is driven in part by the urgency to abandon fossil-fuel dependence and mitigate climate change (Hallgren and Hansson, 2021).

In ecotoxicology, aquatic invertebrates are considered good indicators to assess contaminants toxicity because they can accumulate high levels of contaminants, including metals, from their habitat (Jeong et al., 2023). Moreover, those invertebrates occupy a key position as intermediate consumers in pelagic and benthic food chains and usually have good reproductive capacities, short life cycles, and are easy handling (Morley et al., 2013). In invertebrates from freshwater ecosystems the toxicity effects from certain trace-metals is well known (Jeong et al., 2023), but this knowledge cannot be directly applied to marine invertebrates from the deep sea which makes impossible to establish threshold levels for metal toxicity for this environment. Deep-sea invertebrates hold some specific characteristics such as slow growth rates and high longevity (e.g. sponges and black corals) with recovery from harm being predicted to take decades to centuries (Marriott et al., 2020; Morrison et al., 2020).

Indices based on community structure provide a global ecological status of the biological communities, although they have limited value as early-warning indicators of contamination (Rodrigues et al., 2019). An alternative strategy to detect cellular changes (at gene level) for acute and sub-lethal responses before cellular or tissue damage is the development and incorporation of potential biomarkers across marine organisms with a special focus on invertebrates (Rodrigues et al., 2019). Biomarkers are indicators of biological state or condition of organisms by measurable parameters at various levels (e.g. biochemical, immunological, histological, and others). An early-warning indicator from contaminant exposure is one of the most useful properties of a molecular biomarker to detect changes before the biological and health effects are noted (López-Barea, 1995).

This kind of information is particularly useful for the International Seabed Authority (ISA), to take actions before an ecosystem collapse into a state from which recovery is difficult or impossible. Thus far, ISA has issued 22 contracts for the exploration of polymetallic nodules, polymetallic sulfides and cobalt-rich ferromanganese crusts in the Mid-Atlantic Ridge, CCZ, Northwest Pacific Ocean and Indian Ocean which are scheduled for a period of 15 years with the possibility of an extension for a further 5 years (ISA, 2023). Therefore, it is imperative to define detailed site-specific baselines (Katona et al., 2023), although the information available related to the biological impacts on marine organisms is still limited.

This review summarizes the existing studies and methodologies on deep-sea invertebrates that applied genetic techniques and bioinformatic tools, with emphasis on the high-throughput profiling, to assess environmental impacts at the transcript, protein, and metabolome levels. In addition, potential biomarkers to assess metal toxicity in marine invertebrates are identified, as well as the currents gaps and future perspectives. This information is essential to better understand the modifications in main biological pathways in deep-sea invertebrates when exposed to higher metal concentrations as expected under a DSM scenario.

2. Literature review search methodology

Publications with relevance to metals ecotoxicology in deep-sea invertebrates and reporting changes using omics approaches were retrieved from the Web of Science (WoS) on the 9 of February 2024. Search items were the following: ("deep sea") AND ("transcriptome" OR "proteome" OR "metabolome") AND ("mineral" OR "mining" OR "metal" OR "omics" OR "toxic"). This resulted in a total of 46 documents published between 2013 and 2024. The title and abstracts of the retrieved references was then screened for adequacy to the theme and only those of interest were kept ($n = 7$). An additional search was performed that included the search terms: ("deep sea") AND ("protein expression" OR "gene expression"); excluding ("bacteria" OR "microbiology" OR "microorganism"). A total of 209 publications were found, and after screening the title and abstracts 19 additional publications, published between 2007 and 2022, were retained. This search was complemented with publications identified as useful from a cross-referencing analysis of retrieved publications which enabled to identify further 24 publications of interest. In summary, only the publications presenting data for deep-sea invertebrates, metals exposure, including comparison with different environments enriched with metals, temperature challenges with invertebrates from these environments, comparisons between tissues, male and female comparisons, and omics approaches were considered. Nine publications reporting gene expression analysis of relevance to this review were also included. Genomic and metagenomic studies were excluded from our analysis as they were mostly related to species identification and microbiome characterization. A total of 50 studies published between 2007 and 2024 were considered in the present review. Forty-one studies reported transcriptomic approaches, followed by 8 studies in proteomic, 4 studies in metabolomic and a total of 5 used multiple omic approaches (Fig. 2, Supplementary Table S1). A total of 118 (genes or proteins) biomarkers were extracted from these studies, with the most interesting that could be explored in the future as potential biomarkers ($n = 41$) highlighted taking into account their prevalence across different omics approaches and various species (Supplementary Table S2). In addition, potential biomarker candidates from the few studies available with different metal challenges were identified ($n = 28$; Table 2).

3. Metals in deep-sea invertebrates

Metals are usually defined as elements that have a high atomic weight and a density at least five times greater than water and exhibit characteristics of metals or metalloids (Tchounwou et al., 2012). Metals can be divided into three classes: essential (e.g. iron, calcium, manganese, magnesium, zinc, sodium, potassium, copper), non-essential (e.g. mercury, cadmium, silver, lead, gold) and borderline (e.g. nickel, chromium, cobalt, arsenic) (Jeong et al., 2023). The essential metals are important in a variety of biochemical processes, such as enzymatic activity and structural proteins, and can be toxic in large amounts or certain forms. Non-essential metals have less involvement in

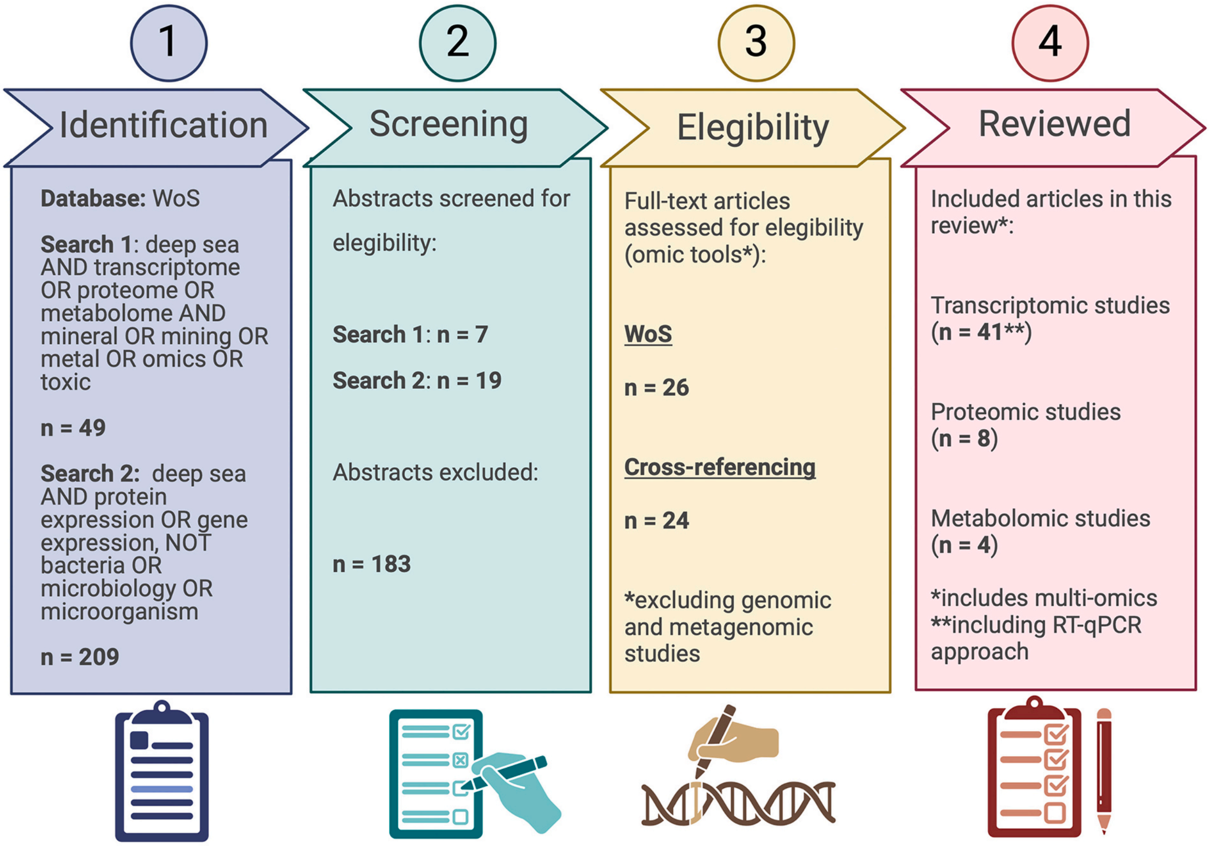

Fig. 2. Methodological screening of papers published until February 2024 related to ecotoxicological studies with omics tools in deep sea invertebrates. This figure was created with BioRender.com.

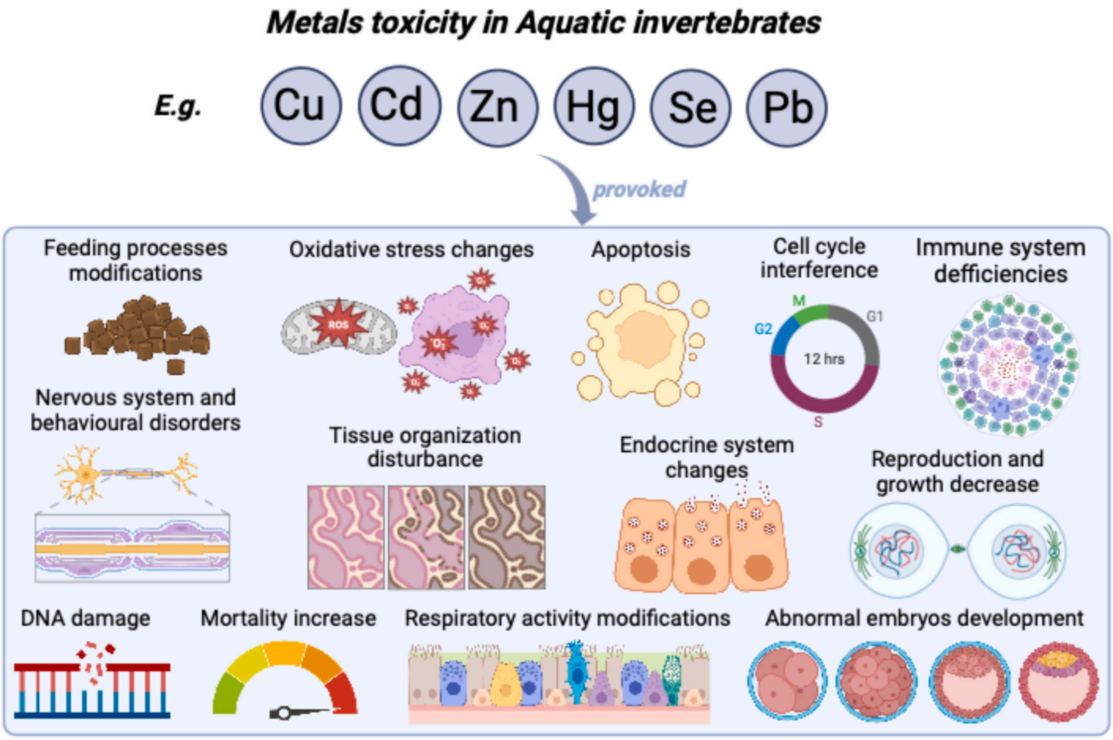

Fig. 3. Principal biological consequences of metals toxicity in aquatic invertebrates. This figure was created with BioRender.com.

physiological processes and can cause severe toxicity even at extremely low concentrations (Ghannem et al., 2023). Toxicity in deep-sea invertebrates under DSM scenario may occur by ingestion of contaminated water and/or food and direct absorption (e.g. skin, gills, and digestive gland) from immersion in contaminated seawater (Chan et al., 2021). Under a DSM scenario several factors may affect toxicity such as the dose, chemical interactions (e.g. synergetic or antagonist effects), organism species, as well as the developmental stage, size, biological sex, genetics background, mobility (e.g. sessile or not) and nutritional status of exposed organisms (Tchounwou et al., 2012).

Current knowledge shows that metals interaction with cellular components and molecular targets in aquatic invertebrates may provoke several negative consequences. For example, metal enriched environments provoked changes in lipid peroxidation and antioxidant-related genes (Cheng et al., 2024; Perić et al., 2020; Zeeshan et al., 2016), modifications in endocrine, immune system, respiration and apoptosis-related genes (Huang et al., 2022; Li et al., 2023; Liang et al., 2022; Stenvers et al., 2023), DNA damage (Barros et al., 2013; Bettencourt et al., 2011; Martins et al., 2017; Wong et al., 2015; Zeeshan et al., 2016), growth and fecundity decrease (Rebolledo et al., 2022), abnormal embryos formation (Jeong et al., 2023; Morroni et al., 2023), and mortality ratio increase (Wang et al., 2017; Jeong et al., 2023) (Fig. 3).

The potential biological disturbance caused by DSM in the organisms from these unique deep-sea environments remains poorly explored which hamper the assessment of metal threshold values (Hitchin et al., 2023). However, current knowledge for deriving potential ecotoxicological threshold values rely mostly on water quality assessments in shallow-waters or freshwater ecosystems. These values should be evaluated also for the deep-sea endemic fauna since changes caused by the multiple and complex exposure conditions may affect entire ecosystems (Hauton et al., 2017; Mestre et al., 2017).

4. The “omics” approach to improve ecotoxicological studies

The study of interdisciplinary system biology based on high-throughput profiling (called omics) combines biology, bioinformatics, and other fields focusing on multiple interactions. This allows a general overview instead of a reduced perspective as that normally obtained in real-time quantitative polymerase chain reaction (RT-qPCR) or ELISA that use previously selected gene candidates (Karahalil, 2016). Advances in the high-throughput sequencing analyzes have allowed simultaneously to uncover the molecular processes and expression profiles of thousands of genes, metabolites and proteins modification involved in each cell, and to correlate data obtained from exposure to metals and other environmental factors in different tissues and organisms (Deidda et al., 2021). At the same time, omics methodologies reveal the shared and unique components of specific biological processes and basic functional aspects that are essential throughout the living world (Dheilly et al., 2014). Since a lot of species are non-identified in such deep-sea habitats, the genomic and metagenomic studies with species identification purposes are slightly more abundant (Cowart et al., 2020; Glover et al., 2016; Gooday et al., 2017; Guan et al., 2022; He et al., 2023; Janssen et al., 2015; Kaiser et al., 2023; Lan et al., 2021; Lim et al., 2017; Osvatic et al., 2023; Quintanilla et al., 2022). Although this topic may lay the foundation of biological baseline information, it was excluded from this review and will not be discussed given the lack of relevance to the development of biomarkers for metal toxicity.

Fifty studies based on omics approaches were eligible for our literature review (Fig. 2). For instance, cold seep mussel *Gigantidas platifrons* transcriptomes (gill, mantle and foot) revealed genes related to metabolism, immune functions (e.g. immune recognition, endocytosis and caspase-mediated apoptosis) and detoxification processes after exposure to various microorganism and three cocktail of metals (Supplementary Table S1) (Bettencourt et al., 2007; Bougerol et al., 2015; Sun et al., 2017). In the shallow-water mussel *Mytilus galloprovincialis* transcriptomes (digestive gland) after sediments exposure were observed genes differentially expressed that are involved in oxidative stress (e.g. catalase, CAT) (Pinheiro et al., 2021).

Transcriptomic and proteomic approaches performed in deep-sea alvinocaridid shrimp *Rimicaris* spp. (*R. exoculata*, *R. hybisae*, *R. kairei* and *R. leurokolos*) and *Alvinocaris longirostris* indicated that most of the genes and proteins involved are related to sulfur (e.g. sulfide:quinone oxidoreductase (SQOR) and rhodanese domain-containing protein (CDC25)), carbohydrate (e.g. c-type lectins (CLECs)) and chitin (e.g. chitinase (CHIT) and mucins (MUCs)) metabolisms (Supplementary Table S1). Also, mechanisms involved in antioxidation (e.g. glutathione peroxidase (GPx), CAT, and superoxide dismutase (SOD), detoxification (e.g. cytochrome P450 (CYP)) and immunity (e.g. CLECs, serine proteases, antimicrobial peptides (AMPs) and heat shock proteins (HSPs)) were found (Mestre et al., 2019a; Pruski and Dixon, 2007; Zhang et al., 2017; Zhu et al., 2020). Other arthropods, such as the squat lobster *Shinkaia crosnieri* from hydrothermal vents and cold seeps revealed up-regulation of genes transcripts expression related to immunity (e.g. HSPs, proteases and complement proteins), antioxidation and detoxification mechanisms (including SOD, peroxidase, dihydrodiol dehydrogenase (DDH), cystathionine gamma-lyase (CTH) and glutathione-S-transferase (GST)) (Cheng et al., 2019). While in the amphipod *Abyssorchomene distinctus*, chitinase, sodium/potassium transporters, SOD, GPx, ferritin (FRI) and proteases were highly modified under copper toxicity (Kwan et al., 2019). On the other hand, the coral *Dentomuricea* aff. *meteors* showed modification in the expression of genes transcripts involved in antioxidant reactions (e.g. α-carbonic anhydrase) and immunity (e.g. toll-like receptors (TLR), lysozyme (LYZ)) after polymetallic sulfides and quartz exposure (Carreiro-Silva et al., 2022). The metabolomic research field is even less explored especially in areas with interest for deep-sea mining exploitation. One study with deep-sea marine sponges (*Geodia barretti*, *Stryphnus fortis* and *Weberella bursa*) from North Atlantic showed the presence of several bioactive compounds (e.g. diketopiperazines Trp-Arg, diketopiperazine Pro-Arg57, bromotryptophane derivatives) that were correlated with their associated prokaryotic community under potential effect of oceanographic conditions (Supplementary Table S1) (Steffen et al., 2022).

4.1. Multi-omics studies towards a better understanding of the biological effects in the organisms

The most recent studies use the combination of two or more different omics approaches that lead to a better comprehension and help overcome certain limitations inherent to each individual methodology (Cheng et al., 2024). The omics integration remains very challenging considering the specific analytical tools, costs associated and experimental designs specificities and variances (e.g. number of biological samples, storage conditions). For example, transcriptomics allow to quantify gene expression changes but are not able to show if the resultant proteins have or not the same function or even if they are absent (Cheng et al., 2024; Mao et al., 2024; Ryu et al., 2023). In the studies reviewed, some potential important information may not be considered because lower number of individuals used ($n < 3$) (Li et al., 2023; Liu et al., 2021; Ryu et al., 2023; Sun et al., 2017; Zeng et al., 2021). For proteomics, protein post-translational modifications and different protein isoforms may be problematic for a proper data interpretation mainly in organisms that are poorly studied and the databases are very limited such as for deep-sea invertebrates (Company et al., 2012, 2019; Zhou et al., 2023). For metabolomics is required an even bigger budget, and similar constraints regarding the database availability to provide an adequate data analysis and identification of unique metabolites, especially for deep-sea invertebrates (Chen et al., 2022). From our review search, these studies only started to appear since 2019 (Vohsen et al., 2019; Zhou et al., 2021, 2023). Protein and metabolite identification by high-throughput technologies are costly and still the major bottlenecks of those omics approaches, despite of constant databases updates. This presumably explains the lower number of proteomic studies available on deep-sea invertebrates (Company et al., 2019; Hui et al., 2018; Zhou et al., 2023). Still, in our opinion and considering the expenses and current available databases of several omics, transcriptomic and proteomic are the best approaches to apply in deep-sea invertebrates for biomarkers discovery. In addition, two important issues were observed from our review literature that should be considered in future experiments:

1) most of the studies reviewed did not measure dissolved metal concentration in water or tissues, what makes arduous to compare metal toxicity effects across different deep-sea invertebrates to further establish threshold levels (Supplementary Table S1) (Carreiro-Silva et al., 2022; Company et al., 2019; Kwan et al., 2019; Martins et al., 2017; Zhou et al., 2021, 2023). The nominal metal concentration (what is added to the seawater) does not necessarily correspond to the measured concentration, and it is not directly proportional to what is measured in the biological tissues (Zhang et al., 2023). This variance can be dependent of type of metal, water properties and species as well (Zhang et al., 2023).

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2) lower number of individuals used or absence of information at all which may compromise the relevance and representativity of the results at species level since wild species variability is higher and their background status is unknown (Supplementary Table S1) (Li et al., 2023; Liu et al., 2021; Ryu et al., 2023; Yuan et al., 2022).

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5. Potential biomarkers for metal toxicity

Metallic elements are considered systemic toxicants capable to induce neural and multi-organ damage, even at low levels of exposure. Marine invertebrates are particularly vulnerable to waterborne metals because they can process large amounts of seawater. Since metals are required cofactors for numerous fundamental processes, physiological and ecological changes can be detected by Ca^{2+} dysregulation, oxidative stress with reactive oxygen species (ROS) production, neurological dysfunctions and sensory, DNA damage, metabolism disturbances and immune defense strategies such as the synthesis of heat-shock proteins, apoptosis, and autophagy, in a dose/time-dependent manner (Ghannem et al., 2023) (Fig. 3). In addition, several metabolites were changed after metal exposure, for example lactic acid, ceramides, betaine lipids which are involved in oxidative stress, inflammation process and metabolism (pyruvate, gluconeogenesis, and glycolysis) (Chan et al., 2022; Hillyer et al., 2022; Lettieri et al., 2023).

In the deep sea, the commonly investigated biomarkers to evaluate metal toxicity effects are related to oxidative stress, such as the antioxidant enzymes (SOD, CAT, GPx), the biotransformation enzymes (e.g. GST), metal detoxification (e.g. metallothionein proteins (MTs)) and those related to oxidative damage (e.g. LPO) (Bebianno et al., 2018; Company et al., 2008; Martins et al., 2009; Mestre et al., 2019a; Zhou et al., 2020). However, most of these enzymatic biomarkers are not specific to metal toxicity and respond to other types of stressors that renders the interpretation of the results difficult. Meanwhile, deep-sea species are dwelling in extreme environments, and they may have unique responses to metals exposure. For instance, the scaly-foot snail has an upregulation of metal-binding protein, a common gene in marine invertebrates but barely studied, to the elevated ambient iron concentration (Sun et al., 2020). In this sense, it is legitimate to search for more specific biomarkers of metal toxicity in deep-sea organisms, and the use of multi-omics technologies may help to achieve this, providing more knowledge and a general overview of the ecological status based on gene expression, protein and/or metabolites alterations (Karahalil, 2016).

Our review focused on invertebrates from the deep sea that are poorly understood but will likely be affected by DSM activities. Here we identified 118 potential biomarkers (genes or proteins) that were frequently modified in several omics' studies related to deep-sea invertebrates, under different situations that can be related to metals exposure or the naturally enriched metal environment where they live (Table 1, Supplementary Table S2). Those biomarker candidates belong mainly to three biological processes: 43 to immune system, 46 to cellular metabolism and 29 to oxidative stress (Table 1, Supplementary Table S2).

Several biomarker candidates that we indicate (e.g. LPO, MTs, SOD, GPx, HSPs) were already being used for many years based on biochemical enzymatic activities assays (e.g. Company et al., 2008). These methods are less accurate and sensitive, although faster and cheaper, compared to high-throughput sequencing technologies (e.g. Zhang et al., 2018). As these were also observed in omics results it is suggested that they can be good candidates, but further studies should confirm their valuable contribution. However, their specific interactions and respective functions with different metal compounds (e.g. synergetic or antagonist effects) in deep-sea invertebrates remain poorly understood. It is also important to keep in mind that specific adaptations in the genes are likely to occur, particularly at the transcription/regulation level, between these organisms and their extreme and unique deep-sea habitats (Wray et al., 2003). Therefore, some of the biomarker candidates highlighted here may not be adequate for all deep-sea invertebrates even when gene/proteins have similar functions. Some of them are well-known and considered references for ecotoxicology, although others are poorly studied. Anyway, it is important to mention that several were not previously reported as potential biomarker candidates as we are suggesting in this work. For immune system process, forty-three potential biomarkers are proposed to assess metal toxicity namely pathogen-recognition receptors (e.g TLRs, PGYLRPs, CLECs) and humoral components (e.g TRY, AMPs) (Supplementary Table S2). For cellular metabolism process are forty-six potential candidates such as those involved in migration (e.g TSPAN11), protein binding (e.g BP10), homeostasis (e.g PSMB5), catabolism (e.g COX5B, ATP1A1), sulfur metabolism (e.g ETHE1, TST) (Supplementary Table S2). While for oxidative stress process are twenty-nine biomarkers, that participate in DNA damage repair (e.g CCD51, TXN1, VMA4) and temperature stress (e.g EXL1, NUP155) (Supplementary Table S2). Overall, the potential biomarkers in immune system participate in foreign particles recognition (as metals) and immune defense of organism. The cellular metabolism allows to have enough energy for the organism be capable to deal against environmental variables, support other biological processes that require a lot of energy and oxidative stress to repair the damages (or try to) for organism survival.

5.1. *Immune system*

The immune system is characterized by a set of processes that defend the organism against foreign potential pathogens (e.g. bacteria, virus, fungi, and parasites) or compounds (e.g. metals) (Marshall et al., 2018; Tort, 2011). However, this biological system is also influenced by both intrinsic factors (e.g. immune cell repertoire), and extrinsic factors (e.g. metals concentration, temperature, stress, food, pH, salinity, oxygen

Table 1
Number of potential biomarker candidates for the different biological processes and pathways identified for deep-sea invertebrates.

Biological process	Biological pathway	Total number			
		Biomarker	Up-regulated	Down-regulated	Both
Immune system	Innate immune response	43	20	3	20
Cellular metabolism	Cell migration	1	1	0	0
	Cell protein binding	10	9	1	0
	Cell homeostasis	1	1	0	0
	Catabolic metabolism	12	12	0	0
	Chitin metabolism	7	6	1	0
	Sulfur metabolism	15	14	1	0
Oxidative stress	DNA damage and repair	24	19	1	4
	Temperature stress	5	5	0	0

availability and hydrostatic pressure) (Marshall et al., 2018; Tort, 2011). Immune system is divided in non-specific (or innate) and specific (or acquired/adaptive) responses, and both immune responses can be subdivided in humoral (e.g. cytokines, hydrolytic enzymes, and complement proteins) and cellular components (e.g. leucocytes, lymphocytes) that vary between innate and acquired immunities (Marshall et al., 2018). It is generally assumed that the innate immune defense is more well developed in invertebrates, and the acquired immunity is practically absent, which seems not entirely true since it is observed several cellular and humoral components from acquired immunity on omics result studies (Canesi and Procházková, 2013; Ghosh et al., 2011). Recent investigations at the molecular level are providing indications about the complexity and diversification of the immune gene repertoire, which may contribute to the plasticity of immune responses in different long-lived invertebrates (e.g. sea urchins, crustaceans, mollusks, and sponges) (Lafont et al., 2019). This allowed them to evolve genetic mechanisms capable of producing thousands of different proteins from a small number of genes. It is known that metals can trigger inflammatory responses which is a physiological event that protects tissues from infection, injury (such as membrane disruption, production of ROS) and chronic exposures in organisms (Anka et al., 2022). The various metals tend to induce dysfunctions and pathological conditions, and therefore lead to proinflammatory and innate immune signaling in a similar manner; however, certain metals can stimulate or inhibit specific pathways compared to others (Anka et al., 2022; Saco et al., 2021). In deep-sea invertebrates, namely mussels, the pathogen-recognition receptors (PRRs) (such as TLRs, CLRs, AMPs) and others are important in the detection of microbial and other foreign antigens (e.g. metals) and participate rapidly and efficiently in systemic responses (Canesi and Procházková, 2013; Wong et al., 2015). TLRs have been identified as responsive to captivity stress in *G. haimaensis* from cold seeps, while CLRs have been found important in mucosal immunity (mainly in digestive gland and gills) of bivalve mollusks (Saco et al., 2023). Other immune components (humoral and cellular) participate in other functions namely in protein degradation, apoptosis controlling inflammation, cell death (e.g. caspases, CASPs), cell signaling (e.g. complement C1q) and nutritional immunity (e.g. ferritin, FRI) (Martínez et al., 2020; Zhang et al., 2022). For example, C1q is still unexplored in invertebrates, but was shown its involvement in pathogen associated molecular patterns (PAMPs) recognition and as opsonin in scallop *Chlamys farreri* (Wang et al., 2012, 2015). Besides the nutritional immunity role, FRI also participates in the transition and storage of toxic metal ions in invertebrates, however its regulation and function are still largely unknown (Chen et al., 2015). Most of those humoral compounds have metal affinity to gold, silver, copper, and zinc among other metals, changing their activity or mode of action. Thus, their deficiency increases the susceptibility to infections (e.g. tissue homeostasis, immune tolerance, and clearance of damaged cells) and lead to high-energetic costs for organisms (Brzeski et al., 2022; Łoboda et al., 2018; Lu and Kishore, 2017).

HSPs are largely studied in deep-sea invertebrates (e.g. HSP70, HSP90) (Carreiro-Silva et al., 2022; Kwan et al., 2019; Pruski and Dixon, 2007; Wang et al., 2022). In the past, they were exclusively classified as thermal stress related genes, but it is currently accepted that these proteins are responsive to other environmental factors (such as metals and high-hydrostatic pressure) (Pruski and Dixon, 2007; Ritchie et al., 2018). Also, they have other important functions namely in immune system, cell function and maintenance and DNA damage (Pruski and Dixon, 2007; Ritchie et al., 2018).

The immune system is a process highly affected by metal toxicity in vertebrates, however it remains unexplored in deep-sea invertebrates. For this reason, we indicate forty strong candidates that are involved in general metal recognition, cell signaling and further activation of inflammatory response (humoral and cellular components) to assess metal toxicity in deep-sea invertebrates under deep-sea stressful conditions (such as high pressure and higher concentration levels of metals) (Fig. 4A, Supplementary Table S2).

5.2. *Metabolism*

The metabolic process is a set of biochemical reactions that converts food into energy that are essential for various life processes (e.g. reproduction, growth, development, and immune system), synthetizes new organic compounds (e.g. proteins, lipids, nucleic acids, and some carbohydrates) and eliminates the metabolic wastes (Fernandez-de-Cossio-Diaz and Vazquez, 2018). After metals absorption into the organisms, the cellular organelles can be damaged and thus, inhibit biochemical and metabolic pathways and impart physiological functions of the organs (Akash et al., 2023). Metal toxicity does not depend on total accumulated metal concentration, but it is related to a threshold concentration of internal metabolically available metal (Rainbow, 2007). The damages caused (such as disruption of metabolism and metabolites) by metals in the organisms can become life threatening and irreversible. This knowledge is well-known in vertebrates (e.g. carcinogenic effects, anemia, nervous disorders, diarrhea, skin burns and vision problems) (Akash et al., 2023; Ghannem et al., 2023; Tchounwou et al., 2012). Contrarily, the metabolites information remains poorly explored in deep-sea invertebrates, although unique metabolites were already found in *B. platifrons* exposed to copper (such as dopamine) (Zhou et al., 2021).

The metabolic rates in deep-sea organisms are widely perceived to be low because of the low temperature that prevails at depth (Brown et al., 2018; McClain et al., 2020). Forty-six potential biomarkers with functions related to cell protein binding, cell homeostasis, catabolic and sulfur metabolisms were found in deep-sea invertebrates (Fig. 4B, Supplementary Table S2).

Cell protein binding mediates protein transport between Golgi complex and endoplasmic reticulum. In the deep-sea squat lobster, *S. crosnieri*, the gene expression of cell protein binding ADP-ribosylation factor GTPase-activating protein 2 (ARFGAP2) was modified (Cheng et al., 2019). Similarly, the candidate laminins (LAMs) were identified in the vent shrimp, *Rimicaris* spp. when they were maintained for 10 days in captivity (Zhang et al., 2017). Whereas cell homeostasis has been associated to embryos development and reproduction, such as sperm

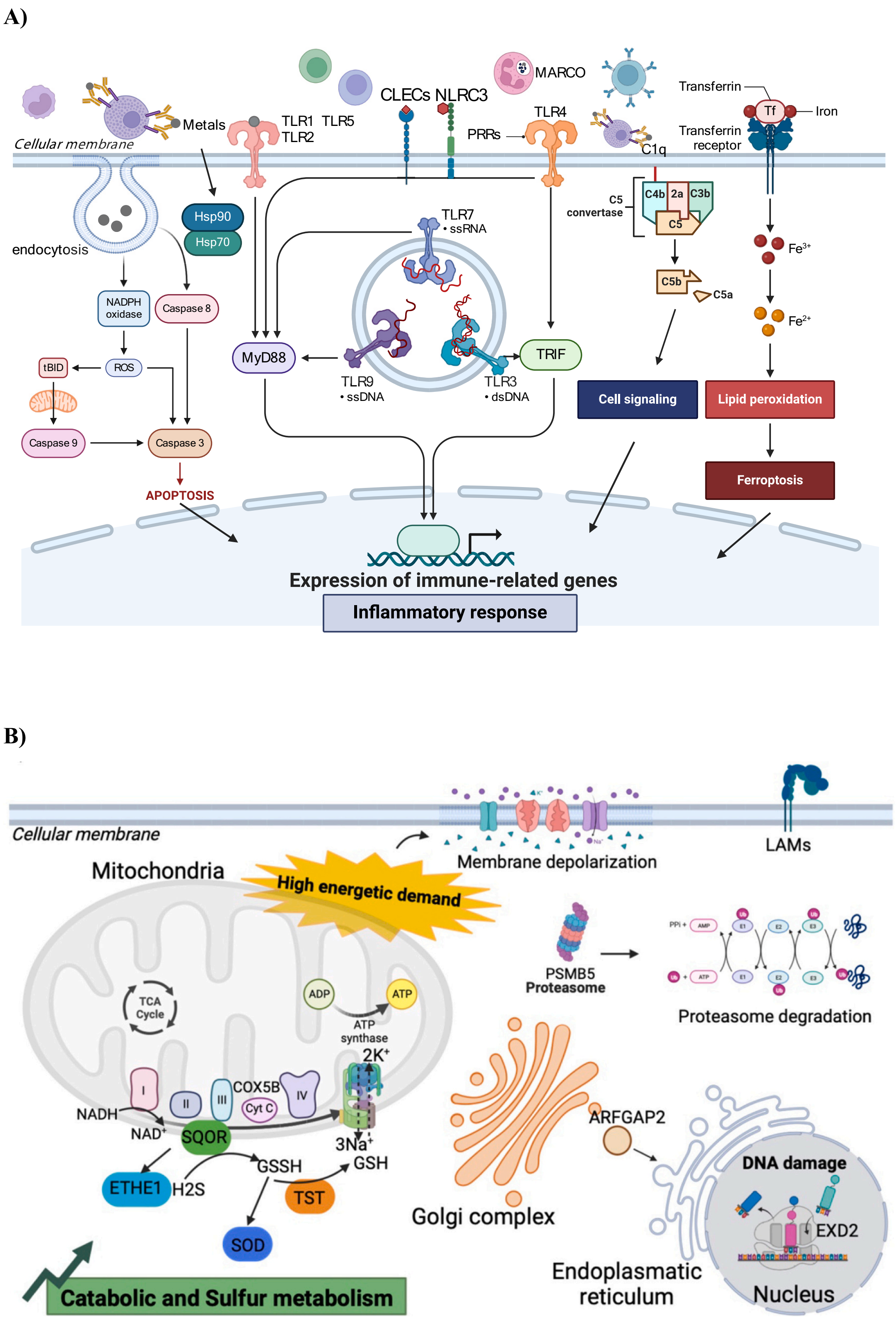

Fig. 4. Principal pathways affected by metal toxicity in deep-sea invertebrates: A) immune system, B) metabolism and C) oxidative stress. ADP; ATP; ARGAFP2 (ADP-ribosylation factor GTPase-activating protein 2); C (complement); C1q (Complement C1q complex); Cyt (cytochrome); CLECs (C-type lectin receptors); COX5B (Cytochrome c oxidase subunit 5B); ETHE1 (Persulfide dioxygenase); EXD2 (Exonuclease 3′-5′ domain-containing protein 2); Fe (iron ion); GSH (glutathione); GSSG (glutathione disulfide); GSSH (Oxidized glutathione); H (hydrogen); H_2O (water); H_2S (hydrogen sulfide); HSPs (heat-shock proteins); K^+ (potassium ion); LAMs (Laminins); MARCO (macrophage receptor MARCO); Myd88 (innate immune signal transduction adaptor); P450 (Cytochrome P450); Na^+ (sodium ion); NAD (nicotinamide adenine dinucleotide); NADPH (nicotinamide adenine dinucleotide phosphate); NLRC3 (NOD-like receptor 3); PSMB5 (Proteasome subunit beta type-5); O (oxygen); ROS (reactive oxygen species); Se (selenium); SQOR (Sulfide:quinone oxidoreductase); tBID (membrane-targeted death ligand); TLRs (Toll-like receptors); TST (Thiosulfate sulfurtransferase); TCA (tricarboxylic acid cycle); TRIF (TIR Domain-Containing Adaptor-Inducing Interferon-β); TXN1 (Thioredoxin 1);

Zn2+ (zinc ion).
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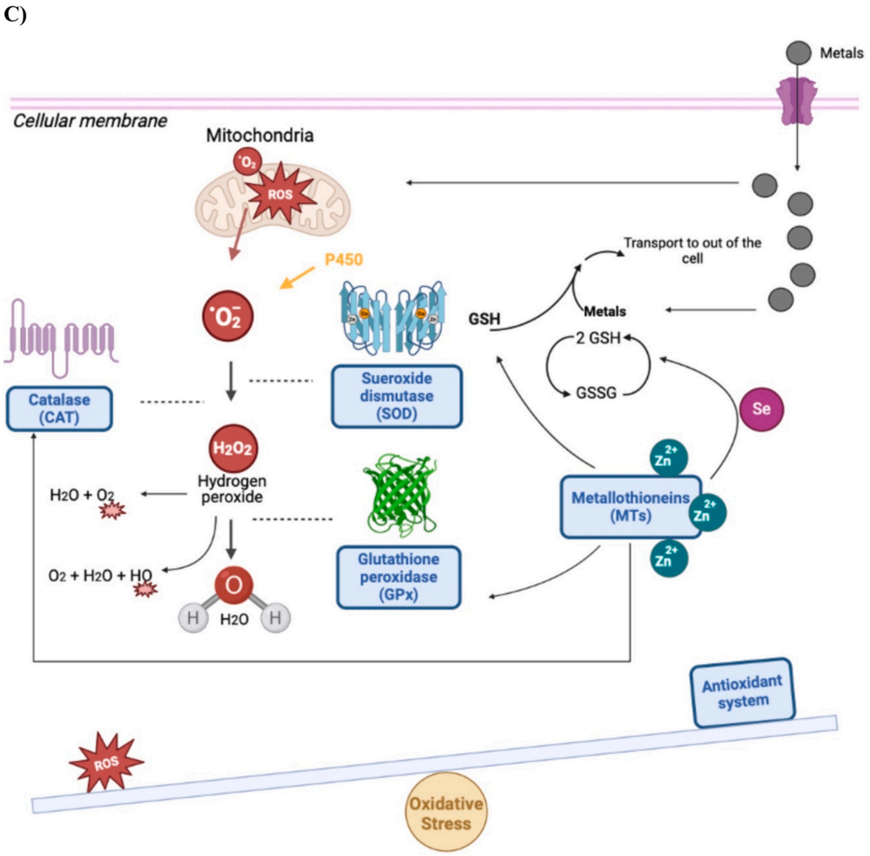

Fig. 4. (*continued*).

function. It has been suggested that the presence of Cu^{2+} ions may inhibit the zinc ion binding-related blastula protease 10 (BP10) and proteasome subunit beta type-5 (PSMB5), such as in deep-sea amphipod *A. distinctus* (Kwan et al., 2019).

In addition, changes in catabolic metabolism reported, involve mitochondrial respiratory chain and oxidative phosphorylation, that are responsible for synthetizing ATP from ADP and reducing nicotinamide adenine dinucleotide (NAD) to NADH needed for anabolic reactions. Some of these examples are the ATP synthase subunit alpha, mitochondrial (ATP5B), cytochrome *c* oxidase subunit 5B (COX5B), exonuclease 3′-5′ domain-containing protein 2 (EXD2) and mitochondrial NADH dehydrogenase (MT-ND1) (Kwan et al., 2019).

Sulfide detoxification has been also demonstrated in deep-sea invertebrates which is crucial for promoting sulfur cycling, reducing, and converting sulfur compounds necessary for symbiotic survival (Bettencourt et al., 2007; Kwan et al., 2019; Shi et al., 2023; Zhang et al., 2017; Zhu et al., 2020). The persulfide dioxygenase (ETHE1), SQOR and thiosulfate sulfurtransferase (TST) are potential candidates whose gene expressions were modified between deep-sea shrimp species from Sakai and Southeast of Desmos Manus hydrothermal vents, in amphipods from polymetallic nodules after copper exposure, and in mussels from Lucky Strike and Rainbow hydrothermal vents (Kwan et al., 2019; Zhang et al.,

Table 2
Potential gene/proteins and metabolite biomarkers after different metal challenges in deep-sea invertebrates.

Organism	Species	Metal measured	Metal concentration in seawater	Potential biomarker candidates	References
Jellyfish	*Periphylla periphylla*	Sediment	0; 16.7; 33.3; 166.7; 333.3 (mg/L)	CLECs, ATP1A1, ATP5A	(Stenvers et al., 2023)
Mussel	*Gigantidas platifrons*	Cd	100; 1000 (μg/L)	NLRC3, CASP9, TXNRD2, NUP133	(Zhou et al., 2023)
Octocoral	*Dentomuricea aff. meteors* and *Viminella flagellum*	Co, Cu, Mn	Co: 0.045–0.26; Cu: 6.6–22; Mn: 0.13–0.26 (μg/L)	HSP70, FRI, TLR, SOD	(Carreiro-Silva et al., 2022)
Mussel	*Gigantidas platifrons*	Cu, Cd	Cu: 100; Cd: 500; Cu: 100 + Cd: 500 (μg/L)	Hypotaurine, betaine, succinate, glucose 6-phosphate, fructose 6-phosphate, guanosine, guanosine 5′-monophosphate, inosine	(Zhou et al., 2021)
Mussel	*Bathymodiolus azoricus*	Cd	0.9 (μM)	PPID, GST, PSMB5, CA	(Company et al., 2019)
Amphipod	*Abyssorchomene distinctus*	Cu	25; 50; 100 (μg/g)	ATPA1, SOD, GPx, FRI, BP10	(Kwan et al., 2019)
Mussel	*Bathymodiolus azoricus*	Cu	300; 800; 1600 (μg/L)	CAT, MT, TLR2	(Martins et al., 2017)

2017; Zhou et al., 2020). Thus, these mechanisms involved in metabolic processes have high-energetic costs for the cells as well as for the whole organism and thus, can lead to a cell membrane depolarization followed by cellular apoptosis (Kwan et al., 2019; Zhang et al., 2017; Zhou et al., 2020). Inhibition of proteasome activity is also induced by metals toxicity (Kwan et al., 2019). The metabolic mechanisms described above that were modified by metals toxicity in deep-sea invertebrates are depicted in Fig. 4B.

5.3. *Oxidative stress*

Accumulation of metals in the body causes suppression or imbalance of the enzymes that are involved in antioxidant defense (such as CAT and SOD) that leads to ROS (Reactive Oxygen Species, Wu et al., 2016). The imbalance of ROS and antioxidants results in: (1) depletion of cell antioxidant defense systems, (2) metabolic reprogramming, (3) organelle dysfunction, (4) perturbations of critical pathways related to carbohydrate, protein, and lipid metabolism, (5) destabilization of cell membranes and (6) DNA damage (cells and tissues) (Hossen et al., 2023). Oxidative stress and metabolism processes are linked, which complicate the aggregation of several genes/proteins candidates in a specific topic (metabolism or oxidative stress) because they normally participate in both biological processes especially in the mitochondria organelle (Raut and Khullar, 2023). Twenty-nine biomarkers are highlighted whose functions are mostly related to DNA damage and repair, and temperature stress (Table 1, Supplementary Table S2). DNA is damaged by ROS (and other factors) generated endogenously because of oxygen metabolism and a complex network of different repair systems has evolved to maintain genomic integrity and minimize adverse consequences by antioxidant processes (Aranda-Rivera et al., 2022). The principal pathways to eliminate those damages (e.g. DNA base or helix distortions) in deep-sea shrimp species are the excision repair pathway (e.g. HIPK2, HEAT3 and RUVB2), cell cycle arrest (e.g. CYP, CCD51, GPx and GSSH) and apoptosis (e.g. TXN1) (Bougerol et al., 2015; Wang et al., 2022; Zeng et al., 2021; Zhang et al., 2022, 2017; Zhu et al., 2020) (Fig. 4C, Supplementary Table S2).

The intracellular ubiquitin-proteasome system is a key regulator of cellular processes involved in the controlled degradation of short-living or malfunctioning proteins. Metals are recognized stressors of the proteasome system in vertebrates, whereas their effects on the proteasome of invertebrates are not well understood. However, we highlight VMA4 since it is involved in proteasome system and was found in the deep-sea limpet *Bathyacmaea lactea* (Hou et al., 2020; Yee et al., 2023) (Fig. 4C, Supplementary Table S2).

Several detoxification processes of metals have been described in deep-sea invertebrates to reduce their intake, enhance their excretion and/or sequestration (Zhou et al., 2023). One example is the trace metal detoxification process involving the participation of the metal binding protein MT that have a strong metal affinity for copper, zinc, cadmium, and silver (Amiard et al., 2006). Differentially gene expression of MTs was observed in the deep-sea mussel *Bathymodiolus azoricus* after several cocktails of metals at different concentrations (Bougerol et al., 2015). The temperature is often a critical ecological factor for species and its variance may be considerable in deep-sea environments which can be high in hydrothermal vents or cold in cold seeps. The thermal stress related gene candidates, (e.g. EXL1 and NU155), were identified and their gene expression was modified in shrimp *R. leurokolos* (living in close proximity to the hot vent chimney fluids) compared to *A. longirostris* (living in the periphery of the vents, where temperature is around 4 °C) (Wang et al., 2022) (Supplementary Table S2). Overall, the mechanisms by which most studied metals exert their toxic effect is through impairment of cellular respiration by the inhibition of various mitochondrial enzymes, and the uncoupling of oxidative phosphorylation (Fig. 4C).

5.4. *Biomarkers responding to metal challenges*

From the biomarkers described above, those biomarker candidates responding to metal challenges are shown in Table 2 (with more detail in Supplementary Table S2 for the case of genes and proteins). From those potential biomarkers, 21 are genes/proteins involved in immune system (e.g. TLR2, NLRC3, CLECs), metabolism (e.g. ATPA1, ATP5A, PSMB5) and oxidative stress (e.g. CAT, MT, SOD) processes and 8 are metabolites that participate in metabolic processes (Table 2). These potential biomarkers were retrieved from the very few studies on deep-sea invertebrates that performed specific metal challenges ($n = 8$) (Carreiro-Silva et al., 2022; Company et al., 2019; Kwan et al., 2019; Martins et al., 2017; Stenvers et al., 2023; Zhou et al., 2021, 2023). From these studies, one does not specify which metals were tested because they used sediment (Stenvers et al., 2023). Other constraint observed is the existence of fewer studies that measured metal concentration in tissues, which complicates the interpretation and further evaluation of specificity of biomarker candidates (Martins et al., 2017; Stenvers et al., 2023).

6. Current gaps and future perspectives

One of the most important goals of ecotoxicological studies involving omics is to understand the general response of an organism to a specific environmental challenge. Omics can provide large results datasets that are helpful to determine whether a contaminant causes biological effects in organisms, the magnitude of the response, and what mechanisms of defense may be stimulated or suppressed (Karahalil, 2016). This holistic view of all the possible biological mechanisms activated when facing toxic DSM plumes will help to identify which are the most reliable and specific biomarkers for this environmental challenge and help to define future threshold values for plume/metal concentrations. An adequate bioindicator/biomarker selection for deep-sea invertebrates requires a greater research effort in order to integrate and obtain meaningful results from data between different omics (Karahalil, 2016). A major gap is the reduced number of omics studies available for these invertebrates, especially related to metabolomics when compared to transcriptomics. Metabolomics has an enormous potential due to its robustness and accuracy to investigate metal toxicities at the cellular, tissue, and organismal levels (Akash et al., 2023) Also, all changes in the proteome and transcriptome are in fact reflected in the final metabolic profile and functions (Rattray et al., 2018). However, the budget required for those analyzes are higher and data interpretation can be complicated, mostly when species are not well known, and very challenging to interpretate, due to the lack of adequate databases (Trapp et al., 2014). Moreover, experimental designs need to be well planned to take into account the number of samples needed since the limited accessibility in deep-sea environment, the amount of tissue(s) available to perform different analysis and most suitable fixation methods that differ between different omics and other analysis (e.g. metal accumulation).

Eight main constraints and gaps can be identified in our review: 1) the determination of which metal, or group of metals, is producing the observed effect, since the organisms may come into contact with a diversity of metals at different concentrations (e.g. sediment plume); 2) the difficulty to perform experimental trials with deep-sea species in situ or using high-hydrostatic pressure aquaria, including long-term monitoring and assessments; 3) the often reduced number of specimens and the high individual variability (e.g. size, weight, age) to study specific challenge effects; 4) few information on metal effects on embryos and larvae in deep-sea species; 5) a large number of species remain unknown limiting the development of potential biomarkers across species; 6) the understudied cumulative impacts effects in deep-sea ecosystems, 7) DNA methylation after metal exposure and subsequent transgenerational effects (epigenome) and 8) lower number of metabolomic studies in deep-sea invertebrates.

Nowadays, it has been applied several metal exposures to shallow-water species to understand and somehow predict behavior, physiological and biochemical responses of phylogenetic similar species from the deep sea (Mestre et al., 2019a, 2019b). However, the species found in the coastal areas and in the deep sea present different physiologies and environmental adaptations indicating that comparisons should be made with caution.

In the future it will be important to address those constraints and gaps by investing more in multi-omics studies to better understand the cellular/molecular processes most affected by metal toxicity under DSM scenario in juvenile and adult deep-sea invertebrates. The application of metabolomics approach is important considering that is already a guiding decision making for drug safety and biomarker discovery in vertebrates.

Embryos and larvae should also be included, as they are good indicators of future population viability and is also particularly important studies with sessile species (Taslima et al., 2022). Behavioral responses should also be considered, along with the potential effects of multiple environmental stressors (e.g. temperature, salinity, pH, noise, light) in DSM scenario, assessing the bioavailability of metals in the tissues of organisms and evaluating the vulnerability across species through the analysis of early warning biomarkers. The future perspectives undergo increasing the knowledge to bridge the main current gaps and to be able to establish metal concentration levels thresholds. This is essential for further develop adequate legislation and regulations and thus, minimize the impacted area from potential DSM activities.

7. Conclusion

Metals disturb DNA repair systems and cell cycle control by diverse mechanisms. In addition, an imbalance of the antioxidant defense may lead to an excessive accumulation of ROS which emerge as key players in pathophysiology of metal toxicity. Furthermore, ROS damages endoplasmatic reticulum and mitochondria cellular organelles, and irreversibly damage cellular biological macromolecules including nucleic acids, proteins, and lipids. Innate immune defense and metabolism processes mostly associated to sulfur and catabolic metabolism were also changed in deep-sea invertebrates. The biomarkers identified here are mostly based on transcriptomic studies because this approach has been more applied in deep-sea invertebrates. Those biomarkers belong to the processes mainly reported to be affected by metals in both shallow and deep waters that are immune system, metabolism, and oxidative stress. However, these potential biomarker candidates need validation with further experiments simulating DSM plume exposure disturbances across deep-sea organisms. Once validated, they can work as early warning biomarkers for potential environmental disturbance caused by the exposure to the toxic metals entrained in DSM plumes. Moreover, the validated candidate biomarkers can be helpful to implement threshold levels and may allow future biomonitoring actions during DSM activities.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.175628.

Funding

This work was funded by Fundação para a Ciência e a Tecnologia (FCT) through the BiDiRisk (PTDC/CTA-AMB/2894/2021) (doi:10.54499/PTDC/CTA-AMB/2894/2021) and grants CEECIND005262017, UID/00350/2020CIMA (doi:10.54499/UIDB/00350/2020; doi:10.54499/UIDP/00350/2020), LA/P/0069/2020 (doi:10.54499/LA/P/0069/2020). JS was supported by Fundamental Research Funds for the Central Universities (202172002 and 202241002).

CRediT authorship contribution statement

Cármen S.V. Sousa: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Conceptualization. **Jin Sun:** Writing – review & editing. **Nélia C. Mestre:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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