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Overview of the effects of heavy metals on the reproductive health of males in the genus *Mytilus* spp.

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ABSTRACT

This review examines the reprotoxic effects of key heavy metals, specifically nickel, chromium, zinc, copper, mercury and cadmium, on the reproductive health of bivalve molluscs of the genus *Mytilus spp*. which serve as important bioindicators in marine ecosystems. Exposure to even sub-lethal concentrations of these metals induces oxidative stress, DNA damage and changes in chromatin structure, which are thought to affect fertility. These metals, known for their persistence and bioaccumulation, interfere with critical reproductive processes by also altering the functions of protamine-like proteins (PLs), the main nuclear basic proteins of sperm chromatin of these organisms. Moreover, Seasonal variations influence the toxicity of these metals, coinciding with critical phases of gametogenesis and amplifying their detrimental effects during reproductive cycles. The study highlights the urgent need for comprehensive biomonitoring programmes, as the sensitivity of *Mytilus spp*. to environmental contaminants provides valuable insights into the health of marine ecosystems. Given their economic importance and ecological role as filter feeders, the impact of heavy metal pollution on these bivalve molluscs goes beyond increased environmental pollution and threatens biodiversity and seafood safety. To mitigate these risks, strict pollution controls and further research into molecular toxicology are essential. Understanding this will help to develop effective strategies to conserve marine life and maintain the ecosystem benefits provided by these sentinel species.

1. Introduction

A realistic view of our world reveals that it is heavily polluted, with the release of various chemicals from anthropogenic processes continuing to increase due to rapid industrialisation and urbanisation in many regions. The marine environment is polluted by agricultural fertilisers, wastewater and industrial effluents, which have an impact on marine life. Terrestrial ecosystems are contaminated by heavy metals and organic chemicals that can be absorbed and accumulated by crop plants, while groundwater is heavily affected by industrial effluents [1]. In addition, an emergent contaminant is represented by microplastic which can act as a carrier for other pollutants such as heavy metals [2]. Similarly, other types of pollution, i.e. particulate pollution poses significant risks to aquatic invertebrates, with impacts manifesting at physical, physiological, and molecular levels. An example is particulate matter (PM), including PM2.5, PM10, and nanoparticles, entering aquatic systems through precipitation and industrial runoff, resulting in developmental abnormalities and multi-organ toxicity in invertebrate species [3,4]. Crustaceans, molluscs, and annelids demonstrate particular vulnerability, with research documenting bioaccumulation of

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heavy metals and microplastics that disrupt physiological processes and behavioral patterns. For instance, PM2.5 exposure correlates with reduced locomotor activity in freshwater gastropods and developmental retardation in crustaceans [3]. Invertebrate species such as the Mediterranean mussel *Mytilus galloprovincialis* function as essential bio-indicators due to their filter-feeding mechanisms, which facilitate the accumulation and reflection of environmental pollutants within their tissues. Scientific investigations have established that exposure to contaminants including pharmaceuticals, personal care products, heavy metals, pesticides, and microplastics induces physiological alterations, reproductive dysfunction, and mortality in these organisms [5]. Microplastics, frequently ingested by invertebrates, accumulate in their tissues, presenting cascading risks throughout the trophic chain with potential implications for human health [3], while simultaneously disrupting osmoregulatory mechanisms, energy metabolism, and inducing oxidative stress responses in bivalve molluscs [5]. All of this causes a serious impairment of all ecosystems and biodiversity, with consequent damage to the general and, in particular, reproductive health of organisms [6]. Among all pollutants, heavy metals, which are widespread in the environment due to their many uses, are of particular concern because of their lethality, persistence and accumulation in the food chain. Heavy metals, which include transition metals, metalloids, lanthanides, and actinides, are natural components of the Earth's crust with significant metallic properties. They have atomic weights between 63.5 and 200.6 g/mol and densities greater than 4.5 g/cm^3 . Although there are over 50 elements classified as heavy metals, only 17 are considered highly toxic and accessible. The term 'heavy metal' is used synonymously with 'trace metal' [7] and are considered trace elements because of their presence in trace concentrations. Naturally found in marine and freshwater environments, heavy metals can result from, erosion, volcanic eruptions, and mineral weathering and are distributed in a dissolved and particulate phase [8]. Marine organisms can therefore be directly exposed to heavy metals through both ingestion and dissolution. Within each of these phases, an organism may be exposed to different physicochemical forms of a metal. Each form, or speciation, may differ in its accessibility and toxicity to an organism [9,10]. To understand the impacts of increasing heavy metal concentrations in the marine environment, it is essential to understand the nature, effects and interactions of heavy metals with marine organisms, populations and communities. All heavy metals can be toxic if their concentrations are higher than certain threshold values, resulting in adverse impacts on organisms. High levels of heavy metals in marine environments can cause sub-lethal effects in organisms, including alterations in physiology, tissue structure, and reproduction [11]. Toxicity is linked to ROS production, DNA damage and protein misfolding [12]. In particular, some heavy metals can produce free radicals that can harm cellular and molecular constituents [13]. The generation of these radicals is specific and differs from metals, e.g., chromium (Cr), in particular Cr(IV), releases mostly hydrogen peroxides [14], while Hg creates oxygen and non-oxygen radicals [15–21]. Lead releases free radicals such as hydrogen peroxide (H_2O_2) and superoxide radicals (O2-) [22]. Moreover, living organisms interact with their environment not just through exposure to individual heavy metals, but typically through a combination of various compounds that can produce synergistic negative effects or antagonist effects on the organism[23] and in recent times it has been widely accepted that environmental chemicals show their toxicity not only as single entities but also as mixtures. The concomitant presence of these contaminants in marine ecosystems has been linked to many adverse effects on the health of several marine organisms [24]. Close links have been observed with immune system dysfunction [25], hematic system [26], neurotoxicity [27], defects in embryogenesis [28], disruption of the balance of sex hormones [29] and metabolism [30,31]. An aspect that should not be underestimated is the potential for heavy metals to biomagnify and accumulate along the trophic chain. in fact, metals can reach humans through the consumption of seafood products, i.e. shrimp [32], mussels [33,34], and areas related to the marine and food sector [35]. Heavy metals also are a major toxicology concern for fertility. Some of them are so-called reproductive toxins and are commonly known as 'endocrine disruptors' (EDCs) [36]. Reproductive health is one of the topics of greatest interest in recent years since the reproductive performance of most animal species is greatly impaired by high exposure to essential and non-essential heavy metals such as lead, cadmium, nickel, mercury, zinc, copper and titanium [37–39]. These compromise various reproductive functions in marine organisms [40] and therefore represent an important aspect of reproductive toxicology [41]. In addition, semen is considered an early sentinel of the health of environment and of the organisms [42]. The review aims to report the literature data relative to the effects of the main six repro-toxic heavy metals (nickel, chromium, zinc, copper, mercury, and cadmium) on the reproductive health of the organisms belonging to the genus *Mytilus spp*. These molluscs are commonly utilized in extensive marine biomonitoring programs to investigate the accumulation of pollutants in their tissues and the resulting impacts on biological processes [43–45]. Concentrations of heavy metals in these organisms reflect levels of pollution in the surrounding environment and provide an indication of the quality of the environment and the general and reproductive health of organisms [46,47].

2. Models System

2.1. M. galloprovincincialis, M. edulis, M. californianus and M. trossulus

The organisms belonging to the *Mytilus* spp. genus, play a critical role in maintaining water quality by filtering out particulate matter from the water column and supporting the nutrient cycle within marine habitats. Additionally, they serve as a vital bioindicator for the entire marine ecosystem. For these reasons, mussels (*Mytilus spp*.) are commonly employed as sentinel species in biomonitoring programmes to monitor environmental concentrations of contaminants and to evaluate the overall health of coastal and estuarine ecosystems [48]. Changes in their overall health are considered to be indicative of biological effects of environmental stressors, including contamination, and are assessed using biological effects approaches at different levels of biological complexity. Biological responses provide information on changes in the overall health of an ecosystem, which are often seen as reflecting the effects of contaminants, but not exclusively. Indeed, biomarkers may depend on environmental conditions such as tidal regime, temperature and seasonality [49], and biological features such as reproductive condition of individuals and food availability [48,50]. This genus *Mytilus* belongs to the family Mytilidae and includes several congeners. Of these, the four: *Mytilus galloprovincialis* (*M. galloprovincialis*), *Mytilus edulis* (*M. edulis*), *Mytilus californianus* (*M. californianus*) and *Mytilus trossulus* (*M. trossulus*), are the main and most common ones. These mussels are filter-feeding bivalves and are sessile organisms. Mussels are a promoted biomonitoring organism of the International Council for the Exploration of the Seas (ICES) and are commonly used for national monitoring programmes [51]. The reproductive health of all *Mytilus spp* species is vital for their population dynamics and sustainability. These species, like other bivalves and marine species such as molluscs and other invertebrates, reproduce by releasing sperm and eggs into the water column, where external fertilization occurs [52]. This reproductive strategy, coupled with their sessile adult life stage, makes them particularly vulnerable to environmental stressors, including pollution by heavy metals [53]. These pollutants can accumulate in the tissues of mussels, leading to potential disruptions in their reproductive processes and overall health [15,17,30,31,54–62].

Mussels of the genus *Mytilus* are among the most extensively studied marine molluscs, with significant research dedicated to their ecology, physiology, and genetics. However, their origins and taxonomic classification remain unclear. The four species of the genus *Mytilus*, taken into consideration for this review, share similar morphological traits and can hybridize in regions where their habitats overlap [63]. They show

differences in response for certain endpoints of biological effects, following exposure to various stressors. Overall, it has been proven that there are variations in bioaccumulation of chemicals and biomarker reactions between species of *Mytilus spp*, with potential implications for mussel exposure studies and biological effects monitoring programmes. In addition, differences in the general physiology and behaviour of the mussels affect on fitness and biological response. For example, divergences have been found in the reproductive strategy of *M. edulis* and *M. galloprovincialis*. It has been shown that thermal stress significantly affects the biogeographic and local-scale distribution patterns of the genus *Mytilus*. These temperature-related distribution patterns may be largely driven by the ability of the species to address damage at the cellular level caused by sub-optimal temperatures. The emerging insight into the responses of mussels to thermal stress is that fast shifts in the activities of signalling proteins, which are frequently the consequence of post-translational modifications of existing proteins, triggers several changes in cellular activity that have the role of re-establishing cellular homeostasis. However, potential physiological variations between species could be balanced by adaptation to particular environmental conditions and lead to similar responses to pollutants and other environmental stressors. Therefore, future research is needed to establish the degree to which species affect biological responses to pollutants, particularly in different mussel species from the same population/sampling point.

The following subsections provide a description of each model organism.

2.2. Mytilus galloprovincialis

M. galloprovincialis shares similar morphological traits with *M. edulis* and *M. trossulus*, a similarity that facilitates hybridization in regions of habitat overlap [63]. Genetic studies, utilizing mitochondrial DNA sequences, reveal a high degree of similarity between *M. galloprovincialis* and *M. edulis* [64], but only *M. galloprovincialis* has displayed the capacity to infest new locations, including South Africa, Japan and California. Native to the Mediterranean Sea, this species is adapted to warm, high-salinity environments and is generally found in intertidal, estuarine, and coastal zones; its northern distribution is limited (up to ~55°N in Great Britain), presumably due to sensitivity to colder conditions. *M. galloprovincialis* is notably more stress tolerant, surviving elevated heat exposures (32 °C for 8 hours) and displaying a robust cellular stress response that involves the increased expression of molecular chaperones (e.g., Hsp70, Hsp90, Hsp60, DnaJ, and the chaperonin TCP1) [65]. Furthermore, cases of gamete plasticity under high hyposaline conditions have been documented in this species [66]. *M. galloprovincialis* shows adaptability to wider temperature ranges compared to its congeners. All this has been observed by DNA damage from a variety of toxic insults and physical and chemical stressors in the environment. DNA damage due to combinations of environmental factors, occurs at a high rate in cells and increases under stress. The amount of SSB (single strand break) and DSB (double strand break) in mussels' cells submitted to heat and cold stress differed between species and with the temperature. Comparative evidence also indicates that *M. galloprovincialis* enhanced resistance to heat-induced DNA damage may be pivotal in its ability to colonize diverse habitats respect to its congeners [67]. The Californian hybrid zone between *M. trossulus* and *M. galloprovincialis* has only recently been reported and has therefore been the subject of relatively few investigations. This secondary contact zone was established by the introduction of *M. galloprovincialis* into Southern California, probably by sea, in the first half of the 20th century. *M. galloprovincialis* has displaced *M. trossulus* over a large area of the Californian coastline, from the Mexican border to the rough latitude of Monterey (37°N). While *Mytilus* blue mussels are generally present on the open coasts of the North Atlantic and parts of the North Pacific (as well as other geographic regions), they are unusual in California's open coast habitats and abundant only in preserved sites, often estuaries. In these protected areas, it is suggested that the mosaic of hybrid zones of *Mytilus* species is indicative of physiological adaptation to local abiotic environmental conditions. Accordance with this hypothesis is the evidence that the distribution of adults is linked to temperature and salinity gradients. *M. galloprovincialis* worldwide invasive growth suggests that this mussel may be the most physiologically adaptable among the Mytilus congeners, with reduced physiological constraints [68].

2.3. Mytilus edulis

M. edulis is believed to have originated from ancestral *M. trossulus* via a trans-Arctic migration, subsequently giving rise to the Mediterranean population that evolved into *M. galloprovincialis*. It co-occurs with *M. trossulus* in northern regions (with both observed at latitudes >66°N) and is also present in southern areas (28–35°N). Recent studies have documented considerable resilience in *M. edulis*, with reported upper lethal air temperatures between 25 °C and 38 °C. Concerning to salinity tolerance, *M. edulis* functions as an euryhaline osmoconformer, maintaining extracellular fluid isosmotic with the surrounding medium from full-strength seawater (35 ‰) to very low salinity conditions (~5 ‰), although such conditions can be tolerated only for limited periods. Molecular data confirm that its ability to acclimatize to decreasing salinity is mediated by stressor-specific HSPA12 genes [69]. In addition to its role in filtering particulate matter and supporting nutrient cycles, *M. edulis* contributes significantly to bioremediation of waste [70]. However, increased temperature decreases the thermal tolerance of marine species including *M. edulis* and may also reduce their filtration rate [71].

2.4. Mytilus californianus

Genetic investigations utilizing mitochondrial DNA have demonstrated that *M. californianus* is the most genetically distinct among the *Mytilus* congeners, a finding further supported by 18S rDNA analyses [64,72]. This intertidal mussel forms dense shoals along the western coasts of North America, where its beds can host up to approximately 300 related taxa. These aggregations play a critical role in maintaining water quality by filtering particulate organic matter and serve as prey for numerous marine organisms, thereby providing essential ecological services to coastal communities. *M. californianus* optimises nutrition and digestion by switching resource acquisition solutions when exposed to different environmental factors and use rate or yield maximisation strategies under varying environmental conditions that result in variable resource uptake efficiency. Shoals of *M. californianus* are like vital chemical reactors within coastal environments because they transform suspended organic material into particulate organic material that goes down in the form of faeces, as well as into soluble organic substances such as ammonium. Their contribution to biogeochemical processes and trophic cascades in coastal regions are proportional to their demography, which is modified over time by physiological processes that respond to the environment (e.g. feeding, growth, reproduction) However, *M. californianus* exhibits a lower high-temperature limit, with exposure to 28 °C for 12 hours resulting in markedly higher mortality compared to its congeners [67]. In line with this, comparative studies of DNA damage under thermal stress indicate that *M. californianus* suffers greater molecular damage than species such as *M. galloprovincialis*.

2.5. Mytilus trossulus

Originating from the North Pacific, *M. trossulus* is considered the progenitor of the two later-emerging species found in the North Atlantic and the North Atlantic/Mediterranean regions (i.e., *M. edulis* and *M. galloprovincialis*). It shares morphological characteristics with these congeners, which facilitates hybridization in regions of overlapping distribution [63]. *M. trossulus* is considered to be the species most tolerant to cold, low salinity conditions, although it is more vulnerable

to elevated temperatures [73]. As the species most tolerant of cold and low salinity conditions, *M. trossulus* reflects its evolutionary adaptation to the North Pacific environment. Laboratory comparisons of larval survival rates between *M. trossulus* and *M. edulis* suggest that *M. trossulus* may be more euryhaline under fluctuating salinity conditions, despite similar growth and feeding rates observed in adults. The recent establishment of a Californian hybrid zone—resulting from the introduction of *M. galloprovincialis*, which has largely displaced *M. trossulus* along significant stretches of the coast (from the Mexican border to around 37°N)—further underscores the differences in thermal tolerance and environmental adaptation between these species. It has been proven that, in response to severe heat stress, *M. trossulus* and *M. galloprovincialis* are highly similar at the transcriptome level. In particular, these species are almost indistinguishable about to the gene expression of the main components of the cellular stress response. The fact that there are relatively few differences in heat stress-induced gene expression between congeners with well-documented disparities in thermotolerance suggest that different regulation of a small subset of genes played an adaptive role in the evolution of these species [65].

2.6. Sperm chromatin organization of the species of Mytilus spp.

The DNA of eukaryotic organisms is associated with proteins, mainly basic, in a structured macromolecular assembly known as chromatin. In these cells, DNA is packaged by an association of core histones, linker histones and non-histone proteins. Nevertheless, there is marked remodelling of chromatin during spermiogenesis, involving significant cellular morphological changes, in conjunction with modifications in the nature and amount of basic nuclear proteins. The accumulating data from previous research on sperm basic nuclear proteins (SNBPs) has helped to categorise them into three main classes: histone-like (H-type) with a content of arginine about 2–6 % and lysine about 25–30 %, protamine-like (P-type) with a content of arginine about 30 %-70 % and protamine-like (PL-type) with a content of arginine about 8–30 % and lysine about 30 %-40 % [74,75]. These latter proteins are structurally highly heterogeneous and represent a molecular intermediate between histones and protamines, with evolutionary and functional implications [76]. Despite having been first discovered in bivalve molluscs, they are widespread across the animal kingdom, having been recognized in phylogenetically different organisms like Cnidaria, as well as in chordates. In molluscs, PLs proteins are usually sub-classified into four basic types based on their respective electrophoretic mobility: PL-I, PL-II, PL-III and PL-IV [77]. Like histones, PLs proteins are rich in arginine and lysine, reflecting their evolutionary relationship to histone H1 [78] and, like protamines, PLs proteins are very basic, with an arginine + lysine contents of at least 35–50 mol%, with the considerable presence of cysteine. These proteins replace somatic histones during spermiogenesis, modifying chromatin structure, particularly in organisms such as *Mullus surmuletus* where PLs proteins exclusively organize sperm chromatin. The PL-I proteins in this species generate a chromatin configuration similar to protamine-based systems in other organisms, though distinct due to the absence of histones. In most organisms investigated, PL proteins are found to coexist in mature sperm with a complex histone complement, accounting for about 30–40 % of the overall SNBPs. Investigations involving electron microscopy to observe the sperm DNA of various bivalve molluscs have found the existence of densely packed chromatin folds with a diameter of around 25–50 nm [79], based on protein composition. The early micrococcal nuclease digestion studies of the sperm chromatin of these organisms revealed the presence of a double chromatin arrangement, in which a part of the genome appears to maintain the somatic nucleosomal organisation, while the part that remains is strongly compacted as a product of the binding of the PLs compound [80,81]. The linker DNA is about 260 bp in length. The winged helix of PL-I matches the winged helix of H1, permitting the N-terminal tail of PL-I to neutralise the charge of a considerable length of linker DNA. Thus, enabling this DNA to wrap itself further around the nucleosome. The two extra coils formed in this way would produce two additional potential binding sites for PL-I and make for a highly compact but uniform structure. *Mytilus* spermatozoa are rich in PLs proteins. Specifically, these proteins—designated as PL-II, PL-III, and PL-IV—constitute approximately 80 % of the protein composition associated with sperm chromatin. PL proteins are found in *M. galloprovincialis* in the following pattern: PL-II comprises approximately 20 % of the total, while PL-III constitutes a predominant 50 %, with PL-IV making up a smaller fraction at approximately 6 %. The remaining 20 % of the chromatin-bound protein structure is composed of core histones [82]. There are probably three PL-III molecules for every for each molecule of PL-II or PL-IV, allowing for the structure of sperm chromatin [82]. *Mytilus* protein PL-II is a member of the histone H1 family, having a conservative globular core of 84 amino acid residues that show high familiarity with the winged helix motif of histone H1. The PL-IV protein is very small (6500 kDa) and has a highly analogous composition to the C-terminal lysine-rich tail of histone H1 [83]. In *Mytilus* sperm chromatin the series of complex and dynamic changes result essential for the successful delivery of genetic material during fertilization. These modifications are tailor-made to ensure the protection, compaction, and appropriate regulation of the DNA within the spermatozoa, which is crucial for the development of offspring, making it a unique mark in sperm chromatin organization. During spermatogenesis, the replacement of conventional histones with PLs proteins, as described by Vassalli et al. [84] is critical as PLs proteins play a pivotal role in the condensation and stabilization of the sperm DNA. The enhanced compaction achieved by the incorporation of PLs proteins serves multiple purposes. Firstly, it reduces the volume of the sperm nucleus, allowing for a better motility. Secondly, this tight packaging provides robust protection to the genetic material against potential physical and chemical damage in which the sperm cell might come across [85]. This protective mechanism is vital, given the vulnerability of spermatic DNA, which could lead to mutations or fragmentation. Moreover, PLs protein replacement is not only a structural adaptation but also a regulatory mechanism. The replacement of histones with PLs proteins changes the epigenetic organization of sperm chromatin, influencing gene expression patterns. Of the three SNBPs in *Mytilus* sperm, PL-III is present in the greatest quantity and research on *M. trossulus* emphasizes the significance of this protein, which constitutes the majority of sperm nuclear proteins and exhibit considerable interspecies variability [74,82]. Much research shows that PL-II/PL-IV and PL-III of *Mytilus* are encoded by separate genes. The PL-II/PL-IV protein is cleaved post-translationally to produce two distinct peptides, PL-II and PL-IV, which exhibit sequence similarities with the globular region and the C-terminal region of a canonical H1, respectively. Although the presence of an intermediate sequence in the coding region of PL-III of the mussel *M. edulis* had been previously described (Ruiz-Lara et al., 1993), but no such intron was found in the genomic sequence of PL-III of *M. californianus*. It is assumed that the discordance may be the reason for the well-known linkage of PL-III with highly variable regions of the genome, which incur regular gene conversions and unequal recombination effects. A comparison of the nucleotide coding sequences of the various PLs groups of molluscs displays, as predicted, that the lowest divergences are reported in PL-II/PL-IV and PL-III. This could be assumed to be an indication of the recent divergence of these genes, as not enough time has evolved to collect high nucleotide divergence [76]. In their exploration of the histone-to-protamine transition, Vassalli et al., 2015 [84] hypothesize a structural arrangement where the PL-III protein acts as a central scaffold around which DNA molecules are intricately wrapped (in a core histone complex) [86]. According to the electrophoretic assay described by Vassalli et al. [84], PL-III protein exhibits a binding behaviour to DNA described as "all or nothing," resembling that of H1 protein [87]. This similarity is attributed to the conserved amino acid sequence between the two proteins, particularly in their lysine and arginine content, which is also conserved across histones and this specific SNBP isoform. This is a

result of their low K/R (lysine/arginine) ratio [88,89], which is the lowest among all three PLs protein isoforms. This protein core is flanked by PL-II and PL-IV proteins, which likely contribute to the overall stability and organization of the chromatin during this crucial biological process. Given the ecological significance of the organisms belonging to the genus *Mytilus* and their sensitivity to environmental pollutants, understanding the molecular mechanisms underlying heavy metal-induced reproductive toxicity is crucial. Research has shown that exposure to metals such as mercury, copper, cadmium, nickel and chromium can lead to alterations in PLs proteins which as mentioned above, are just such key proteins for reproduction, as they are essential for chromatin stability and DNA protection in the sperm of these organisms [15–17,31,62]. These molecular alterations can lead to a decrease in reproductive success and can threaten the sustainability of mussel populations. These proteins, which also showed antibacterial activity against several bacterial strains and even against clinical isolates [90], are so sensitive to environmental pollutants that exposure of mussels to chromium and mercury led to a reduction in their antibacterial activity [91].

3. Heavy metals

Heavy metals are naturally occurring elements that are found in the Earth's crust and are present in soils, water bodies, sediments, atmosphere, and living organisms. Natural phenomena such as volcanic eruptions and biomass fires contribute to their release. However, human activities significantly exacerbate the problem. Practices such as mining, fuel combustion, industrial discharges, urban sewage, and agriculture have led to widespread contamination of the environment with heavy metals, affecting locations globally, from polar regions to deep oceans [92]. Heavy metals are well known as environmental risks due to their environmental toxicity persistence and bioaccumulation throughout life organisms. A higher concentration of one or a combination of some heavy metals could be the cause of the poor larval survival in mussels [93]. In particular, the aquatic compartment is particularly susceptible to contamination by heavy metals, which can accumulate in sediments and organisms, as well as being a risk to human health, given the large source of food that the seas represent [94,95]. Little is known about how heavy metals affect invertebrate reproduction. For these reasons, in this review we collected all the information relative to the molecular effects of some reprotoxic heavy metals (nickel, chromium, zinc, copper, mercury, cadmium) on the mussels of the genus *Mytilus* spp. (Fig. 1).

3.1. Chromium

Chromium usually exists in two oxidative states, i.e., Cr(III) and Cr (VI); and Cr(VI) is 100 times more toxic and 1000 times more mutagenic than Cr(III) [96]. In the aquatic environment, Cr(VI) is the predominant chemical form of chromium in aquatic ecosystems, where it is released from domestic and industrial sources and has the potential to cause a variety of adverse effects in biological systems [97,98]. As regard the toxic effect of chromium on the reproductive health of these mussels, Moriello et al., 2022 [14] investigated on the effects of 24-hour of exposure to different concentrations of hexavalent chromium (VI) (1, 10 and 100 nM) on the reproductive dynamics of *M. galloprovincialis*. Their findings revealed that lower concentration of chromium (1 nM) produced notable alterations in the properties of PLs proteins. Specifically, at 1 nM chromium exposure, PLs proteins exhibited an bioloired ability to bind DNA, indicating reduced DNA protection to oxidative stress mechanisms compared to control samples. The study then elucidated the interaction between chromium and PL proteins using ANS (8-Anilinonaphthalene-1-sulfonic acid) dye, which brought to light that chromium molecules bind to the hydrophobic domains of PLs proteins, causing conformational changes in these proteins [14]. Considering that PARP-catalysed ADP-ribosylation is also important in the defence against heavy metal toxicity and the maintenance of genomic integrity, and that PARylation is involved in chromatin remodelling, but its role in

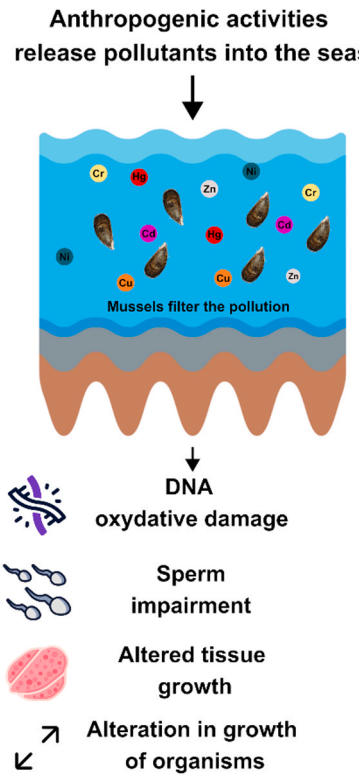

Fig. 1. Principal effects on reproductive health following the exposure of *Mytilus spp* to thesee heavy metals, released into the sea by human anthropogenic activities.

sperm chromatin remains to be elucidated, it was also investigated the effects of chromium(VI) at the same doses on this aspect [31]. Damage to the gonads was determined by morphological analysis and the indices of damage PARP and ɣH2A.X were quantified. The possible poly (ADP-ribosyl)ation of PLs proteins was also assessed. The results of this work demonstrated that mussels exposed to the highest dose of chromium(VI) showed gonadal accumulation and damage [99]. In particular, the highest levels of gonadal ɣH2A.X and PARP were found at 100 and 10 nM Cr(VI), respectively [99]. Interestingly, poly (ADP)-ribosylation was detected for the first time under all exposure conditions on PL-II, which together with PL-III and PL-IV are the major nuclear basic proteins of *M. galloprovincialis* sperm chromatin [99]. Since PL-II is involved in the final high level of sperm chromatin compaction, this post-translational modification altered the binding of the PLs protein to DNA. These studies provide new insights into the effects of chromium(VI) on the reproductive system of *M. galloprovincialis* and proposes a molecular mechanism hypothesis describing the toxic effects of this metal on PLs -DNA binding, sperm chromatin and gonads [99]. Zaroogian et al., 1983 [100] also confirmed that bivalve molluscs, especially *M. edulis*, can accumulate chromium in their tissues, including reproductive tissues. During a spawning period [100], observed a decrease in chromium concentrations, which coincided with gamete production and subsequent releases. This suggests that during the gamete production period, the organism uses most of its energy to produce gametic cells by reducing chromium intake. The long-term interaction of chromium with reproductive tissues, however, results in a considered metal stress that, in combination with high energy metabolism due to gamete production, may have deleterious effects on reproductive tissue and gamete quality. Finally, the few data available on the consequences of chromium (VI) on the reproductive health of these mussels highlights the toxic effects of this metal which cause oxidative damage by affecting enzyme activity and gene expression. The histological abnormalities observed also suggested that Cr(VI) caused

developmental delay and disruption of spermatogenesis by damaging sperm structure and generating reactive oxygen species (ROS) [101]. These results are consistent with those obtained in other bivalves, where alterations in a several antioxidant biomarkers and gene transcription were detected in sperm [102]. There is no information on the effects of this metal on the reproductive health of *M. trossulus* and *M. californianus*.

3.2. Nickel

Nickel is a hard, ductile, silvery-white transition metal. It can exist in several oxidation states (from −1 to +4), but the + 2 oxidation state (Ni2 +) is the most common in the environment and biological systems. Although it can occur in several different oxidation states, the predominant oxidation state under environmental conditions is Ni(II), nickel in the + 2 valence state. Valences (-1, +1, +3 and +4) are also found, although they are less common. Nickel is a ferromagnetic element and occurs naturally in the Earth's crust, usually in combination with oxygen and sulphur as oxides and sulphides. In combination with other elements, nickel can be found in the soil, in meteorites and erupted from volcanoes. There are about eight billion tonnes of nickel in the oceans. Due to its unique physical and chemical properties, nickel is used in modern metallurgy in a wide range of metallurgical processes such as alloying, electroplating, nickel-cadmium battery production and as a catalyst in the chemical and food industries. The widespread use of products containing this metal inevitably leads to environmental pollution from nickel and its by-products at all stages of production, recycling and disposal. It is recognised as an essential element for several important biological processes, such as the healthy growth of plants, animals and soil/water microbes. Nickel is linked to reproductive toxicity, but there is little knowledge about the molecular mechanisms behind nickel-induced impacts on the sperm chromatin and PLs. Carbone et al., 2023 [62] conducted a series of experiments to elucidate the effects of nickel exposure on *M. galloprovincialis* spermatozoa. The study assessed the impact of nickel on PLs proteins and their DNA binding capabilities after exposing *M. galloprovincialis* specimens to 5, 15, and 35 μM $NiCl_2$ for 24 h. They analyzed PLs by urea-acetic acid polyacrylamide gel electrophoresis (AU-PAGE) and SDS-PAGE and assessed the binding of PLs to DNA by Electrophoretic Mobility Shift Assay (EMSA). In addition, to evaluate possible changes in sperm chromatin, a time course of digestion with MNase and the release of PLs from sperm nuclei by the NaCl gradient was also performed. For all doses, an additional migrating band was observed on AU-PAGE between PL-III and PL-IV, which corresponded to a fraction of PLs in the peptide form detectable by SDS-PAGE. This was indicative of the formation of protein fragments and peptides. This phenomenon suggests that exposure to nickel leads to modifications of PLs proteins. Existing literature hints at a possible model wherein nickel binds to specific amino acids, particularly serine, which are abundant in PLs proteins. This binding may induce the formation of peptides. Changes in the DNA binding ability of PLs were found by EMSA after exposures to 5 and 15 μM $NiCl_2$, in fact, a higher amount of PLs was necessary to obtain DNA saturation and enhanced accessibility of MNase to sperm chromatin was found at all doses of $NiCl_2$, meaning a more decondensed structure [62]. The effect was particularly pronounced at 15 μM $NiCl_2$, a dose at which there was also increased release of PL-II and PL-III from sperm nuclei and the highest level of accumulation of nickel in the gonad [30]. In line with other findings from other heavy metals (i.e. mercury), this impairs spermatic motility and, consequentially, fertilizing capability. The decrease in DNA-binding capacity by PLs after mussel exposure to nickel, is in agrees with other papers in which nickel has been demonstrated to inhibit specific DNA-protein interactions [103]. On the other hand, nickel has also been reported to influence the proper assembly of human protamines with DNA [104]. Considering that protamine-like proteins belong to SNBP together with protamines, probably could be a key target for the toxicity of this metal, since nickel can interact with arginine residues which are crucial for the binding of these proteins to DNA [105,106]. In addition, the enhanced accessibility of sperm chromatin to MNase could be explained by the capacity of nickel to induce conformational changes in PLs that in turn bind diversely the DNA, generating an improper sperm chromatin structure.

As a matter of fact, fluorescence analyses of PLs of mussels exposed to these doses of $NiCl_2$ produced increased fluorescence compared to the unexposed condition, indicative of PLs structural changes that modified interaction with the fluorescence probe. In particular, the results obtained after $NiCl_2$ exposure indicated the formation of a more constrained macromolecular aggregate between PLs and ANS [57]. On the other hand, it has been reported that nickel interferes with the interaction between protamines and DNA by altering the functional structure of protamines [107] and making sperm DNA "loosely packed" [108] and more susceptible to damage caused by ROS. The molecular data on altered condensation of sperm chromatin was supported by morphological data from Toluidine Blue (TB) -stained sperm smears. In fact, while in the spermatozoa of the control mussels, a clear staining of the nucleus was seen, indicative of an appropriate chromatin compaction, the spermatozoa of mussels exposed to nickel were found nuclei stained in a deep blue [57]. This latter result revealed poor chromatin compaction most likely due to the nickel hindering the action of the PLs, whereby DNA sites become more accessible to TB by staining them deep blue [109]. Moreover, morphological analyses showed severe gonadal damages which was also supported by increased Poly ADP-ribose polymerase (PARP) expression, by Western blotting, and sperm DNA fragmentation, by comet assay Marinaro et al., 2024 [57], in line with Carbone et al., 2023 [62].PARP activation detect DNA damage and initiate repair mechanisms to ensure genomic integrity. Then, in *M. galloprovincialis*, nickel exerts a dual harmful impact on sperm cells: it affects both the properties of PLs proteins and induces DNA damage, as suggested by PARP activation. In Marinaro et al., 2024 [57] it was also investigated the expression of stress genes, *gst*, *hsp70* and *mt10*, in gonadal tissue after $NiCl_2$ exposure and it was found that exposure to this metal produced an altered expression of these genes. Very recently, the impact of nickel exposure to the same doses, on the metabolic profile of *M. galloprovincialis* spermatozoa was determined [31]. This pioneering research represents the first exploration into the metabolic changes occurring in *M. galloprovincialis* spermatozoa when exposed to heavy metals. The findings of this study revealed alterations in the levels of some metabolites, which play crucial roles in various pathways essential for sperm function. These preliminary results were obatained using a metabolomics approach based on GC–MS analysis as described in Lettieri et al., 2023 [31]. These authors found that the level of urea was higher compared to the control after exposure to nickel. Elevated urea concentrations above normal physiological levels had adverse effects on important aspects of sperm function, including sperm membrane and acrosome integrity and mitochondrial membrane potential. All of these parameters play a vital role in the success of the fertilisation process. In contrast, myo-inositol was most abundant in the control compared to nickel exposure. Myo-inositol is a safe compound that has been shown to be useful in fertility issues. Myo-inositol is involved in several signalling processes, including the insulin and gonadotropin pathways, and is therefore likely to have a positive effect on fertility. Glucose and L-serine were found to be higher after nickel exposure compared to the control condition. This could be explained because serine is considered a non-essential amino acid involved critical biological activities ranging from protein synthesis to cell signalling, the latter mainly through post-translational modification by phosphorylation [110,111]. Serine is a precursor to glycine and cysteine and can be used to synthesise glutathione. The synthesis of glutathione reduces oxidative stress and increases the activity of antioxidant enzymes [112,113]. Finally, the malic acid (MA) amount increased after exposure of mussels to nickel compare to the control condition. MA is an excellent chelator of toxic elements [114] and has been found to enhance the expression of several genes that are associated with antioxidant enzymes. In another study, it was evaluated the level of the same metabolites in the male gonads of

mussels exposed to the same doses of Nickel [30]. All metabolites tested in this study resulted lower in comparison with the control condition, after nickel exposure. In particular, the lactic acid. The reduction in lactate resulting from the exposure of mussels to nickel could probably cause fertility problems, as nickel inhibits the enzyme lactate dehydrogenase, which catalyses the reaction from pyruvate to lactate [41]. Another metabolite which decreased after nickel exposure was Myo-inositol. In this study also other heavy metals were considered. It was observed that exposure to copper and cadmium metabolites in gonads showed an opposite trend compared to nickel, possibly due to the dose of nickel chloride used. These data were in line with those obtained exposing for 24 h to individual chlorides of Ni, Cu, 5, 15, 35 μM and Cd 1,5; 5, 10 μM in artificial sea water. Inductively coupled plasma mass spectrometry revealed that metal levels in both tissues accumulated in a dose-dependent manner with exposure, except for Ni in gonadal tissues, where a low level was found at the highest exposure. Nickel was the most efficient metal to produce π-gst expression increase in both tissues; The lowest δ level of π-gst expression was obtained at 15 μM, indicating a possible hormesis effect. Hormesis is an adaptive function observed in all living organisms, including plants, and may be due to changes in physiological state or modification of regulatory mechanisms [115]. The effects of nickel on *M. trossulus* and *M. edulis* have been explored in a limited number of studies, revealing some concerning insights into the impact of this metal on mussel development. In a notable study by Nadella et al. (2009) [116], researchers focused on the toxicity of nickel during the early life stages of *M. trossulus*, conducting a 48-hour development test on embryos and larvae. Their findings indicated that exposure to nickel resulted in significant abnormalities in larval development, with an established EC50 value of 150 μg/l (0.15 mg/l) and an EC20 of 82 μg/l (0.082 mg/l). This highlights the sensitivity of these organisms to nickel contamination, particularly during critical developmental phases. Similarly, Martin et al. (1981) [117] investigated the impact of nickel on the larvae of *M. edulis* in a separate 48-hour experiment. Their research determined the 48-hour EC50 for nickel exposure to be 891 μg/l (0.891 mg/l), further underscoring the detrimental effects of this metal on marine bivalves. Together, these studies contribute to our understanding of how nickel pollution can threaten the development and survival of mussel populations, emphasizing the need for ongoing research and monitoring of heavy metal contaminants in marine environments. There is no information on the effects of this metal on the reproductive health of *M. californianus.*

3.3. Copper

Copper is used in construction, engineering, energy, transport, electronics, agriculture and, in more recent years, in the animal feed industry. In the 1880s, fungicide (based on a mixture of copper sulphate, lime and water) was used on a large scale to control powdery mildew in vines. In the 1950s, there was laboratory evidence that copper had antifungal properties. After 1987, the use of copper in agriculture increased worldwide [118]. Copper is an essential element, but it can become toxic when found in excessive quantities within the ecosystem. A European Union initiative, titled Marine Ecosystem Evolution in a Changing Environment, has found that significant amounts of copper are discharged into the marine environment [119]. It is often detected at elevated concentrations along coastlines and in the water column of the open sea. Notably, its levels are particularly high in sediments and interstitial waters [120]. Various studies on the effects of copper exposure on the reproductive system of *M. galloprovincialis* have highlighted its detrimental impacts. Zitoun et al., 2019 [121] observed a reduction in embryonic development in the larvae of *M. galloprovincialis* after exposure to copper. Copper also produces sex reversal in female mussels as demonstrated in *M. galloprovincialis* in the coastal waters of Crimea [122]. In particular, these authors indicated that copper can lead to considerable alterations in sex ratios, with the most pronounced effect observed for Cu^{2+} (19.5 μg· L^{-1}), resulting in 56.3 % of females experiencing sex reversal. The findings imply that environmental factors significantly impact mussel populations, because Cu^{2+}, determined to be highly toxic, causing 20 % mortality during the experiments. Fitzpatrick et al., 2008 [123] conducted a study on egg and sperm motility, fertilization success, and embryo development of *M. galloprovincialis* after exposure to copper. Sperm exposed to high copper concentrations (100 μg/L) exhibited notably reduced swimming speeds and impaired fertilization capacity, although their morphology remained unaffected. Eggs, on the other hand, showed no loss of viability even at elevated copper levels, suggesting that sperm function is more vulnerable to copper toxicity than eggs. Embryo development was susceptible to copper, with a marked increase in abnormalities occurring at concentrations above 10 μg/L. At 100 μg/L, most of embryos developed abnormally. The embryotoxicity of copper was confirmed also by Boukadida et al., 2019 [124], on larvae of *M. galloprovincialis* finding an increase of malformed larvae and double strand break DNA. In the 2019 study by Lettieri et al., 2019 [59], copper exposure was found to alter the expression levels of *mt10* and PLs protein genes in the gonads and spermatozoa, respectively. In addition, PLs proteins, from mussels exposed to copper (15 μM $CuCl_2$), exhibited higher DNA binding affinity and required increased NaCl for release from sperm nuclei, suggesting structural changes. Moreover, PLs proteins also showed a change in DNA binding from all or nothing to intermediate forms [87]. Additionally, these effects promoted oxidative DNA damage in the presence of H_2O_2, highlighting the genotoxic potential of copper [59]. On the other hand, the involvement of arginines in copper/H_2O_2-induced DNA breakage was already demonstrated [125]. These changes rendered the spermatozoa unable to prevent DNA damage, indicating a broader impact on reproductive health [59]. The study by Lettieri et al., 2019 [59] confirmed these findings, showing alterations in *mt10* and *hsp70* gene expression. Further elucidations were obtained in a study of 2023 by Lettieri et al., 2023 [31] utilizing metabolomics approaches to reveal complex interactions in metabolic pathways and oxidative stress metabolism. Specifically, levels of Malic Acid (MA), in spermatozoa, increased denoting an antioxidant response. This comprehensive approach provided detailed insights into how copper disrupts the physiological functions of spermatozoa [31]. The same metabolomic analyses were performed also on the gonadal tissue of *M. galloprovincialis* [30]. The exposure of *M. galloprovincialis* to 15 μM of $CuCl_2$ produced an increase in lactic acid, and this increase in lactic acid production could be due to a defensive state on the part of the tissue to avoid cell apoptosis [30]. Regarding *M. trossulus*, in a study conducted by Fitzpatrick et al., 2008 [123] it was investigated the effects of copper on early life stages of this mussel. The authors evaluated the effects of increasing copper concentrations on embryo development, oocyte viability, sperm fertility and, in particular, sperm swimming speed using computerized sperm analysis. Sensitivity to copper followed the pattern: embryos> sperm> eggs. A drastic increase of embryo abnormal development was found after exposure to copper at concentrations above 10 μg/L. The swimming speed of spermatozoa was significantly reduced by exposure to 100 μg/L copper, but sperm swimming speed was not affected by lower doses [123]. Exposure to copper (at each concentration tested) did not influence sperm flagellum length. Based on these findings, it is suggested that sperm exposure to copper may impair mitochondrial activity, reducing sperm swimming speed [123]. The negative effects of copper on spermatozoa motility were observed also by Earnshaw et al., 1986 [126] on *M. edulis*. The authors observed a reduced motility of spermatozoa after exposure to copper at concentrations in the range of 0.1–3 mM. The decrease in sperm motility could be due to improper functioning of the spermatozoa's mitochondria as copper is can alter the electron transport chain and consequently alter the energetic production of the spermatozoa. In addition, Hoare et al., 1995 [127] found that copper has significant effects on the embryonic development of *M. edulis*, with variation in tolerance observed among populations exposed to different levels of pollution. In fact, embryos from unpolluted sites exhibit higher rates of abnormal development and

lower yields of normal larvae when exposed to copper concentrations as low as 8 ppb. In contrast, embryos from polluted environments show greater resistance, likely due to maternal and some genetic influences. This could be an indication of a possible physiological or genetic adaptation to pollution, allowing these mussels to better survive in conditions of high copper pollution. The increased abnormalities in embryos from unpolluted areas highlight the toxic nature of copper, even though it is an essential element in small amounts. In a similar manner, a decrease in spermatozoa motility after exposure to 100 μg/L copper for 100 min of these cells was observed for sperm in *M. trossulus* [123]. In addition, these spermatozoa showed a reduced ability to fertilize the oocyte of unexposed spermatozoa. Finally, several authors reported that the presence of copper also affected embryonic development, causing an increase in abnormal embryos [116,123]. In *M. californianus* instead, larvae exposed to copper concentrations as low as 2–25 μg/L showed suppressed gene expression, in particular gene implicated in polysaccharide binding and metabolism, at lower doses, affecting developmental pathways, including nervous and muscular systems. Higher doses triggered gene induction related to stress responses like oxidative stress and metal detoxification. Downregulated genes were particularly sensitive markers of copper exposure, providing early warning signs of toxicity, even at sublethal concentrations. Biomineralization pathways, essential for shell formation, were notably suppressed, contributing to morphological abnormalities in developing larvae [128]. While copper is necessary for normal physiological functions, its dysregulation can have deleterious effects on male reproductive health. Therefore, careful monitoring of copper levels in the environment and within the body are essential to prevent these adverse outcomes. Collectively, these studies indicate that even subtoxic levels of copper can significantly disrupt the reproductive health of this species.

3.4. Zinc

One of the most important nutrients for animal health is zinc (Zn). Many proteins, important enzymes and transcription factors bind to Zn and are thought to be dependent on Zn for their functions. Zn has a role in many biochemical processes that are essential for life. They include cellular respiration, cellular utilisation of oxygen, DNA and RNA expression, maintenance of cell membrane integrity, scavenging of free radicals, and protection from lipidperoxidation. A central component of metalloenzymes is the trace element Zn, lactate dehydrogenase, carboxypeptidase and DNA and RNA polymerases and RNA polymerases [129]. The increase of Zn in the environment is mainly related to human activities such as mining and metal smelting [130]. In the genus *Mytilus spp*., zinc is an essential trace element for the development of the gonads and proper production of gametes, both male and female. In addition, zinc plays an important role in the development of larval shells [131]. Like other metals, however, zinc, despite its important biological function, is capable of inducing problems in the organism if present in excess. Bayne et al. [132] examined stressors affecting different stages of gonadal development in *M. edulis*, using various indices of condition and maturity to assess physiological stress. Of the metals observed, zinc inhibited gamete maturation but with minor detrimental effects that were not immediate (unlike copper, which prevented mussel development at very low concentrations, damaging gonadal development). In *M. edulis*, concentrations of zinc between 0.1 and 3 mM, caused a reduction in sperm motility. In addition, by transmission electron microscopy, zinc was found to be the metal capable of inducing greater mitochondrial damage in *M. edulis* spermatozoa and ultrastructural damage to the spermatozoon, compared with untreated spermatozoa [126]. In addition, Akberali et al. [133], reported a decrease of mitochondrial activity of spermatozoa in the presence of zinc in the sea water, for both sperm and egg cells. This effect is linked to the shifting of seawater pH [126]. Hanna et al., 2013 [134] instead, investigated the effects of engineered zinc oxide nanoparticles (ZnO ENPs) on *M. galloprovincialis* mussels, focusing on physiological and demographic responses. Mussels exposed to ZnO ENPs for 12 weeks displayed increased respiration rates and zinc accumulation in tissues, with small mussels accumulate zinc up to 10 times faster at 0.5 mg/L concentrations in the gonadal tissue. Growth declined by approximately 40 % for mussels exposed to 2 mg/L ZnO, while survival rates dropped significantly at this concentration, particularly for smaller mussels. The findings suggest size-dependent vulnerability, with smaller mussels exhibiting heightened sensitivity to ZnO ENP toxicity. These effects were attributed to energy expenditure in mitigating zinc toxicity, limiting resources available for growth and reproduction. While these concentrations likely exceed typical environmental levels, the study highlights the potential risks of ZnO ENPs to marine ecosystems, emphasizing the need for further research on their long-term impacts, especially under food-limited conditions [134]. Regarding *M. californianus*, no studies on the reproductive effects of zinc have been reported. Nadella et al. [116] studied the effects of metal toxicity on *M. trossolus* embryos, focusing on four dissolved metals, including Zn at various salinity levels. Their results indicate that *M. trossolus* embryos show increased sensitivity to Cu and Zn, raising concerns about their vulnerability in both acute and chronic exposure scenarios. Thus, Zn pollution poses significant risks, highlighting the importance of understanding the effects of this metal on marine organisms.

3.5. Mercury

Mercury (Hg) is a toxic and persistent non-essential metal that can be found in various chemical forms in the environment, including elemental, organic, and inorganic. It is released into the environment from both natural and anthropogenic sources. These different forms of Hg can interconvert, contributing to their toxicity and bioavailability [135]. Organic mercury compounds include methylated forms such as monomethyl mercury (MMHg), dimethyl mercury (DMHg), and phenyl mercury (PhHg). Elemental mercury (Hg_o), a highly volatile liquid at room temperature, is emitted as vapour into the atmosphere, whereas inorganic mercury exists as salts like mercuric ($HgCl_2$, HgS) and mercurous (Hg_2Cl_2) compounds. Gaseous elemental mercury has an atmospheric lifetime of approximately one year, and once released, it can be transported on hemispheric and global scales [136]. In recent decades, environmental mercury contamination has become a major global health concern. This heavy metal, primarily produced by industrial activities, mining and the burning of fossil fuels, finds its way into bodies of water, where it is transformed into methylmercury, a highly toxic form. The contamination cycle begins with the accumulation of mercury in aquatic ecosystems, and from there it expands through the food chain [137]. Hg is a notable heavy metal that is prevalent in the air, soil, and water. In the case of mercury, mussels are also an excellent solution for biomonitoring studies [138].

Mussels, are notably vulnerable to the accumulation of methylmercury, an exceedingly toxic organic form of mercury. Thriving on the seabed, these organisms filter particles from the water column, leading them to absorb and concentrate substantial amounts of mercury from their surroundings. This process of bioaccumulation presents serious health risks, not only to the mussels themselves but also to the broader marine ecosystems in which they reside. The consequences extend beyond the aquatic environment, posing potential threats to human health through the consumption of contaminated mussels, which are a vital dietary staple in many cultures [139]. Regular monitoring of mercury levels in bivalves is essential, as it could play a pivotal role in protecting marine biodiversity and public health. As regard the molecular alterations of the components of sperm chromatin in mussels induced by mercury, Lettieri et al. [16] investigated the impact of $HgCl_2$ exposure on the male gonads of the mussel species *M. galloprovincialis*. This research explores changes in gonadal morphology and gene expression tied to stress responses. After exposure to varying concentrations of mercury (1, 10 and 100 pM $HgCl_2$ for 24 h), significant

hypo-expression of stress-related genes (*mt10*, *π-gst*, and *hsp70*), by RT-qPCR, was documented, accompanied by notable disruptions in gonadal structure. In addition, the altered expression levels of the *mt10* and *hsp70* genes in the sperm were also measured [17]. At the same mercury exposure doses inductively coupled plasma-mass spectrometry indicated an accumulation of this metal in the gonads of exposed mussels [17]. In addition, the expression of PLs protein genes resulted in decreased expression, especially for PL-III. These findings point to significant cellular stress and raise serious concerns regarding reproductive health. The study shed light on the complex interactions between mercury exposure and the biological mechanisms governing spermatogenesis and hormone production, particularly concerning key enzymes (3β-HSD and 17β-HSD) involved in testosterone synthesis. After exposure of mussels to these doses of $HgCl_2$, fluorescence spectroscopy measurements indicated mercury-induced conformational changes in PLs proteins. In addition, turbidity assays revealed that these doses of mercury induced PLs protein aggregates [15], in fact, these proteins appeared partly as aggregates on SDS-PAGE and showed a reduced DNA-binding capacity that rendered them unable to prevent DNA damage in the presence of $CuCl_2$ and H_2O_2. [17]. Micrococcal nuclease digestion showed that all doses of $HgCl_2$ exposure resulted in increased accessibility of sperm chromatin to this enzyme, indicating improper sperm chromatin structure [16]. On the other hand, alterations in PLs -DNA binding after mussels exposure to $HgCl_2$ at these doses, was also evidenced by DNA absorption spectra and by decreased release of PL-II and PL-III from sperm nuclei [15]. Moreover, another group of authors conducted an analysis of Hg levels in Baltic mussels (*M. trossulus*) from Puck Bay, Poland. Their study focused on both total mercury and its labile and stable forms. Researchers measured Hg concentrations in mussels, along with their diet (suspended matter and phytoplankton) and surrounding sediments. The results indicated that total mercury concentrations in mussels are influenced by individual characteristics related to growth and reproductive cycles, as well as the quality of their food sources [140]. Agnieszka Jędruch et al., 2019 [140] instead, observed a negative correlation between bioaccumulation of mercury and weight of soft tissue of *M. trossulus*. This data is indicative of a negative effect of mercury on *M. trossulus* development [140]. Regarding *M. californianus,* Robert P. Eganhouse and David R Young 1978 [141] reported an excess of bioaccumulation of mercury until 30 m of sampling, in all tissue, including the gonads. The bioaccumulation of mercury in the gonads tissue was already reported by Lettieri et al., 2021 [17] for *M. galloprovincialis*. Many studies emphasize the variable and often detrimental effects of mercury on the survival and development of mussels, demonstrating a pronounced sensitivity of their early life stages to mercury exposure. For instance, Nelson et al. (1988) [142] identified lethal concentrations of mercury (LC50 and LC95) for juvenile *M. edulis* at 161 μg/L and 284 μg/L, respectively. In terms of developmental impacts [143], determined a 48-hour embryotoxicity EC50 of 5.8 μg/L for *M. edulis*. Additionally, Beiras and His 1995 [144] observed significant reductions in larval growth at a concentration of 4 μg/L, with an EC50 for abnormal larval development calculated at 10 μg/l [117]. Moreover, exposure to methylmercury has been linked to diminished feeding rates in *M. edulis* at concentrations ranging from 400 to 2800 μg/L, without causing immediate mortality [117]. In contrast, Pelletier 1988[145] reported alarming mortality rates of 30–67 % at a considerably lower concentration of 3 μg/l over a 32-day exposure period. These findings collectively highlight the critical and varied consequences of mercury exposure on mussel populations, particularly during their vulnerable early life stage [145].

3.6. Cadmium

Cadmium (Cd) is a non-essential element, very toxic and present in all environmental compartments [146]. An enormous amount of cadmium is released naturally into the environment, around 25,000 tons annually, causing structural changes. Fifty percent of this cadmium is found in rivers from rock weathering, and some is released into the air from fires and volcanoes. Several human activities, such as the manufacturing industry, contribute to increased emissions of cadmium to the environment [147]. The main source of cadmium pollution in the aquatic environment is anthropogenic inputs [148]. In marine organisms like *M. galloprovincialis*, cadmium can bioaccumulate in tissues over time, posing significant risks to both physiology (hepatopancreas accumulation) and reproduction (gonadal accumulation). While in the first case it poses a threat to metabolism and similar dynamics, gonadal accumulation compromises the reproductive mechanism [149]. Surprisingly, cadmium bioaccumulation in mussel tissue is very difficult to reverse: as the experiments from Scudiero et al., 2014 [149] show, also after a long period of metal-free seawater, levels of cadmium continue to be significant, indicating unreversible threats to its reproductive system. Cadmium is recognised as a reproductive toxicant that can alter fertility through effects on reproductive hormones and oxidative stress. Indeed, one of the primary consequences of cadmium toxicity is its impact on the gonads, where it impairs gametogenesis and leads to reduced reproductive output. Seasonal studies have highlighted a clear pattern of cadmium bioaccumulation, with the lowest accumulation rates in spring, coinciding with the onset of the reproductive season. This seasonal fluctuation suggests that cadmium accumulation is closely linked to the mussels' physiological state and reproductive cycle. Furthermore, cadmium exposure also affects the gonadosomatic index (GSI), a measure of the proportion of gonad tissue to the total soft tissue mass, which decreases during the reproductive season when cadmium is present. The reduction in GSI during peak cadmium exposure is an indication of impaired gonadal development and a reduction in the production of gametes, ultimately affecting population dynamics. Interestingly, there's also a correlation with Metallothionein (MT) accumulation, which are proteins involved in metal detoxification [150]. In fact, as MT levels decrease, mussels become more prone to bioaccumulate cadmium particles, compromising reproductive capacity. This statement is strengthened by the observation of the GSI index about MT levels: in reproductive cycles, a high MT levels positively correlate with high GSI index, which reflects the mussels' reproductive status. The metal detoxification action of MT protects reproductive cells from damage avoiding ROS signaling. Another study investigated the effects of cadmium bioaccumulation in the gonads of *M. galloprovincialis*, mainly comparing specimens from a polluted site in Bagnoli with reference specimens from Capo Miseno. During a 24-hour exposure to various concentrations of cadmium chloride ($CdCl_2$), analyses revealed a significant increase in cadmium accumulation in the gonads of mussels. The study focused on increased expression of the *mt20* isoform of MT, known to respond to heavy metal exposure suggesting negative repercussions for the reproductive health of these mussels [148]. Concerning to mussels, concentrations of 100ppb in seawater cause a delay in follicular development in both males and females [151]. In addition, studies conducted on *M. galloprovincialis* exposed to sub-toxic doses of cadmium (1.5 and 5 μM $CdCl_2$) showed accumulation of cadmium in protamine-like proteins with a linear increase with the exposure dose. Exposure to sub-toxic levels of cadmium has been shown to result in the accumulation of cadmium in PL proteins, potentially functioning as transporters of cadmium to DNA. This mechanism could exacerbate the toxic effects of cadmium and suggests that even at sub-toxic concentrations, cadmium may alter the structural organization of sperm chromatin, consequently impacting reproductive fitness [152]. In addition, after exposure of mussels to 5 μM $CdCl_2$, the mobility of the PL-II band changed in SDS-PAGE, suggesting a structural rearrangement in the presence of cadmium. Structural analysis using fluorescent probes showed that complete conformational change of the PL-II protein was achieved at 5 μM $CdCl_2$. In the same condition of exposure of mussels to 5 μM $CdCl_2$, the PL-II protein changed its DNA binding mode, which determined a tighter DNA binding, because a higher amount of NaCl was required for PL-II protein release from sperm nuclei [152]. Considering that mussels reproduce seasonally and cadmium is a common stressor in

estuarine and coastal environments, the possibility that the effects of cadmium on PLs proteins could be seasonal was also analysed. It was found that cadmium exposure did not produce the same effects on summer mussel PLs proteins as on winter mussel PLs proteins, i.e.: cadmium bioaccumulation, changes in (AU-PAGE) and SDS pattern, reduced DNA binding affinity and ability to induce DNA oxidative damage. This study reveals clock-related seasonal responses to cadmium in *M. galloprovincialis* [153]. Another work analysed cadmium-based quantum dots effect on the gonads of the marine mussel *M. galloprovincialis*. In particular, in this work, a 14-day exposure to CdTe QDs (Cadmium Telluride Quantum Dots) and dissolved Cd (10 μg Cd L-1) was performed and Cd accumulation, oxidative stress, biotransformation, MT and oxidative damage in the gonads were analysed. The biochemical impairments in the gonads of *M. galloprovincialis* demonstrate that the reproductive toxicity induced by CdTe QDs in mussels was sex-dependent and mediated by oxidative stress and lipid peroxidation. Both forms of Cd caused significant antioxidant alterations, with QDs being more pro-oxidant, leading to oxidative damage, with females being more affected [154]. While marine invertebrates do not have a biological requirement for nonessential metals, the accumulation of these toxic elements can lead to serious biochemical and physiological disturbances [155]. Although heavy metals are not directly linked to testosterone levels, they can disrupt hormonal balances and adversely affect reproduction and development, resembling the effects observed in mammals [156]. Other authors have investigated sex reversal in the mussel *M. galloprovincialis* resulting from exposure to heavy metals, highlighting its contribution to alterations in the sex ratio of the mussel population along the Black Sea coast of Crimea [122]. This research provides direct evidence that heavy metals drive female *M. galloprovincialis* mussels to transform into males during post-spawning gonadal development. The degree of effect of heavy metals on sex change in female mussels was different and decreased in the following order $Cu^{2+} \rightarrow Cd^{2+} \rightarrow Hg^{2+} \rightarrow Pb^{2+} \rightarrow Zn^{2+}$. Copper ions had the greatest effect, causing sex inversion in 54 % of the females. The heavy metals Hg^{2+} and Pb^{2+} were also quite toxic, causing mortality in 13 % and 10 % of individuals, respectively. This work also demonstrates that it is possible to use *M. galloprovincialis* as a model organism to examine the mechanism of environmental sex reversal in bivalve molluscs. Another study examined metal levels and intersexuality in *M. galloprovincialis*. Samples from two areas of Gamak Bay in May 2010 showed Al was the highest metal ion concentration, followed by Zn. Co had the lowest concentration. Intersex was at 26.4 %, with males (38.8 %) exceeding females (12.9 %). Four types of intersex were observed [157]. Consequently, *M. galloprovincialis* can serve as an effective model organism to study the mechanisms underlying environmentally induced sexual reversal in bivalves [122]. Metabolic analyses conducted on *M. galloprovincialis* spermatozoa revealed that after exposure to 1.5 μM cadmium, a decrease in malic acid, an important metabolite in the chelation of toxic xenobiotics. This decrease could result in increased accumulation of this metal in the body and gametes [31]. Similarly, metabolomics studies conducted on the gonadal tissue revealed an increase in lactic acid after exposure to cadmium. This metabolite is reported in the literature to increase in the presence of anaerobic metabolism resulting in tissue damage [30]. As regards *M. edulis*, data obtained after exposure to 100 ppb of cadmium in seawater showed that this concentration significantly inhibited follicle development in the male and female gonads, but subsequently had a stimulating effect on egg-laying frequency [151]. Serotonin has a crucial role in the mussels' survival and reproduction, such as sexual differentiation, gamete production and spawning. Consequently, serotonin system dysregulation may impair several key processes, including mussel reproduction, and exposure to heavy metals can be damaged this system. In this contest, Fraser et al. [158] demonstrated the chronic exposures to low, environmentally relevant concentrations of Mn, Pb and Cd, in ion form, can disrupt the Serotonin system in the blue mussel, *M. edulis*. Regarding *M. californianus*, Lares et al., 2005 [159] found an inverse correlation between bioaccumulation of cadmium and grow of shell of *M. californianus*.

3.7. Other metals

For other metals, there is no information in the literature that specifically refers to effects on spermatozoa, male gonads and sperm chromatin components in *Mytilus*, but effects on embryonic development have been reported. The following information can be found in the literature.

Aluminium: Pagano et al. [160] investigated the toxicity of aluminium on early development, fertilization, and offspring quality of *M. galloprovincialis*. Using $Al_2(SO_4)_3$ at concentrations $> 10^{-6}$ M, these researchers observed severe embryotoxicity. Most larvae developed abnormally when exposed to a concentration of 3×10^{-6} M, and only 2–4 % of larvae showed normal development. Concentrations between 10^{-5} and 10^{-4} M produced only abnormal larvae. Exposure to aluminium did not impair fertilisation success or significantly increase the percentage of abnormal larvae after sperm were exposed to aluminium, with the exception of the 10^{-4} M treated group.

Arsenic: Martin et al. [117] found that the 48-hour EC50 for arsenic to cause abnormal embryo development was > 3 mg/L.

Barium: The effects of barium on the development of *M. californianus* embryos and larvae were studied by Spangenberg and Cherr [161]. Barium at concentrations between 200 and 800 μg/L had a marked effect on the development of larvae, resulting in abnormal and delayed development after 48 h of barium exposure. The NOEC (No Observed-Effect Concentration) was determined to be 0.1 mg/L of barium and the EC50 was determined to be 1890 μg/L of barium.

Chromium: The 48-hour EC50 for chromium causing abnormal embryo development was determined to be 4469 μg/L by Martin et al. [117].

Dysprosium: Freitas et al. [162] found that 28-day exposures to a range of concentrations of dysprosium up to 40 μg/L had no effect on the survival of *M. galloprovincialis*. However, there were metabolic and oxidative effects.

Gadolinium. Henriques et al. [163] reported that gadolinium concentrations up to 120 μg/L did not affect *M. galloprovincialis* survival during a 28-day trial. *M. galloprovincialis* biochemical performance was, in contrast, affected by gadolinium exposure.

Manganese: Morgan et al. [164] showed that the manganese metal caused abnormal development and mortality in *Mytilus* larvae during a 48 h exposure period. Larvae were subjected to concentrations ranging from 1 to 560 mg/L and an EC50 of 30 mg/L manganese was found. With 320 mg/L, the development of 100 % of the larvae was abnormal and the survival rate was 1 %. The highest concentration tested, 560 mg/L, resulted in 100 % larval mortality.

Molybdenum: The metal molybdenum was found to cause abnormal development and mortality in Mytilus larvae during a 48-h exposure period by Morgan et al. [164]. An EC50 of 147 mg/L molybdenum was found when larvae were exposed to concentrations between 1 and 560 mg/L. At 320 mg/L, 76.9 % of the larvae showed abnormal development and survival rate was 7 %. Survival was 1 % and 100 % of the larvae were abnormal at the highest concentration tested, 560 mg/L.

Strontium: The effects of strontium on the development of *M. californianus* embryos and larvae were investigated by Spangenberg and Cherr [161]. Strontium did not impair larval development at a dose of up to 20 mg/L.

Vanadium. Miramand and Unsal (1978) [165]. found 50 % mortality in *M. galloprovincialis* within nine days of exposure at an exposure concentration of 6500 μg/L vanadate.

3.8. Metallothioneins

MT are a class of low molecular weight, cysteine-rich proteins present in all organisms and bind with high affinity various divalent and

monovalent, essential and toxic transition metals [166]. MTs provide multiple biological roles linked to their metal-binding abilities, with key roles in Zn(II) and Cu(I) homeostasis, impounding and protection from environmentally toxic metals such as cadmium, mercury and lead. In addition, MTs have a crucial role in protecting from oxidative stress, in particular reactive oxygen and nitrogen species and other free radicals [167]. It suggests that some organisms may have evolved an efficient metal stress response, increasing MT levels to reduce the toxic effects of environmental pollutants that accumulate during their life. The exposure of marine organisms, including mussels, mainly to cadmium, copper and zinc, is linked to the induction of metallothioneins, indeed, their detection content are applied for assessing the negative biological effects of metals contamination [168]. It is well known that reproductive tissues and organs react to oxidative stress by the synthesis of stress proteins, such as metallothioneins. In many studies, the heavy metals concentration and MT content have been determined in mussels located in polluted areas, to assess the amount of metal pollution, and therefore MTs have been proposed as biomarkers of trace metal exposure in numerous species from different zoological groups. Focusing on the reproductive sphere, MTs are found mainly in the cytoplasm of germ cells, suggesting a possible involvement in spermatogenesis and protection of the gonads in marine organisms [169]. In the tissues of *M. edulis* ([170]), *M. galloprovincialis* [171], Bathymodiolus azoricus, and Bathymodiolus thermophilus mussels [172], MT belongs to two distinct classes of genes, *mt10* and *mt20*, which manifest different expressions both under basal conditions and in the presence of heavy metals [173]. The *mt20* gene is responsive to cadmium in *M. galloprovincialis* testes, several investigations have proposed that Cd-induced MT mRNA levels are positively related to exposure and concentration of this metal. The exposure of mussels to 1.5, 5 and 10 μM $CdCl_2$ under laboratory conditions increases in mt20 expression, respectively [148]. Furthermore, the study documented increased activity in *mt10* across all chromium (VI) exposure levels, suggesting a stress response (as observed also in studies conducted on mercury). Conversely, the expression of *hsp70*, associated with heat stress, did not show significant changes. These findings underscore the potential of even minimal concentrations of chromium (VI) to disrupt reproductive processes in mussels, emphasizing ecological concerns regarding its impact on marine organisms. Data confirmed by Vincent-Hubert et al. [174], here show that Cd is a strong inducer of MT and HSP70 genes. Cd-induced alteration of cells by many mechanisms, such as the production of ROS (the mussels MT gene promoter could be activated by ROS [175], lipid peroxidation, disruption of protein structure, and mutations in DNA. It has been suggested that spermatozoa are highly sensitive to oxidative damage. This is due to the abundance of polyunsaturated fatty acids, which act as substrates for ROS [40]. Other studies show, as mentioned, that Cd is not the only metal capable of inducing the expression of *mt10* and *mt20*, so much that *mt10* genes can respond to both essential (Cu, Zn) and non-essential (Cd, Hg) toxic metals; as demonstrated in Lettieri et al. [17], mussels revealed higher expression levels of mt10 after mercury contamination, in particular, the 100 pM treatment showed an approximately three-fold increase in expression level compared to the control condition. Overall, the results demonstrated that transcriptional activation of MT during metal exposition is affected in a dose- and/or time-dependent way and seems to be conservative between vertebrates and invertebrates [175]. Considering these results MT mRNA levels in gonads can be employed to examine the effects of heavy metals on reproduction of marine organisms.

4. Conclusions

Increased heavy metal release adversely affects marine organisms, particularly those residing in coastal areas exposed to anthropogenic pollutants (Fig. 2). This review highlights the profound impact of 6 heavy metals: nickel, chromium, zinc, copper, mercury, and cadmium, on the reproductive health of the main bivalve molluscs of the genus

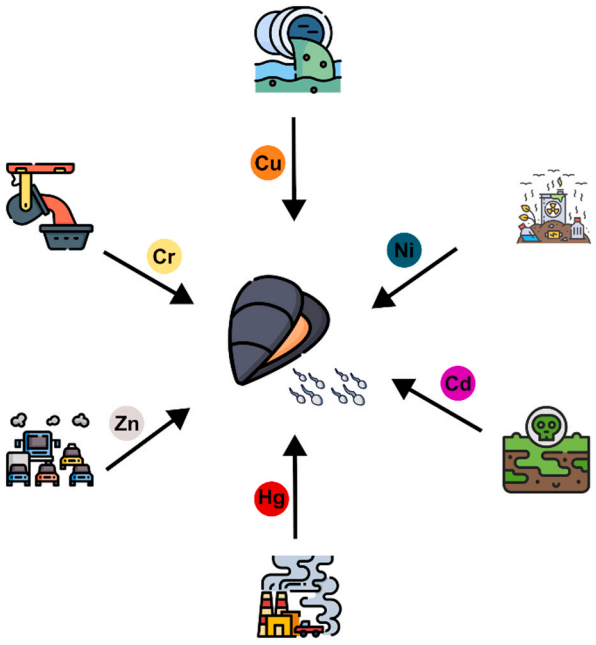

Fig. 2. The principal source of these heavy metals released into the environment.

Mytilus spp. These metals not only bioaccumulate in the tissues of these organisms but also induce profound molecular changes, such as impairments in PLs properties such as their DNA-binding capacity, and in turn sperm chromatin organization. They induce DNA oxidative damage and impairments of larval development and a decrease in the growth of organisms. The study underscores the seasonal variability in the response of *Mytilus spp*. to heavy metal exposure, with reproductive toxicity often aligned with key phases of their reproductive cycle. This seasonal sensitivity suggests that bioaccumulation and its effects on PLs proteins and DNA integrity can vary depending on environmental conditions, reproductive stages, and stress levels. Furthermore, the differential impacts of these metals highlight the importance of investigating their combined and synergistic effects, as marine organisms are often exposed to mixtures of pollutants. *Mytilus spp*.'s ecological role as filter feeders and their economic importance as a food source makes these findings particularly significant. The potential of mussels as bioindicators for environmental monitoring is particularly valuable, given their sensitivity to pollution and their economic and ecological importance. They are a source of food for several organisms - crustaceans, fish, birds and mammals, as well as humans. In many countries, mussels represent an important part of the diet. The EU average annual mollusc consumption is 1 kg per person, while in Spain, Belgium, Denmark and France it is much higher (FAO Food and Agriculture Organization of the United Nations, 2014). Their ability to accumulate and respond to environmental contaminants positions them as effective bioindicators for monitoring marine pollution and its broader ecological implications. The documented alterations in metabolic pathways, antioxidant responses, and reproductive proteins provide a detailed molecular blueprint for assessing environmental stressors. To mitigate these harmful effects, it is imperative to adopt stricter pollution control measures, enhance monitoring programs, and promote interdisciplinary research. Future studies should focus on exploring the long-term impacts of heavy metal exposure, the interaction of multiple stressors, and the potential for adaptation and resilience in *Mytilus spp*. By addressing these challenges, we can safeguard not only these critical sentinel species but also the health and balance of marine ecosystems as a whole.

CRediT authorship contribution statement

Lucia Rocco: Writing – review & editing, Software. **Gennaro Lettieri:** Writing – review & editing, Writing – original draft, Supervision, Software, Conceptualization. **Marco Trifuoggi:** Writing – review & editing, Writing – original draft. **Filomena Mottola:** Writing – original draft, Visualization. **Marina Piscopo:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Conceptualization. **Luigi Montano:** Writing – review & editing, Project administration. **Mirko Cardillo:** Writing – review & editing, Writing – original draft, Software. **Maria Grazia Guarnieri:** Writing – review & editing, Writing – original draft. **Carmela Marinaro:** Writing – review & editing, Writing – original draft, Software, Conceptualization. **Rosaria Notariale:** Writing – review & editing, Writing – original draft, 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 article.

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