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REVIEW ARTICLE

Comprehensive review of *in vitro* approaches for environmental heavy metal exposure

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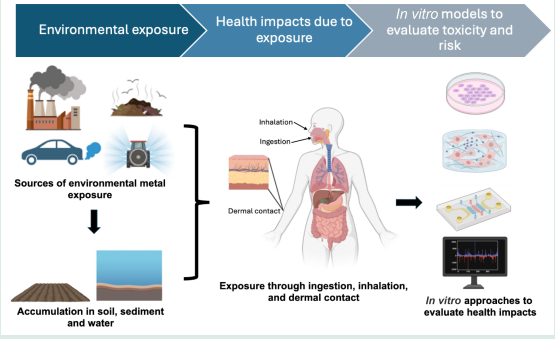
HIGHLIGHTS

- Environmental exposure to toxic metals is a major human health concern.
- Inhalation, ingestion, and dermal contact are the major pathways of exposure.
- Limited studies are available using environmental samples *in vitro* approaches.
- 3D microfluidic organ-on-chip devices can improve evaluation of toxicity.



ABSTRACT: Heavy metals are ubiquitous environmental pollutants, contaminating air, soil, and water via the erosion of natural deposits, as well as originating from anthropogenic sources, such as agriculture, industries, transportation, and landfills. The increasing utilization of heavy metals over the years, combined with the persistent nature of metals in the environment poses a direct threat to human and environment health. Although regulatory limits have been established for toxic metals, assessing the associated health risks using real-life exposure scenarios remains challenging. In this review, we summarize the development and use of *in vitro* models based two- and three-dimensional cell culture systems, focusing on exposure to heavy metals via the dermal, inhalation, and ingestion routes using environmental samples. We also highlight recent developments in three-dimensional cell culture

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techniques and their potential for implementation in evaluating environmental samples for heavy metal toxicity. In addition, we assess the comparative strengths and specific applications of different modeling approaches, emphasizing the value of integrating advanced *in vitro* systems in environmental toxicology.

KEYWORDS: *In vitro* models, Environmental exposure, Human health

1 Introduction

Heavy metals are ubiquitous in the natural environment, found in air, soil, and water. These metals are derived from geological processes such as weathering and volcanic eruptions, as well as from anthropogenic activities, such as mining, industrial plants, transportation, landfills, and agricultural practices (USEPA, 2023). Heavy metal pollution is being increasingly reported worldwide. At several superfund sites in the U.S, the average concentrations of lead (Pb), cadmium (Cd), and arsenic (As) in soil are higher than the limits set by the United States Environmental Protection Agency (USEPA) (Chen et al., 2022), whereas in the Silesia region of Poland, the concentrations of heavy metals in the soil exceed the European Union (EU) soil standards by up to 33-fold (Qu et al., 2016; Tomczyk et al., 2023). Similarly, in the Hunan region of China, the soil concentrations of Cd (85 mg/kg), As (120 mg/kg), and Pb (2000 mg/kg) exceed the national quality standards. The accumulation of heavy metals in the environment has often been reported in clusters or hotspots near mines, industrial areas, and areas with heavy urbanization (Kravchenko et al., 2025; Yu et al., 2025). Mining and industrial activities are considered to be the most significant contributors to the environmental presence of these metals (Xue et al., 2017), with refineries, power plants, petroleum combustion, plastics, textiles, micro-electronics, wood preservation, and paper processing plants being notable sources. Combustion processes and increased exposure to naturally occurring toxic metals in soils, such as those found in industries including mining and drilling, heighten the risks associated with exposure to these metals. In addition, heavy metals are commonly used in pesticides in commercial agriculture, thereby increasing the risk of human exposure via the food chain (Bencko and Foong, 2017; USEPA, 2021). Leachates from landfills contain high concentrations (from highest to lowest) of iron (Fe), zinc (Zn), Cd, Pb, nickel (Ni), chromium (Cr), and As (Essien et al., 2019). Heavy metals have a wide range of commercial applications that contribute to their prevalence in the environment. Moreover, consumer products, such as cosmetics, toothpaste, jewelry, glass products, and home fixtures, including plumbing and lighting, are common sources of contamination, whereas these metals also have applications as color additives and abrasives that are used to add weight to products and stabilize or soften plastic (Akhtar et al., 2022). Although some elements such as Zn and copper (Cu) are essential micronutrients for human well-being, all heavy metals are toxic when present in sufficiently high concentrations. Notably in this regard, the reference dose value (RfD), defined as an estimated daily exposure level to a toxic substance that is without appreciable risk of harmful effects over a lifetime (USEPA, 1993), may not adequately represent the exposure risk. For example, in areas with high industrial activity, chronic exposure to heavy metals could well be higher than the recommended RfD levels. Moreover, new chemical formulations enter the market each year, for which risk values need to be determined.

The most common modes of human exposure to heavy metals are ingestion, inhalation, and dermal contact. Human health risks typically differ depending on the route of exposure owing to associated differences in biological and environmental factors. Given the limited availability of robust therapeutic strategies for managing heavy metal toxicity, exposure prevention through mitigation and awareness campaigns remains the most effective public health strategy.

Both *in vitro* and *in vivo* studies have provided extensive evidence to indicate the toxic effects of metals through ingestion, inhalation, and dermal exposure, many of which have been based on the use of model animals, such as rats, mice, zebrafish, and monkeys, used to evaluate the effects of As, Cu, Cr, and Pb (Briffa et al., 2020). Such studies have employed a number of experimental approaches, including the use commercial solutions to spike media, as well as estimating metal concentrations in environmental samples and spiking media at similar concentrations or performing risk assessments. Contrastingly, however, there has been comparatively little research focusing on the direct use of environmental samples to determine their toxicity.

Despite their widespread use, animal studies are expensive and time-consuming, and the outcomes are often of limited relevance, in that they do not provide human-specific data. Additionally, these experiments utilize representative samples to assess exposure.

Comparatively, *in vitro* cell culture systems are less expensive and provide more relevant models. Assessing the effects of heavy metals on cellular activities *in vitro* under environmentally relevant conditions could contribute to gaining a better understanding of the risks of exposure and the development of remediation strategies. By incorporating scaffolds, co-cultures, and other recent advances in tissue engineering, it is becoming increasingly possible to assess the responses of cells in a more physiologically relevant settings than in traditional monolayer cultures, thereby enhancing our understanding of heavy metals and their mechanisms of action at the cellular level. Moreover, organotypic cell cultures based on three-dimensional (3D) co-cultures of relevant cells and homeostatic environments can provide more relevant models (Hartung, 2018).

Given the aforementioned limited research on the use of direct environmental samples on human-derived tissues, physiologically relevant cell responses to heavy metals in the environment remain largely undetermined. Moreover, as cells can show differential responses when exposed to different heavy metals, it is not possible to generalize the toxicological effects of these metals. However, with recent advances in physiologically relevant *in vitro* models and new approach methodologies (NAMs), there has been a growing shift from *in vivo* bioavailability testing to *in vitro* approaches (Schmeisser et al., 2023; Silva et al., 2024). This in turn has highlighted the need for enhanced *in vitro* models that accurately reflect human physiology and provide more relevant insights than traditional animal-based methods (Jensen and Teng, 2020).

In this comprehensive review, our objectives are to (1) summarize the biological impacts of environmental (dust, soil, and water) heavy metal exposure; (2) highlight environmentally and physiologically representative approaches that mimic real exposure conditions; (3) identify the research gap in *in vitro* models that focus on evaluating environmental samples; and (4) compare the 2D and 3D *in vitro* approaches designed to identify the best approach for studying the effects of human exposure to environmental pollutants.

2 Cellular effects of metal toxicity

In biological systems, heavy metals affect the cellular organelles and enzymes responsible for metabolism, detoxification, and repair. The harmful effects of heavy metal exposure have long been recognized, and these elements are classified as “toxic candidates” (USEPA, 2023). Table 1 outlines the current regulatory limits for eight Resource Conservation and Recovery Act (RCRA) metals (USEPA, 1976a), and Table 2 summarizes the effects of heavy metal exposure on cellular pathways. Different metals have distinct physicochemical properties and may elicit different cellular responses in environmentally relevant situations compared with traditional laboratory conditions (Yi et al., 2015), thereby highlighting the importance of assessing environmental samples directly and thus providing relevant data for determining responses to actual environmental biochemical conditions, rather than approximating concentrations or undertaking incomplete analyses of mixtures. With the increasing potential for airborne contamination, it is essential to develop regulatory measures that reflect environmental responses. Although no drugs have been released into the market without the requisite clinical trials, the FDA Modernization Act 2.0, signed in December 2022, amended the Federal Food, Drug, and Cosmetics Act of 1938, which formerly required animal testing for all new drug development protocols. With the development of new models, *in vitro* options have become increasingly more applicable. The new Act enables the FDA to approve drugs based on studies using non-clinical tests (Han, 2023).

2.1 Oxidative stress

Cells utilize oxygen to generate usable energy, although as byproducts of ATP generation also produce free radicals, typically reactive oxygen species (ROS) or reactive nitrogen species, both of which result from cellular redox processes (Pham-Huy et al., 2008). Other natural cellular processes also generate ROS, which are integral to cellular signaling (Snezhkina et al., 2019). These free radicals are generally neutralized by antioxidants, which donate electrons to free radicals, inhibiting their reactivity and significantly reducing their capacity to cause damage (Lobo et al., 2010). Without antioxidants to neutralize these free radicals, cell damage can occur, leading to dysfunction in organs, metabolism, development, hormones, and the immune system, as well as an increased risk of cancers (Balali-Mood et al., 2021). Chronic exposure to metals can result in ROS generation, which can overwhelm antioxidant mechanisms such as the glutathione system and superoxide dismutase, often resulting from activation of the NLRP3 inflammasome (Tchounwou et al., 2012; Snezhkina et al., 2019; Jomova et al., 2023).

Table 1 Source, exposure, biological samples, and regulatory limits of RCRA8 metals/metalloids in the environment

Heavy metal/metalloid	Exposure pathways	Source of exposure	Biological sample	Regulatory limits		
				Air	Drinking water	Oral
Arsenic (ATSDR, 2007b)	Dermal, oral, inhalation	Drinking water, natural erosion, volcanic activity, mines, soil, cigarette smoke, chemical factories, food	Urine, hair, fingernails	1.5×10^{-3} μg/m^3 (WHO) 10 μg/m^3 (OSHA)	0.01 mg/L (WHO) 0.05 mg/L (EPA)	–
Lead (ATSDR, 2019)	Dermal, oral	Lead paint, e-waste, cosmetics.	Blood-lead levels	0.5 mg/m^3 (OSHA) 0.05 mg/m^3 (NIOSH)	0.015 mg/L (EPA); 0.01 mg/L (WHO)	–
Selenium (ATSDR, 1999)	Dermal, oral, inhalation	Food, coal combustion, cigarette smoking	Blood, urine, fingernails	0.2 mg/m^3 (OSHA)	0.05 mg/L (WHO) 0.01 mg/L (EPA, FDA)	0.9 μg/kg (WHO)
Cadmium (ATSDR, 1999)	Dermal, oral	Electroplating, pigment industries, food, soil	Blood, urine	5 ng/m^3 (WHO) 5 μg/m^3 (OSHA)	0.005 mg/L (EPA, FDA 0.003 mg/L (WHO)	–
Mercury (ATSDR, 1999)	Dermal, oral	Seafood, diet, industrial and household waste	Urine, bodily waste	0.05 mg/m^3 (OSHA, NIOSH)	0.002 mg/L (EPA) 0.001 mg/L (WHO)	5 μg/kg (WHO) 1 ppm (Seafood, FDA)
Chromium (ATSDR, 1999)	Dermal, oral, inhalation	Wastewater, soil, air	Blood, hair, fingernails	1 μg/m^3 (WHO) 0.005 mg/m^3 (OSHA) 0.001 mg/m^3 (NIOSH)	0.05 mg/L (EPA, FDA) 0.01mg/L (WHO)	–
Silver (ATSDR, 1999)	Inhalation	Air	Blood, urine, Body tissues	0.01 mg/m^3 (OSHA, NIOSH)	0.05 mg/L (EPA)	–
Barium (ATSDR, 2007a)	Dermal, oral, inhalation	Drinking water, diet, contaminated soil	Hair	0.05 mg/m^3 (OSHA)	1 mg/L (EPA) 7 mg/L (WHO)	–

2.2 Apoptosis

The generation of ROS can also lead to endoplasmic reticulum stress, disrupting protein folding, and triggering the unfolded protein response, which can initiate cell death (Lin et al., 2007; Sano and Reed, 2013; Luo et al., 2016). This in turn results in the activation of caspases and upregulation of proinflammatory markers, ultimately inducing cell death (Ahn et al., 2018; Mou et al., 2023). Caspases 3, 8, and 9 are important mediators of apoptosis that are generated following the metal-induced release of cytochrome *c* from the mitochondria (Korotkov, 2023)**.** Pro-apoptotic proteins, such as Bax, and anti-apoptotic proteins, such as Bcl-2, may also be disrupted by exposure to heavy metals, further contributing to apoptosis (Sharifi et al., 2010). Necroptosis signaling factors, including those in the RIPK family, contribute to cell death and inflammation (Zhang et al., 2021).

2.3 DNA damage

Among of the consequences of the redox imbalance resulting from heavy metal exposure are increases in DNA damage, DNA–protein crosslink formation, cellular toxicity, apoptosis, and the aberrant activation of cellular signaling pathways (Wang and Shi, 2001). It is well established that metal ions interact with DNA leading to conformational changes that modulate the cell cycle, induce apoptosis, and potentially contribute to carcinogenesis (Tchounwou et al., 2012). Heavy metals can also promote the upregulated expression of specific microRNAs that play pivotal roles in gene regulation and contribute to tumorigenesis and other pathologies (Nail et al., 2022; Wallace et al., 2020). Although variable, these mechanisms lead to the disruption of numerous enzymatic pathways, ultimately causing phenotypic changes (Witkowska et al., 2021). In addition to their effects on metabolic enzymes, by promoting the generation of ROS, heavy metals can also have adverse effects on the functioning of different cellular organelles, including mitochondria, endoplasmic reticulum, lysosomes, and nuclei (Wu et al., 2024).

2.4 Cellular signaling

Voltage-gated calcium channels (VGCCs) are the primary components of excitable cells (Marchetti, 2013) that are responsible for muscle contraction, neuronal excitation, gene expression, and hormone regulation (Catterall, 2011). The presence of heavy metals can induce the opening of VGCCs when a certain charge is reached, which can be ascribed to similarities between the charges heavy metal and calcium ions (Marrero-Rosado et al., 2013). Similar ionic and molecular mimicry can occur when metal ions

Table 2 Effect of heavy metals on cellular pathways

Function	Marker	Associated Metals	Biological effect	References
Inflammasome	NLRP3 Activation	Lead, Cadmium, Mercury, Chromium, Barium	Increased cytokine production leads to inflammation	Huang et al., 2015; Ahn et al., 2018; Mou et al., 2023
Redox System	Reactive Oxygen Species (ROS) Production	Arsenic, Lead, Cadmium, Mercury, Chromium	Increased oxidative stress, lipid peroxidation, protein modification, and DNA damage	de Vries et al., 2021; Teschke and Xuan, 2022; Rahaman et al., 2023 Briffa et al., 2020 Nain and Kumar, 2020; Zhang et al., 2020 Jan et al., 2015; Akter et al., 2018 Koedrith and Seo, 2011; Tchounwou et al., 2012 Singh et al., 2011
	Glutathione (GSH) System	Arsenic, Lead, Cadmium, Mercury, Chromium	Disruption affects detoxification, antioxidant defense, and ferroptosis	
	Superoxide Dismutase (SOD)	Arsenic, Lead, Cadmium, Mercury, Chromium	Increased ROS leads to cell damage	
	Mitochondrial ROS Generation	Arsenic, Lead, Mercury, Chromium	Induces mitochondrial dysfunction, apoptosis, and cell death	
Ion Channel Disruption	Potassium Channel Blockade	Barium	Hypokalemia, muscle weakness, paralysis, cardiovascular effects	Catterall, 2011; Marchetti, 2013; Bhoelan et al., 2014 Koch et al., 2003
	Calcium Channel Blockade	Lead, Cadmium, Mercury, Silver	Altered neuromuscular and cardiovascular signaling	
Apoptosis	Caspase 3	Mercury, Cadmium, Chromium, Selenium, Silver	Apoptosis induction	Sun et al., 2022a; Korotkov, 2023; Koyama et al., 2024 Eichler et al., 2006
	Caspase 8	Lead		
	Caspase 9	Lead, Cadmium, Selenium		
	Bax	Lead, Cadmium, Selenium, Silver		
	Bcl-2	Lead, Cadmium, Selenium, Silver	Decreased Apoptosis regulation	
	p53	Chromium, Selenium, Silver	Increased apoptosis, mitochondrial dysfunction	
Necroptosis	RIPK1	Cadmium	Necroptosis induction	Koyama et al., 2024 Zhang et al., 2021 Sano and Reed, 2013
	RIPK3	Lead, Cadmium		
	MLKL	Lead, Cadmium		
ER Stress	GRP78	Lead, Cadmium, Mercury, Chromium	Increased ER stress induction, apoptosis, mitophagy	Yan et al., 2023; Koyama et al., 2024 Luo et al., 2016 Mao et al., 2016 Lin et al., 2007; Sano and Reed, 2013
	PERK-eIF2α-ATF4	Lead, Cadmium, Chromium	Increased ER stress induction, apoptosis, autophagy	
	IRE1α-XBP1	Lead, Cadmium, Mercury, Chromium	Increased ER stress induction, apoptosis	
	ATF6	Chromium		
miRNA Regulation	p53	Chromium, Selenium, Silver	Increased apoptosis, mitochondrial dysfunction	Nail et al., 2022; Koyama et al., 2024; Wallace et al., 2020
	miR-193	Cadmium		
	miR-221/222	Cadmium		
	miR-33-5p	Cadmium	Autophagy regulation	
	miR-101	Cadmium	Respiratory damage	
	miR-144	Cadmium		
	miR-146a	Lead	Inflammation regulation	
	miR-155	Arsenic/Lead		
Tumor and Senescence Pathways	p16/p21/p53	Cadmium, Lead, Chromium, Selenium, Silver	Cellular stress response, apoptosis, DNA damage, epigenetic modifications	Hafsi and Hainaut, 2011; Vielee and Wise, 2023; Koyama et al., 2024

or molecules resemble the shape of natural endogenous ions/molecules, occupying the active sites of carrier proteins, channels, structural proteins, enzymes, and transcription factors (Bridges and Zalups, 2017). Furthermore, exposure to heavy metals can influence tumor senescence pathways, potentially preventing the normal aging and death of damaged cells, thereby contributing to the progression of cancer (Hafsi and Hainaut, 2011; Vielee and Wise, 2023). At the cellular level, heavy metals can bind to enzymes and proteins via thiol (-SH) groups (Tchounwou et al., 2012), which are primarily found in cysteine, one of the two sulfur-

containing amino acids. This binding disrupts cell redox status and often causes the inactivation of proteins (Bridges and Zalups, 2017), the latter of which can lead to an antioxidant imbalance, resulting in organ damage, particular in the liver (Jan et al., 2015; Wu et al., 2016). Other established metabolic effects of heavy metals include the overexpression or downregulation of numerous proteins, many of which are essential for the metabolism of steroid hormones (Mir et al., 2017).

3 *In vitro* cell culture models using environmental samples

By facilitating the independent examination of organs and organ systems under controlled conditions, the use of *in vitro* models can contribute to simplifying experimental variables. In this section, we focus on current *in vitro* models that use environmental samples. The goal of *in vitro* models is not to duplicate *in vivo* conditions, but to assess different levels of cellular physiology and behavior. These models have proven valuable for gaining an understanding of the pathological, physiological, and biological processes in cells (de Carvalho et al., 2025). Kastury et al. (2017) have outlined the advantages of an *in vitro* approach over *in vivo* studies. Although many of the currently used models employ simple monocultures, the development of 3D models has facilitated assessments under more complex conditions. Despite limitations associated with restricted crosstalk and inter-relationships between different cell types, *in vitro* model systems can be effectively used to determine immediate responses to environmental pollutants and aid in understanding clinically relevant cellular changes (Li et al., 2024). However, whereas all models attempt to mimic cellular conditions within the human body, none are able to fully replicate the *in vivo* cell responses (Nguyen et al., 2024). Nevertheless, ongoing developments are contributing to a new generation of more advanced models with *in vitro* environments that more faithfully mimic the human *in vivo* environment, which could lead to a more comprehensive understanding of the mechanisms of heavy metal toxicity.

Villegas et al. (2019) simulated dermal exposure to metals, in which standard solutions of metals were added to artificial sweat and sebum solutions to assess their *in vitro* bioaccessibility, whereas other studies have estimated the concentrations of pollutants such as Cd, Pb, and As, and spiked media solutions with commercial salts in food crops and soils (Xue et al., 2017; Lin et al., 2023). Furthermore, Zuo et al. (2022) have evaluated the effects of Cd on Caco-2 and MDCK cells by exposing these cells only to bioaccessible fractions of Cd, whereas Aziz et al. (2015) treated Caco2 and HL-7702 cells with *in vitro*-digested rice grown in Cd-contaminated soil, and Sieg et al. (2018) followed a practical approach to mimic the conditions of aluminum (Al) exposure, focusing on the behavior of three Al species after treatment with saliva, bovine serum albumin, and digestive enzymes to simulate oral exposure. In addition, a number of studies have performed health risk assessments based on the presence of metals in soil and crop samples (Swati et al, 2017; Fernández-Landero et al., 2021). For example, Kafaoglu et al. (2016) have determined the bioaccessible fractions in nuts and seeds and performed risk assessments of metals to simulate oral exposure. However, although the effects of metals have been elucidated to some extent, the development of experimental procedures using field environmental samples would aid in toxicological investigations and contribute to determining the human health risks associated with different heavy metal species.

3.1 Oral exposure

Among the different routes of exposure to heavy metals in water, air, and soil, the oral route has been the most well characterized. Previous studies in this regard have reported the use of food products, sediments, and soil samples to demonstrate the effects of ingesting contaminated soil. For humans, the average daily intake of soil has been estimated to be 100 mg (Stanek and Calabrese, 1995), whereas exposure through water and air is an occupational or incidental occurrence. The three most common approaches to simulating human digestion *in vitro* are the simplified bioaccessibility test (SBET), physiologically based extraction (PBET), and the unified BARGE method (UBM), each of which was developed based on a specific physiological functions. The UBM and PBET approaches simulate the physiological conditions within the human digestive tract to estimate the bioaccessible fraction of metals (Li et al., 2015), whereas in contrast, the SBET focuses on risk assessment and seeks to maximize metal extraction, often overestimating bioaccessibility (Li et al., 2015). Most enzymatic metal solubilization occurs in the intestinal environment, and simulating intestinal digestion is thus essential for accurate bioaccessibility assessments (Billmann et al., 2025). The SBET simulates only the gastric phase, whereas PBET includes both the gastric and intestinal phases, and by incorporating oral, gastric, and intestinal digestion phases, the UBM provides the most comprehensive

approach (Gaberšek and Gosar, 2024). *In vitro* bioavailability assessments using the UBM are commonly conducted to determine metal bioavailability from soil and food samples on Caco2 cells (Boim et al., 2020; Gaberšek and Gosar, 2024; Billmann et al., 2025)

Boim et al. (2020) reported that in multi-metal soil samples, there is no clear association between the intracellular accumulation and bioavailabilities of Cd, Cu, Zn, Mn, and Pb, whilst highlighting the significance of using Caco-2 cells as a model approach for assessing the bioavailability of heavy metals in soils. They also emphasized the importance of assessing urban environments using an *in vitro* cell culture models for relatively more rapid and simpler evaluation of the hazards posed by contaminated soil (Boim et al., 2020). Furthermore, Yin et al. (2017) have used a combination of PBET and the simulator of human intestinal microbial ecosystem (SHIME) method to simulate gastric, small intestinal, and colon phases, reporting that the gut microbiota release Fe/Al-bound As from the soil, thereby rendering it bioaccessible for absorption by intestinal cells. Uptake was found to be dependent on factors such as the species of As, glucose transporters (GLUT5 and SGLT), aquaporins (AQP9), and phosphate transporters, although determining the specific mechanisms will necessitate further studies.

The versatility of *in vitro* digestion enables evaluation of the bioaccessibility of metals in food crops and market vegetables. Yin et al. (2017) adapted the same approach as Boim et al. (2020), using the UBM method to assess the bioavailability of metals in market vegetables, whereas Lee et al. (2018) assessed the bioaccessibility and effects of As in rice based on different cooking methods to determine which is conducive to the least As uptake. Furthermore, Yao et al. (2021) compared the *in vitro* and *in vivo* results of rice cooked with $CdCl_2$, finding that these results of these analyses were poorly correlated. However, given that ions derived from $CdCl_2$ are freely available for uptake, this could plausibly account for the inconsistent results. Notably, however, replicating the digestion process *in vivo* presents considerable challenges.

Chen et al. (2020) determined the bioavailabilities of As, Cd, and Pb in mineral clay soil using Caco2 cells and soil samples with metal concentrations of 4–17, 24–61, and 6–20 ppm, respectively. The final daily intake of As (45 μg/d) and Pb (52 μg/d) was determined to be higher than the stipulated daily intake limits. Although the concentration of Pb in the clay samples was below the regulatory limit of 400 ppm, the daily intake of this metal was almost five times higher. This indicates the value of *in vitro* bioavailability studies for determining metal intake and adsorption. In addition, Ma et al. (2022) obtained water-extractable metals from soils at an e-waste dismantling site and determined the effects of exposure to the extractable metals Cd, Cu, Ni, Pb, and Zn on Caco2 cells, reporting increases in ROS activity, suppression of antioxidant enzymes (superoxide dismutase and catalase), and the elevated expression of inflammatory and apoptotic genes. A similar approach has also been adopted for dermal exposure (Warke et al., 2022; Wang et al., 2022).

In further studies using Caco-2 cells, Fu and Cui (2013) evaluated the bioaccessibility Cd and Pb in raw and cooked vegetables following *in vitro* digestion. Notably, whereas a higher bioaccessibility of Cd was observed in the gastric phase compared with the small intestinal phase, that of Pb showed the opposite trend. Moreover, the bioavailability of Cd and Pb in raw vegetables was shown to be significantly higher than that in cooked vegetable, leading to daily intakes that exceed to recommended values, with differences found to be influenced by factors such as additives, plant species, heavy metal species, and the digestive phase.

3.2 Dermal contact

Exposure to different toxic metals (As, Pb, Cd, Cr, and Cu) via dermal contact has been shown to cause damage to the renal, respiratory, neurological, skeletal, and digestive systems, leading to cancers (Wang et al., 2022). A typical scenario for dermal exposure is through contact with contaminated soil or water. Wang et al. (2022) determined the toxicity of Cr, As, Cu, Pb, and Cd from a multi-metal-contaminated soil by simulating the dermal exposure from sweat on HaCaT cells. The soils were acid-digested and then combined with artificial perspiration solutions to initiate immersion experiments. Although the bioaccessible values were within regulatory limits, cytotoxic effects were observed following exposure to the soil samples. Warke et al. (2022) simulated contact exposure from As-contaminated soils by extraction of the water-soluble fraction followed by treatment of HaCaT and HDFa cells. The levels of As in the extracts were found to be significantly higher than the regulatory limits, which could be due to the high concentrations of soil-As treatments and the sandy nature of the Immokalee series soil used in the study. Whereas Wang et al. (2022) focused on the exposure of children and workers to contaminated soil, Warke et al. (2022) assessed exposure among farmers working in agricultural fields with contaminated soils. Despite certain limitations in adopting both approaches, the studies demonstrate the utility of a rapid and efficient method for simulating contact exposure to soils. There is, however, a need to

develop a more extensive range of bioaccessibility approaches to determine the effects of environmental toxins through contact exposure. For example, Wang et al. (2023a) combined the UBM method and artificial sweat solution to determine dermal exposure using face paint.

In further developments, Verdin et al. (2019) used a reconstructed human epidermis (RHE) 3D model to evaluate the cytotoxicity of trace metal-contaminated $PM_{0.3–2.5}$ particulates. The particulate matter was sonicated with culture media, potentially extracting the bioaccessible fraction, and the trace metal content was found to influence cell differentiation, adhesion, and apoptosis. The underlying principle of exposure via dermal contact was the same as that used in Wang et al. (2022) and Warke et al. (2022). The 3D RHE model preserves the morphologies of cells and membranes and, compared with 2D cell cultures, more closely mimics exposure *in vivo* (Costa et al., 2016; Verdin et al., 2019).

3.3 Inhalation

With respect to exposure via inhalation, Zhang et al. (2022) assessed the toxicity of dust particles contaminated with metals such as Pb, Cu, and Cr. Following sample preparation, the water-soluble fraction was extracted by sonication using ultrasound. The media was prepared by combining the extract with serum-free DMEM to a concentration of 80 μg/L. A similar approach was followed by Chen et al. (2018) and Pang et al. (2020), who sought to assess the toxicity of contaminated dust. Similarly, Huang et al. (2015) used a salt solution of the same concentration to spike the media, whereas Dankers et al. (2018) determined the dosage rate to establish the concentration of metal nanoparticles for spiking salt solutions. A similar approach could be adopted to simulate environmental exposure through inhalation.

To determine the effects of exposure to metal oxide nanoparticles via inhalation, Dankers et al. (2018) prepared fully differentiated bronchial epithelial MucilAir™ cultures. Although the effects of these nanoparticles were found to be minimal, the levels were representative of those likely to be encountered in *in vivo* exposure, and the overall approach could accordingly be implemented for evaluating the *in vitro* exposure to metals from inhalation. The use of 3D air–liquid interface (ALI) models is becoming increasingly more commonplace in toxicological studies (Lynch et al., 2013; Baldassi et al., 2021), with ALI culture systems being used to assess the toxicity of a wide range of metal ions and metal oxide nanoparticles using commercial salt solutions, although not environmental samples (Herzog et al., 2013; Rach et al., 2014; Mistry et al., 2020; Bessa et al., 2021; Mallek et al., 2024). The most commonly used cell models are BEAS-2B, A549, and 16HBE14o-. Although numerous *in vitro* models have been used to evaluate the inhalation of metals, these tend to be based on a "fit for purpose" designs rather than using a standardized approach (Di Ianni et al., 2024). However, notable efforts have been made to standardize exposure studies, based on the use of systems such as the electrostatic aerosol *in vitro* exposure system (EAVES) and CelTox© *in vitro* air–liquid interface exposure systems (Zavala et al., 2018; de Bruijne et al., 2009). Nevertheless, these and similar systems still need to be further refined to develop effective approaches for environmental samples.

3.4 Computer modeling (*in silico* research)

Ongoing advances in technology and computer modeling are increasingly enabling researchers to employ predictive models to assess human health risks from chemicals based on comprehensively assessed analog substances and a wealth of knowledge accumulated from earlier toxicokinetic studies. These quantitative structure–activity relationship (QSAR) models utilize previously obtained data and iterative analysis to produce representative regressions illustrating the association between chemical features and resulting biological activities (Pradeep et al., 2020). Such *in silico* modeling can be used to quantify certainty and categorize chemical substances according to their toxicity (Firman et al., 2022). These models incorporate numerous parameters representing exposure pathways, chemical mixtures, and other toxicological-associated factors deemed important for gaining an understanding of the effects of trace metals on human health (Gong et al., 2020). In this regard, the USEPA CompTox Chemical Dashboard includes details of the chemistry, physicochemical properties, and in vivo toxicity data for over one million chemicals obtained using predictive models. These models are effective in prioritizing environmental pollutants. Moreover, the USEPA maintains a Toxic Substances Control Act (TSCA) inventory that includes potential environmental pollutants that pose a threat to human health (USEPA, 1976b).

In addition, Hoang et al. (2021) have developed a chronic simulation model that can be used to predict heavy metal toxicity using an Adaptive Risk Modeling System (ARMS), which uses environmental values of metal concentrations to predict accumulation in fish

tissues. However, the shortcomings of the model highlighted by these authors include the use of reference dose values of methyl mercury for As, Cr, and mercury (Hg). Simulation models are dependent on available data and often incorporate assumptions to fit the model or dataset, which may or may not accurately replicate environmental conditions. This accordingly implies the need for a more systematic approach for the rapid assessment of environmental pollutants. The limitations of modeling approaches have been highlighted in a number of studies (Duval et al., 2017; Yaseen, 2021; Zhou et al., 2021). Among other more recently developed models, Xie et al. (2024) developed a data-driven model for 33 different soil types for risk assessment and prediction of heavy metal bioaccessibility, whereas Lombardo et al. (2022) developed a QSAR model based on OECD guidelines to predict half-lives, bioaccumulation, and toxicity, and Pu et al. (2024) sought to estimate general toxicity using quantitative ion character–activity relationships (QSIER), and thereby gain an understanding of differences in the toxicology of different metals in the soil.

However, although these models are far from being mainstream assessment options, there are numerous applications in which the potentially available *in vitro* options could replace animal models. In this context, it is essential to enhance our understanding of the transport and fate of environmental pollutants to accurately assess the risks posed to human health. However, whereas QSAR and QISAR models have been implemented for assessments using aquatic organisms, fish, and invertebrates (Li et al., 2022a), the toxicity data are not directly applicable to humans. Moreover, even though predictions obtained using of these models have been reasonably close to experimental data, they are heavily dependent on already available data, as well as external conditions. Combining NAMs to screen, evaluate, and prioritize chemicals that can be harmful to humans promotes community-engaged research and informed decision-making. (Silva et al., 2024). Thus, *in vitro* models combined with computational models can provide a streamlined approach for evaluating and estimating risks, thereby enabling a better understanding of the health-related implications.

4 Developments in *in vitro* models

Toxicity is determined using a range of different methods that examine the biological response to substances, and these responses will differ depending on the type of culture used. Although current *in vivo* options are not human-specific, the complexity is representative of fully functioning tissues. Nevertheless, whereas numerous animal models have been used to approximate the whole-organism toxicological response, these models are still only approximations, as they are not necessarily representative of human responses due to differences in organism metabolism and lifespan. Here, we have categorized methods as *in vivo*, and 2D and 3D *in vitro* techniques.

In the past, *in vivo* models were the primary method for collecting data considered representative of more complex tissues. However, given the ethical hesitancies surrounding the use of animal models, *in vitro* cell culture methods have proven to be physiologically more representative. In addition, the complexity of whole organisms, although more biologically relevant, means that the response seen in one organism may not translate to another, even if they are very similar (Eaton et al., 2007; Toutain et al., 2010).

Two-dimensional cell culture is an *in vitro* method of cultivating cells in which the cells are grown in a monolayer on the bottom of a cell plate or culture flask. Although this option enables all cells to receive equivalent amounts of nutrients and growth factors from the media, the cells only have two-dimensional contact with one another, which can thus prevent cells adopting their functional morphology, limit intercellular communication, and contribute to growth patterns that are atypical of those in tissues *in vivo* (Hoarau-Véchot et al., 2018). Accordingly, these models tend to be too simplistic and may thus not be conducive to enhancing our understanding of complex biological processes.

Given these limitations, *in vitro* 3D cell culture methods have attracted increasing interest, particularly as a consequence of more stringent controls and ethical concerns regarding experiments on animals. Although animal experiments were once the gold standard for assessing the response of whole organisms, 3D culture models have been proven to be highly informative and are advantageous in pharmaceutical, toxicological, and physiological studies (Costa et al., 2016; Langhans, 2018). Table 3 highlights the key differences between 2D and 3D cell culture techniques.

Replicating the cellular conditions in an i*n vivo* microenvironment highlights the necessity of taking into consideration all factors associated with the extracellular matrix (ECM) of cells, including surrounding cells, bioactive agents, such as hormones and cytokines, and mechanical forces, both internal, such as cell migration, and external, as in local

Table 3 Comparison of 2D and 3D cell culture methods

Criterion	2D	3D	Case studies/examples	References
Time of culture formation	Cells adhere withing minutes to few hours Cells can grow two dimensionally in a single monolayer	Culture takes about a few hours to days to adhere and grow 3D shape of cell is preserved Cells can grow into 3D spheroids in multiple layers	3D hydrogels for Cd, Pb, and Hg toxicity (Tasneem et al., 2016)	Achilli et al., 2012; Breslin and O'Driscoll, 2013; Costa et al., 2016; Langhans, 2018
Exposure to medium	Nutrients and growth factors distributed evenly for all cells in the culture. A high number of cells are in the same stage of cell cycle. Similar changes in growth/morphology	Nutrients and growth factors can be distributed evenly. Significant number of cells are deprived of oxygen availability. Different effects observed within the culture	3D skin on chip model for Ag (Chen et al., 2019)	Imamura et al., 2015; Costa et al., 2016; Langhans, 2018
Comparison with in vivo	Show similar effects for isolated cells but not tissue	Mimic the effects seen *in vivo* due to the 3D form of arrangement	Air-Liquid Interface for metal oxide nanoparticles (Di Ianni et al., 2024)	Bray et al., 2015
Cell differentiation	Cell differentiation is poor due to limited space and area to grow	Cells are well differentiated	3D dermal model for multi-metal particulate matter (Verdin et al., 2019)	Loessner et al., 2013; Rebelo et al., 2018; Getova et al., 2019
Protein expression levels	Expression levels vary with different setups and not always comparable to *in vivo* treatment	Expression levels are similar to *in vivo* treatments	3D scaffolds for optimum protein expression for tumor models (Fontoura et al., 2020)	Ravi et al., 2015; Costa et al., 2016; Langhans, 2018
Sensitivity to toxins	Low resistance to non-media substances. Little scope for treatments to metabolize within the cells	Metabolism of substances is efficient, allowing for more informed toxicity and functionality assessments	3D scaffolds for skin (Sun et al., 2006)	Haisler et al., 2013; Costa et al., 2016; Langhans, 2018
Usage and analysis	Highly replicable, easy to culture and maintain long-term culture, easy to interpret the data	Difficult to replicate similar conditions, difficult to interpret data due to varying levels of effects within the same culture	3D perfusion bioreactor for complex cell culture models (Jun et al., 2025)	Kapałczyńska et al., 2018; Fontoura et al., 2020
Apoptosis	Treatment including drugs, toxins or metals can easily induce apoptosis due to high sensitivity	Treatment including drugs, toxins or metals show higher rates of resistance; ability to survive cytotoxic agents	3D models for better tumor cells (Fontoura et al., 2020)	Costa et al., 2016
Cost	Cheaper than 3D cultures and *in vivo* studies	Cheaper than *in vivo* studies		Ravi et al., 2015; Costa et al., 2016; Langhans, 2018; Urzi et al., 2023

topography (Barthes et al., 2014). This range of conditions at the microenvironmental level has a pronounced influence on cellular behavior (Mouw et al., 2014). Accordingly, minimizing the differences between the *in vivo* and *in vitro* microenvironments can make it easier to determine whether cell behaviors are associated with different substances or differences in the micro-environment. Moreover, it will provide data that is more representative of cell behaviors within tissues *in vivo*.

Immortalized cell lines can provide organism-specific information and are compatible with high-throughput assays, while primary cells may offer a more specific response. Conducting *in vitro* assessment offers direct control over culture conditions and may be exploited to enhance specificity and facilitate modifications to reduce limitations, thereby enabling more reliable toxicity assessments (Madorran et al., 2020). However, both options fail to represent the complexity of the *in vivo* multicellular organization of cells, which is essential for a better understanding of the whole-organism response. Additionally, population variability introduces a layer of uncertainty. Recent advances in tissue engineering have enabled the development of models that facilitate the observation of *in vivo* responses in humans, thereby providing more relevant, organism-specific data. Assays used to measure cell migration, angiogenesis, ROS generation, and protein expression are the primary procedures that contribute to enhancing our understanding of cellular behaviors. Numerous assays have been developed to quantify these processes, although are still based on the assumption that cells cultured *in vitro* behave similarly enough to those *in vivo* to present a similar exposure response (Alam and Kurohmaru, 2013). However, compared with 2D cultures, by facilitating inter-cellular commu-nication, 3D culture systems can replicate cellular behavior that is more comparable to that which would occur *in vivo*. Accordingly, prioritizing the relevance of cellular interactions in these types of assays will contribute to yielding data that are more informative with respect to the responses in the tissues being modeled.

The use of 3D models has become increasingly more

prevalent, as is seen as bridging the gap between 2D cell cultures and animal studies. Differences in the responses observed using 2D and 3D culture techniques have been well established for a wide range of applications and cell types, which is assumed to reflect the complex structure of the 3D models that more closely mimic *in vivo* characteristics (Kapałczyńska et al., 2018). For example, in cases in which scaffolds are utilized, researchers can control ECM features, such as permeability, mechanical stability, porosity, and a range of other characteristics, using more than 100 types of synthetic and natural scaffold materials. By mimicking tissue structures, cells cultured in three dimensions can exhibit more phenotypically relevant behaviors, thereby yielding data that is likely to be more reflective of *in vivo* scenarios (Ravi et al., 2015; Nguyen et al., 2024). These extracellular structures support and enhance bioactive interactions, thereby facilitating phenotypic proliferation, differentiation, communication, and metabolism. The optimal treatment duration in 3D culture systems is approximately 21 d, significantly longer than that using 2D cell culture systems, thus providing better physiological insights into the effects of environmental pollutants (Habanjar et al., 2021). Consistently, microfluidic flow conditions has revealed closer physiological similarities to those obtained in *in vivo* studies, and a constant flow of media has been established to be conducive to cell proliferation and facilitates the removal of unwanted metabolic waste (Zoio and Oliva, 2022). Ghobadi et al. (2025) used bioactive glass nanoparticles to develop a high-throughput microfluidic device that contributed to enhancing the mechanical properties of tissue-engineered scaffolds, whereas Ko et al. (2024) demonstrated that primary cells in a high-throughput 3D cell culture system can replicate patient-specific behavior and response in cells. However, given that most of the system developed in this regard are of a purpose-driven design, their more widespread application is currently limited. Among other models developed to date, skin-on chip models have shown high resistance, better cellular integrity, and replicability with respect to *in vivo* tissues, whereas among intestinal models, Transwell devices have been more commonly used for 3D cultures. Moreover, the recent development of gut-on-a-chip and flow cell bioreactors has opened up the possibility of in situ imaging and better tissue localization (Shim et al., 2017; Shin and Kim, 2022; Li et al., 2022b; Cho et al., 2024).

In the past decade, the potential application of skin-on-chip models, which show changes in morphology and differentiation and display *in vivo*-like characteristics, has been widely assessed (Zoio and Oliva, 2022; Fernandez-Carro et al., 2022). These models, which can overcome the limitations posed by 2D culture in terms of replicating physiological conditions, have been successfully implemented to replicate several pathological pathways (Ren et al., 2021; Jones et al., 2022), and a few studies have investigated their application for assessing metal exposure (Koning et al., 2022). For example, Michielon et al. (2024) demonstrated the effects of Ni exposure using novel skin-on-chip models.

Despite their multiple advantages, the application of 3D models tends to be considerably more expensive than 2D culturing, in terms of the higher costs of materials and maintenance. In addition, 3D cultures take longer to develop, thus making them less reproducible. Nevertheless, although 2D models can serve as an efficient means of obtaining preliminary data, the cellular interaction and characteristics observed using 3D culture are more physiologically representative (Kapałczyńska et al., 2018). Three-dimentional culture systems utilize scaffolds, non-adherent plates, microfluidics, and gel-like mediums to culture cells, thereby results in more complex 3D structures, such as organoids or tissues, which are subject to nutrient and oxygenation gradients, as well as greater cell–cell and cell–ECM communication and other features found in *in vivo* tissues. These interactions drive the structural organization of tissues, which is directly associated with tissue function (Lelièvre et al., 2017). Closer mimicry of the phenotypic *in vivo* ECM features facilitates observations of more relevant cell functions and more pertinent cell responses.

5 Discussion

Heavy metals are ubiquitous in the environment and non-biodegradable. Numerous studies have reported the adverse biological effects of individual metals. However, given that there are significant differences between exposure to single and multiple metals, evaluating the effects of exposure to mixtures of heavy metals in the environment is of particular importance (Mesquita et al., 2015). Compared with organic pollutants, metals are generally resistant to degradation over short timescales and can accumulate throughout the food chain, leading to serious health concerns. Currently, however, there is a paucity of information regarding how environmental samples influence local and systemic toxicity in humans. In this regard, most of

the established regulatory limits are based on total concentrations of metals (Wang et al., 2022), as opposed to the more relevant bioaccessible fractions. Hence the use of environmental samples to study the toxicity of metals is essential for gaining an understanding of the actual health-related effects of exposure to metals in the environment.

A majority of the toxicological methods developed to date are based on animal models. However, despite the valuable data obtained using *in vivo* models, this option requires more resources and time and poses potential ethical problems. An alternative approach is the use of *in vitro* models. Although 2D cell culture techniques have served as a basis for the development of *in vitro* models, cells in 2D cell cultures lack structural organization and many typical cellular characteristics. However, with advances in cell culture techniques, less labor, and fewer ethical debates, *in vitro* models based on 3D cell cultures have developed rapidly over the past few decades. Advances in 3D technology, including microfluidic chips, hydrogels, and scaffolds, have demonstrated that *in vitro* models can closely replicate the *in vivo* physiological conditions of exposure (Hoarau-Véchot et al., 2018; Nikolova and Chavali, 2019; Leung et al., 2022). Environmental exposure to toxic substances, including heavy metals, is characterized by low-dose, chronic, and multi-component mixtures. The 3D organization of cells growing in the basement membrane has been established to provide additional information on drug delivery and tumorigenesis mechanisms (Forsthuber et al., 2022; da Silva et al., 2024).

Among the 3D culture models developed to date are those that have focused on perfluorinated compounds (PFAS/PFOS), bisphenols, airborne pollutants, nanomaterials, and other organic compounds (Wang et al., 2023b). For example, studies have reported the toxicological effects of PFOS on mouse cardiomyocytes, including adverse developmental effects and the induction of excess ROS. Notably, when using 3D and 2D culture systems, the toxicity of PFOS affecting human cardiomyocytes were measured as 30 and 6.3 μmol/L in the former and 60 μmol/L in the latter (Cheng et al., 2013; Yang et al., 2021; Davidsen et al., 2021). Compared with 2D monolayer cells, chronic exposure to PFOS has been found to be more effectively simulated using 3D spheroids (Calitz et al., 2019; Sun et al., 2019), and multiple cells combined to form a close replicate of *in vivo* liver tissues, have enabled a reliable confirmation of the toxicity of PFOS over a 28-d long-term treatment (Sun et al., 2019, 2022b). Furthermore studies based on air–liquid interface models have replicated condition of exposure to particulate matter with physiological similarities to *in vivo* approaches (Lenz et al., 2013), and compared with a 2D culture system, fewer false negative results were obtained using an air–liquid interface approach. In addition, using 3D models, human bronchial epithelial and A549 cells exposed to aerosols comprising nanoparticles of Cu and Zn have been shown to be characterized by significant cytotoxicity, along with elevated levels of ROS and interleukin-6 and -8 (Jing et al., 2015). However, further studies are necessary to assess the extent to which 2D and 3D cultures replicate the different pathways.

Given that the effects of environmental mixtures is often unknown, the combined effects of metals should also be examined (Luo et al., 2022), and in this regard, it is anticipated that the integration of exposure modeling with high-throughput microfluidic 3D cell culture will facilitate the development of more reproducible assays for environmental samples. Such platforms could be used to evaluate sample-specific conditions and thereby pave the way for studying underlying effects on pathological pathways. Using high-throughput cell culture techniques, along with standardized sample collection and processing methods that incorporate multi-omics and NAMs, can contribute to providing more realistic data on the health-related risks posed by exposure to toxic metals in the environment.

6 Conclusions

In this review, we have examined the different types of *in vitro* models that have been developed to assess the toxicity of environmental heavy metals. Despite rapid advances in the development of *in vitro* 3D cell culture models, the integration of environmental samples has yet to be sufficiently assessed. Given the likelihood of increasing levels of exposure to heavy metals within the environment, further investigations are imperative for gaining a more comprehensive understanding of the associated health implications. The primary effect of toxic metal exposure is the induction oxidative stress. However, although the effects of toxic metals on biological functions have been extensively studied using animal models, there remains a pressing need to develop relevant toxicokinetic and toxicodynamic models to accurately evaluate human health risks associated with environmental exposure to these chemicals. By exploiting ongoing advances in cell culture technology, it should be feasible to replace *in vivo* animal studies with effective *in vitro* alternatives

that can provide reliable data more efficiently and at a lower cost. Although 2D and 3D cell culture techniques offer several advantages, these methods can be further improved. Indeed, recently developed models, such as organ-on-chip, scaffolds, and microfluidic devices, offer significant potential to mimic environmental exposures and are accordingly anticipated to yield data that will contribute to gaining a better understanding of toxic mechanisms.

Conflict of Interests The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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