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In vitro testing of hazardous mineral fibres: The issue of the concentration metric

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ABSTRACT

This communication addresses the matter of the appropriate concentration metrics for the *in vitro* testing of mineral fibres, a specific technical issue affecting the correct determination of their toxic/carcinogenic potential. The exposure to certain mineral fibres (e.g., asbestos and erionite) is well-known for its detrimental effects on human health, with caution exposure limits set to 0.01 ff/cm^3 by the European Council in 2023. In this regard, *in vitro* tests have a crucial role in the preliminary determination of the hazardous potential of mineral fibres, although selecting the appropriate concentration metrics and doses is currently controversial. Here, we address the complex technical issues of the current normalisation methods (i.e., mass normalization and fibre number normalization) with their advantages and disadvantages, ultimately concluding that mass normalisation should be recommended. In fact, considering two fibrous species with the same chemical composition, mass normalisation guarantees that the concentration of atomic species and ROS-inducing metals remains equal, while this parameter becomes an additional variable with fibre number normalisation.

1. Introduction

According to the Society of Toxicology (2015), "Toxicity testing is performed to assess the safety or hazards presented by substances such as industrial chemicals". Within toxicity testing, *in vitro* assays play a pivotal role since "*in vitro* toxicology consists of using cells maintained or grown in controlled laboratory conditions to examine the toxic properties of compounds and mixtures" (Quinn, 2014). The U.S. Environmental Protection Agency clarifies that "*in vitro* experiments or tests (are) done under controlled experimental conditions outside of the body, such as in a test tube or laboratory dish. These tests tend to focus on organs, tissues, cells, cellular components, proteins, and/or biomolecules" (U.S. EPA, 2010). In all cases, the purpose is to "evaluate activity in toxicity pathways elucidating the modes and mechanisms of action of toxic substances" (Krewski et al., 2020).

Since *in vitro* toxicity assays have become a priority for natural raw materials and industrial products, numerous attempts have been made to create guidelines, protocols and systematic programs for different classes of *toxicants* so far. A remarkable example is the *ToxCast* research program of the U.S. EPA to predict the potential toxicity of environmental chemicals based on *in vitro* bioactivity profiling (Gangwal et al., 2011). Design and application of toxicological screenings require the characterization of materials, the selection of testing concentrations, and the analysis of the resulting data. The selection of the appropriate concentrations plays a key role to such an extent that a bad choice may cause the failure of *in vitro* models in predicting *in vivo* responses (Sayes et al., 2006; Warheit et al., 2009; Hinderliter et al., 2010). In this regard, human exposure information is deemed essential when selecting the doses for toxicity testing to facilitate the development of environmentally relevant hazard information (NRC, 2007). Nonetheless, according to Oberdörster et al. (2005), *in vitro* concentrations are often chosen to be very high to ascertain a toxicological endpoint without consideration of the real-world exposure.

The selection of concentrations may become an issue when comparing the toxicity potential of different agents. Say we want to compare the toxicity potential of two production batches of TiO_2 powders, with the same chemical composition and particle shape (spherical) but different size: TiO_2-A has a mean diameter W=1 μm and TiO_2-B has a

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mean diameter W=0.1 μm. If mass is used as concentration normalization metric, with 10 μg/mL of agent to a 5×10^5 cells in 1 mL culture medium, there will be ca. 9 TiO_2-A particles per cell and ca. 9034 TiO_2-B particles per cell, showing a critical mismatch between few "large" particles vs. numerous "small" particles. On the other hand, if particle number is used as concentration normalization factor (metric), 10 μg/mL of TiO_2-A added to the culture medium corresponds to 0.01 μg/mL of TiO_2-B, leading to a very low concentration of the latter that could ultimately have no effect on the investigated cells.

The situation is even more intricate in the case of mineral fibres, namely asbestos, because these particles have a fibrous shape with a micrometric length and a sub-micrometric/nanometric width. Since it binds efficiently to various materials enhancing their strength and durability, asbestos has been used in a variety of commercial products over the years, leading to the manufacturing of more than 3000 asbestos-containing materials (Virta, 2002). In particular, asbestos was generally used for roof shingles, floor and ceiling tiles, cement pipes, insulators, and sealants in the construction industry field, as well as for brake linings and gaskets in the automotive industry field (Thives et al., 2022). Due to the hazardous effects of asbestos on human health (IARC, 2012), the periodic assessment of the concentration of airborne asbestos fibres is crucial. To this aim, various techniques have been developed, including phase contrast optical microscopy (PCOM), polarized light optical microscopy (PLOM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), scanning transmission electron microscopy (STEM), and X-ray diffraction (XRD) (Baron, 2001; Millette and Compton, 2005; Gualtieri et al., 2009).

Although threshold limits for environmental asbestos exposure have been defined for individual states (see for example, Lippmann, 1988 for the Unites States), there are currently no globally accepted international threshold limit values (Ghorbani et al., 2020). Chrysotile, the predominant fibre detected, displays typical concentrations of 0.01 ff/L in outdoor air in rural locations, about 10-fold higher in urban locations, and about 1000 times higher in close proximity to industrial sources of exposure. Elevated levels of chrysotile fibres have also been detected at busy traffic intersections. In indoor air (e.g., homes, schools, and other buildings), measured concentrations of asbestos are in the range of 0.03–6 ff/L (WHO, 1998). Nonetheless, numerous studies have shown a substantial variation in airborne asbestos fibre concentrations worldwide (Lim et al., 2004; Gualtieri et al., 2009; Bourgault et al., 2014; Oskierski et al., 2016; Fathi Fathabadi et al., 2017; Taghizadeh et al., 2019; Ghorbani et al., 2020; Moteallemi et al., 2020; Leocat, 2020; Hajizadeh et al., 2021).

Oberdörster (1994) noticed that chrysotile appears to have the same toxicity towards macrophages as amphibole asbestos fibres when concentrations of equal mass are administered. However, based on the number of fibres, which are higher in chrysotile than in amphibole asbestos for the same sample mass, chrysotile appears to be less toxic to macrophages. Oberdörster (2000) later reported that the dose of a particulate compound delivered to the lung by inhalation is generally expressed as particle mass, although other dose-metrics have been used in particle toxicology (i.e., particle volume, particle overload responses, particle surface area, and particle number). Moreover, Oberdörster (2000) affirmed that fibre number is the best dose parameter to characterize responses in the case of fibrous particles, that an exposure concentration (number of fibres/cm^3) is not a dose, and that the term "exposure dose" should be avoided. However, Bernstein (2022) pointed out that mass normalization concentration can result in a very high amount of fibres per cell, significantly exceeding the quantities expected to occur in human occupational or environmental exposures, as reported in the anomalous experiments of Huang (1979) where ca. 500,000 fibres were applied to each cell.

In this communication, the issue of the concentration metrics for *in vitro* studies of mineral fibres, with special attention to asbestos, is addressed. In particular, we discuss what is the best concentration metric, considering the underlying differences between mass normalization and fibre number normalization when performing *in vitro* tests on mineral fibres. What is the scientific rationale to justify that there should be few fibres per cell in the culture medium? An interpretation and evaluation of other possible solutions is given.

2. Results and discussion

2.1. Is it possible to base in vitro concentrations on occupational exposure for mineral fibres?

Ziemann et al. (2024) recently observed that the measured burden of airborne respirable fibres at workplaces is usually < 1 ff/cm^3 and that occupational exposure concentrations have not increased in the last decades (Marchant et al., 2021). The current 8-h OSHA PEL-TWA limit is 0.1 ff/cm^3 (OSHA, 2025) and in Europe the maximum limit for exposure to asbestos fibres is 0.01 ff/cm^3 (European Council, 2023). In the field of mineral fibre toxicology, some authors (see for example, Bernstein, 2022) claim that *in vitro* toxicological studies should be based on the aforementioned threshold concentrations to accurately reflect the actual levels of human exposure to mineral fibres, and to avoid the use of excessively high and unrealistic doses in cellular toxicity studies. Although an exposure concentration (e.g., ff/cm^3) is not considered a dose (Oberdörster, 2000), these exposure limits were translated into actual *in vitro* concentration metrics (ff/cells or mass/cells) and reported in Table 1 to assess whether exposure limit doses could represent suitable *in vitro* concentrations for toxicological cellular studies. The number of fibres (ff) deposited in the alveolar space in a 8 h/d working month (22 working days) was calculated from the different exposure fibre burdens and compared to the total number of alveolar macrophages (AM). Then, the ff/AM ratio was translated into the corresponding ff/cells and mass/cells suggested for *in vitro* testing, assuming asbestos fibres to be crocidolite. All the details of the calculations and references relative to the values used can be found in the Supplementary Materials.

The accuracy of the results was assessed comparing the calculation for 1 ff/cm^3 crocidolite exposure with the calculations made using a commercial software for multiple path particle dosimetry (MPPD) by Ziemann et al. (2024) for the supposedly ca. 1 ff/cm^3 amosite exposure case (also reported in the **Supplementary Materials**). The estimates (Table 1) show that, because the ratio between the number of fibres accumulated in the alveolar space and the number of alveolar cells is extremely low, the exposure limits in working environment (0.1–0.01 ff/cm^3) require mass concentrations (3.9×10^{-2}–3.9×10^{-3}) for the *in vitro* testing too low to be perceived by cells and to record any detrimental effect. The mass concentrations are even smaller in the case of chrysotile fibres, and/or for fibres smaller than the ideal WHO limits. For example, in case of crocidolite WHO fibres with mean L=10 μm and W=0.3 μm, instead of W=3 μm, the expected mass (μg/mL) for the *in vitro* culture medium to mimic the 0.1 ff/cm^3 exposure limit (OSHA, 2025) reduces from 3.9×10^{-2} to 3.9×10^{-4}. In the case of chrysotile WHO fibres with mean L=10 μm, W=0.1 μm and ideal density of 2.53 g/cm^3 (https://webmineral.com/data/Chrysotile.shtml) the expected mass (μg/mL) for the *in vitro* culture medium to mimic the 0.1 ff/cm^3 exposure limit (OSHA, 2025) further reduces to 3.2×10^{-5}. The assumption that few fibres per cell are observed following exposure to ca. 250 ff WHO standard per cm^3 of chrysotile (Bernstein et al., 2021) is plausible but unjustified as that concentration is unrealistically high for the actual limits and concentrations of fibres in the working environment. Thus, the working exposure limits/concentrations can hardly be translated into actual *in vitro* concentration metrics. The low fibres/cells ratio would also require mass concentrations (μg/mL) too small to handle accurately and to reveal, if any, toxicity effects. Furthermore, we should keep in mind that *in vitro* cell models are approximations of the real human physiology and that, for the two situations, *in vitro* or *in vivo*, the sensibility to external challenges may present different threshold doses triggering cell and/or tissue responses. Hence, actual fibre concentrations for *in vitro* testing should point to measure toxic endpoints

Table 1
Calculation of the actual *in vitro* concentration metrics needed to fulfil exposure limits in the working environment.

	0.01 ff/cm³ (European Council, 2023)	0.1 ff/cm³ (OSHA, 2025)	1 ff/cm³	10 ff/cm³	250 ff/cm³
ff accumulated in the alveolar space in 1 working month*	253,440	2,534,400	25,344,000	253,440,000	6,336,000,000
ff/AM**	6.5×10^{-5}	6.5×10^{-4}	6.5×10^{-3}	6.5×10^{-2}	1.6
expected ff in 1 mL of *in vitro* culture medium***	16.2	162.5	1625	16,250	153,882
Expected μg/mL of crocidolite**** fibres for the *in vitro* culture medium	3.9×10^{-3}	3.9×10^{-2}	0.4	3.9	37.0

* 1 working d=8 h and 1 working month=22 d; 1 month has been selected because it is a time span generally shorter than the estimated biodurability time *in vitro* of chrysotile and much shorter than that of amphibole asbestos species and erionite (Gualtieri et al., 2018). **accumulated ff in the alveolar space/total number of alveolar macrophages (AM) in the lungs (i.e., 3.9×10^9), as detailed in the Supplementary Materials. ***it is assumed that a 1 mL well contains 250,000 cells. ****it is assumed a crocidolite ideal density of 3.40 g/cm³ (https://webmineral.com/data/Riebeckite.shtml) a mean WHO L=10 μm and a mean WHO W=3 μm.

and dose-response relationships, while avoiding both a fibre overload situation (large ff/cells ratio) or metrics that are not representative of the real *in vivo* case (very small ff/cells ratio).

2.2. Mass vs. number of fibres concentration metrics

Some authors (e.g., Bernstein, 2022) suggest that the fibre number is the best concentration normalization factor to compare the toxicity effects of different fibrous species (e.g., chrysotile vs. amphibole asbestos) because, for the same mass, some species contain more (or much more) fibres than others. In fact, if specimen A displays single fibres of much smaller thickness compared to specimen B, for the same mass a much greater number of fibres of A, with respect to B, will be in contact with the cells. Hence, it would be reasonable to compare these different fibrous species by normalizing for the number of fibres. Nonetheless, a literature search of the concentration selection mode for the *in vitro* testing has been conducted for the key case of asbestos fibres, highlighting whether mass normalization or fibre number normalization metrics were employed. The consensus is that *in vitro* toxicity studies on asbestos fibres have been generally performed comparing mass doses to date. In detail, a search of the literature data in *Google Scholar* (data accessed on January 14, 2025) of the first 50 displayed entries, regardless of publication year, shows the following outcome.

For the search keywords "*in vitro* toxicity asbestos":

- 26 papers (52.0 %) report mass normalization data;
- 6 papers (12.0 %), reporting both mass normalization/fibre number normalization data, are uncertain or cannot be accessed;
- 2 papers (4.0 %) report fibre number normalization data.

For the search keywords "*in vitro* toxicity chrysotile":

- 26 papers (52.0 %) report mass normalization data;
- 8 papers (16.0 %), reporting both mass normalization/fibre number normalization data, are uncertain or cannot be accessed;
- 6 papers (10.0 %) report fibre number normalization data.

For the search keywords "*in vitro* toxicity crocidolite":

- 29 papers (58.0 %) report mass normalization data;
- 6 papers (12.0 %), reporting both mass normalization/fibre number normalization data, are uncertain or cannot be accessed;
- 1 paper reports (2.0 %) fibre number normalization data.

Now, say we normalize the *in vitro* concentration with respect to the fibre number for two fibrous specimens with the same chemical composition (i.e., same content of reactive oxygen species (ROS)-inducing metal species) (left case in Fig. 1). An example could be the short vs. long chrysotile fibres described in Gualtieri et al. (2023). The nominal concentration (Groothuis et al., 2015) in terms of number of fibres is the same between the two specimens but the nominal concentration of mass is different, resulting in a different nominal concentration of atomic species, including the content of ROS-inducing metals. Size and surface area of the two specimens are also different. When normalizing the *in vitro* concentration with respect to the mass for two fibrous specimens with the same chemical composition (median case in Fig. 1), the nominal mass concentration (Groothuis et al., 2015) is the same, but the number of fibres is different as well as the size and surface area of the two specimens. In this case, at least the nominal concentration of atomic species, including the content of ROS-inducing metals, is

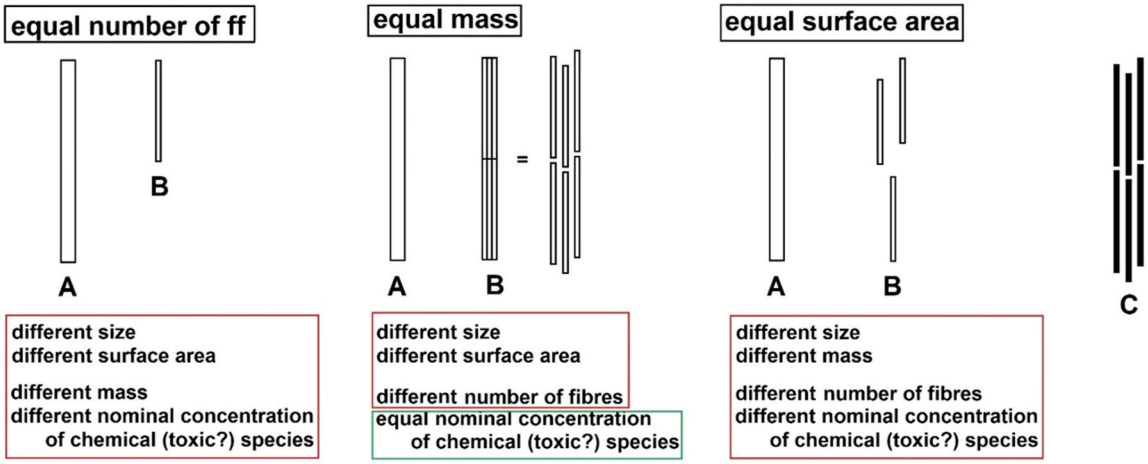

Fig. 1. Comparison of the normalization with respect to the fibre number (left case), mass (central case) and surface area (right case, with the example of the geometric area of the particle A = sum of the geometric area of the 3 particles B) for two fibrous specimens with the same chemical composition for *in vitro* testing. Specimen C is the case of a fibre species with different chemical composition than A and B. See text for details.

equal. All these factors (fibre size, surface area and chemical composition, with special attention to ROS-producing metal species) influence the cellular uptake, phagocytosis, total internal (intracellular) concentration (Groothuis et al., 2015), and toxic activity of the particles in general (e.g., Kettler et al., 2014 and Harik, 2017 for how the parameters of fibrous agents influence their toxic effects *in vitro*). Hence, why normalize for the number of fibres if the different chemical composition (concentration of atomic species) is added as variable of toxicity to the system? It should also be remarked that the content of metals, namely iron, is one of the major parameters responsible for fibre toxicity (Gualtieri et al., 2019; Gualtieri, 2021). Of course, if the fibre type changes (e.g., chrysotile vs. amphibole asbestos), the chemical composition of the specimen changes but not the relative proportions considering mass normalization (see example C in Fig. 1). In this case, the aim of the *in vitro* testing is just to assess the toxicity effects due to the different crystal-chemistry, besides the physical parameters.

2.3. Difficulties in the normalization with the fibre number metric

For fibres like asbestos, relying on fibre number as the normalization metric has two major issues: (i) apparent vs. actual fibre number and (ii) fibre agglomeration.

(i) Asbestos single fibres (fibrils) are generally bound by van der Waals forces, hydrogen bonding or stronger chemical bonds due to intergrain crystallisation (Langer et al., 1974) to form fibre bundles (Ross et al., 2008). The number of individual fibres with defined length (L) and width (W) can be accurately determined using high-resolution imaging techniques, such as SEM or TEM. However, due to the fibres propensity to aggregate into bundles, the actual number of discrete fibrous particles per unit mass is substantially lower than the theoretical estimate based on single-fibre counts. In Gualtieri et al. (2023), it was evidenced that for chrysotile the measured mean W of the single fibres was 0.4 μm, while the actual mean W of actual fibre bundles observed in the cell cultures was about 2.8 μm. Using a concentration of 50 μg/mL in the cell culture, at equal L and mass, these numbers yielded very different concentrations: 207,272 actual fibrous particles (fibre bundles) in contact with the cells (ca. 1 ff/cell) vs. 5,185,797 apparent chrysotile single fibres (ca. 21 ff/cell). Moreover, cells do not differentiate among single fibres or bundles and interact with all of them, as shown in Fig. 2 (modified after Gualtieri et al., 2023) in which THP-1-derived macrophages are internalising a bundle of chrysotile composed of presumably 25 single fibres.

(ii) Single fibres or fibre bundles can agglomerate in suspension, both *in vitro* and *in vivo* (Kettler et al., 2014). Pollastri et al. (2014) observed that the zeta potential of asbestos fibres at pH 4.5 and 7.0 displays values in the range −10 to −26 mV. At such values of zeta potential, agglomeration is favoured. Agglomeration induces enhanced biological responses because cell uptake is slower and can be hindered (see for example, Sharma et al., 2014).

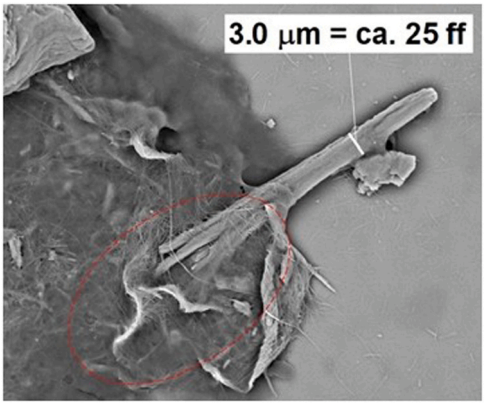

Fig. 2. THP-1-derived macrophages uptake of chrysotile fibre bundle. Macrophages, indicated by red dashed lines, uptake a fibre bundle with W=3 μm composed of presumably 25 single fibres of mean W=0.4 μm (modified after Gualtieri et al., 2023).

Moreover, it should be remarked that, if size and shape allow it, macrophages can engulf numerous targets at the same time, destroy them and survive, e.g. in case of bacterial outbreaks or in the scavenging of dead/damaged cells (Cannon and Swanson, 1992). Alternatively, if the internalised targets (numerous or not) prove to be biodurable, macrophages may undergo apoptosis and die, as in the case of mineral fibres. Therefore, for toxicological studies it seems more important to take into consideration the shape of the target, in this case fibrous, and possibly the size, although tests are always performed on respirable fibres, rather than focusing strictly on their number.

2.4. Are there other possible normalization metrics?

In the past, Gualtieri et al. (2023) have considered normalising on the volume to compare *in vitro* the toxicity of different asbestos species. This procedure is equivalent to normalising on the mass except that the values are rescaled according to the densities of the fibrous materials. For silicate mineral fibres, the density values are not so different (e.g., 2.53 g/cm^3 for chrysotile and 3.40 g/cm^3 for crocidolite) to justify this procedure other than the mass normalization selection.

The concept of surface area ratio has been used in the past to compare cellular responses to various particles in a standardized manner (see for example, Shanbhag et al., 1994). Governa et al. (1998) considered the surface area for dosing mineral particles with different fibre size aiming at solving the problem of standardising heterogeneous mineral particles and thus obtaining comparable responses in short-term *in vitro* experiments. Assuming cells of a fixed size, elongated particles with a large surface area are more likely to interact with the cell membrane respect to an equal mass of particles with a smaller surface area, as shown in Fig. 3a. Indeed, a larger surface area of the particles promotes a higher degree of membrane-mediated surface contact with the cells, while also providing more opportunities for their interaction.

Kaw et al. (1982) reported that the cytotoxic action of chrysotile could be attributed to its greater surface area in comparison to crocidolite or amosite, while following *in vitro* studies highlighted the role of surface area in the harmful effects of various particle types (Monteiller et al., 2007). However, the increased cellular interaction caused by a large surface contact is only partially responsible for particle toxicity due to the additional influence of other parameters, such as shape, biodurability, chemical composition, and size exceeding the phagocytic capacity. In this regard, it has been reported that fibre shape impacts the macrophage phagocytic capacity and efficiency more than size does (Champion and Mitragotri, 2006; Paul et al., 2013). For example, the rate of the phagocytic process is influenced by the angle at which the fibres are engaged during their internalisation in the cells (i.e., the shorter or the longer fibre axis), eventually leading to frustrated phagocytosis (Fig. 3b) when the fibre size exceeds the cell membrane ability to fully wrap around and engulf the target. This has been demonstrated with simple experiments (Champion and Mitragotri, 2006) showing that if macrophages firstly interact with fibres from the shorter axis they will rapidly progress towards the engulfment of the entire fibre in less than half an hour, if the fibre length allows complete internalisation (case B in Fig. 3b). On the contrary, if the same fibre, with the same size, is engaged by the cell membrane along its longer axis, the attempt at phagocytosis will be slowed down and, if completed at all, it may take more than one hour (case A in Fig. 3b). Thus, the same fibre could cause or not frustrated phagocytosis, and consequently cell death, depending on which side is engaged by the cell upon first contact, complicating the issue of the toxicity assessment when considering the variables of size, shape, surface and number of fibrous particles.

Normalizing the *in vitro* concentration with respect to the surface area for two fibrous specimens with the same chemical composition (right case in Fig. 1), the nominal mass concentration (Groothuis et al.,

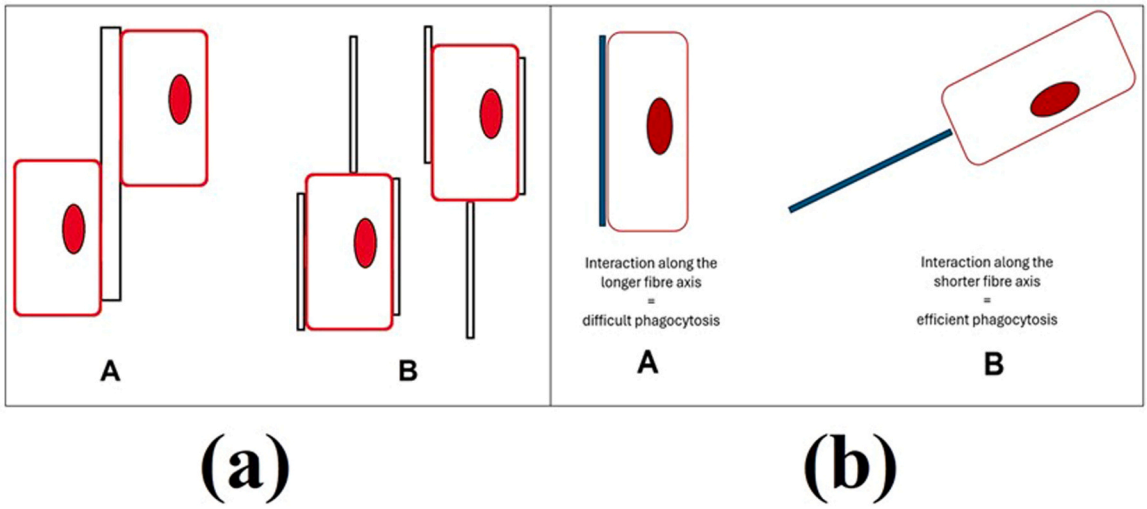

Fig. 3. **(a)** Assuming cells of equal number and size and equal mass of particles in two ideal *in vitro* systems **A** and **B**, elongated particles with greater surface area (**B**) display more contacts (in red) with the cells than those with smaller surface area (**A**). Particles are all vertically aligned for simplicity. **(b)** Macrophage phagocytosis efficiency depending on target shape. (**A**) example of hindered phagocytosis: first interaction with fibre occurs along the longer axis of the target; (**B**) example of efficient phagocytosis: first interaction with fibre occurs engaging the shorter axis of the target.

2015) changes as well as the number of fibres, their size and the nominal concentration of atomic species. Since the different concentration of atomic species — including the content of ROS-inducing metals — is added as variable when evaluating the particle toxicity, a normalization metric based on the surface area, also in this case does not seem to be advantageous.

2.5. Can we establish an appropriate fibre concentration range for toxicological in vitro studies?

The primary parameter for determining the toxicity of a substance is the dose; in fact, almost all substances can be toxic in certain doses or under specific circumstances. From this principle, it follows that any chemical substance can be toxic once the so-called "threshold dose" is exceeded. Thus, a clear consequence is that, if we already know from epidemiologic data that a particular substance/mineral has been recognized as toxic and/or carcinogenic, as in the case of asbestos, does it really make sense to try to spasmodically reproduce *in vitro* the average exposure doses presumably happening in controlled occupational environments? Or should a toxicological study focus more on determining the "threshold dose" in the chosen *in vitro* model by providing dose-response relationships at the cellular/tissue level and elucidate the mechanisms of action of the toxic substance? Since *in vitro* models are necessary extrapolations of the real structure and physiology of a living organism, including humans, the threshold dose eliciting cellular responses indeed may be quite different from the environmental exposure dose causing symptoms, as already stated above. Even in the case of substances of unknown potential hazard, the matter is actually not much different, because in this case the aim is performing predictive toxicological studies where, once again, we try to establish what the threshold dose of the substance is (i.e., the dose at which it becomes toxic) by performing dose-response biochemical/molecular studies, and to predict the potential toxic effects on living organisms including humans, as well as to assess the safety and potential risks associated with its exposure. Thus, we should overturn the paradigm that *in vitro* doses should always be as close as possible to the real exposure doses of living organisms in the environment. Instead, we suggest to adopt the idea that, based on the results from predictive toxicological studies, if certain cumulative doses of exposure are reached over time, especially for biodurable substances, we will have to expect toxic responses and possible damage to the organism, as observed *in vitro*.

Regarding mineral fibres, a good *in vitro* experimental plan should include preliminary cytotoxicity assessments by several standard methods (e.g., MTT, LDH, DNA, or ATP/ADP quantification tests, to cite a few), in different cellular models, and in a wide range of fibre concentrations spanning several orders of magnitude (e.g., from 1 to 100 μg/mL), to initially quantify the level of cellular resilience or toxicity to the fibres and to establish dose-response cytotoxic relationships. Then, a concentration range should be chosen to perform biochemical and molecular analyses aiming at clarifying the cellular effects and the mechanisms of action. To evaluate the latter, the selected concentrations should span from the one causing minimal effect, to one inducing sufficient toxicity to trigger cellular responses. The highest dose of this concentration range should anyway ensure that most cells are still alive in the culture (i.e., 60–70 % compared to the initial number) to observe and measure said responses in a reliable context (see for example, Mirata et al., 2022; Mirata et al., 2025; Scarfì et al. 2025). This kind of mechanistic studies, if correctly postulated according to the New Approach Methodologies (NAMs) strategy for *in vitro* toxicological studies, will be of fundamental importance to design efficient pharmacological therapies once the environmental exposure to the substance or mineral reaches the no-turning point of body tolerance to the challenge.

3. Concluding remarks

In vitro tests have a crucial role in the assessment of the hazardous potential of mineral fibres; in fact, they evaluate whether the exposure to mineral fibres has the potential to trigger toxicity pathways with damaging effects to human health, eventually contributing to the elucidation of the complex mechanisms underlying fibre toxicity. However, the selection of the appropriate fibre metrics for *in vitro* tests is currently controversial, especially when attempting to administer fibre doses close to environmentally relevant exposure scenarios. Thus, in the present study, the working exposure limits/concentrations established for asbestos fibres (European Council, 2023; OSHA, 2025) — which some authors propose as the correct doses for *in vitro* toxicological studies — were experimentally translated into actual *in vitro* concentration metrics, resulting in fibres/cells ratios too low to handle accurately.

Regarding the appropriate normalization metric for *in vitro* tests, most of the literature on asbestos fibres relies on mass normalisation. In fact, considering two fibrous species with the same chemical composition, mass normalisation guarantees that the concentration of atomic species and, in particular, of ROS-inducing metals remains equal, while

this parameter becomes an additional variable with fibre number normalisation. Moreover, some natural fibres (e.g., chrysotile) typically occur as fibre bundles hindering their precise counting for fibre number normalisation.

Among the other possible metrics, SSA normalization focuses only on fibre size/width, leading again to a different concentration of atomic species and toxic metals.

Finally, since *in vitro* models are necessary extrapolations of the human organism, the determination of the correct "threshold dose" is fundamental to stimulate significant cellular responses to obtain a more in-depth understanding of the fibre toxicity mechanism, while also ensuring that most of the cells are still alive to guarantee the analysis reliability.

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CRediT authorship contribution statement

Mario Passalacqua: Writing – review & editing. **Anna Maria Bassi:** Conceptualization. **Sonia Scarfi:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Alessandro F. Gualtieri:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Serena Mirata:** Writing – review & editing, Writing – original draft, Investigation, Data curation.

Declaration of Competing Interest

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

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2025.104813.

Data availability

Data will be made available on request.

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