Clay-induced DNA double-strand breaks underlay genetic diversity, antibiotic resistance and molecular basis of asbestosis

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Abstract

Some natural clays and synthetic nanofibres present in the environment have a severe impact on human health. After several decades of research, the molecular mechanism of how asbestos induce cancers is not well understood. Different fibres, including asbestos, can penetrate and transform both, bacterial and eukaryotic cells. We found that sepiolite and asbestos cause double-strand breaks in bacteria when the incubation occurs under friction forces. Since antibiotics and clays are used together in animal husbandry, we propose that this mutagenic effect constitutes a pathway to antibiotic resistance due to the friction provided by peristalsis of the gut from farm animals. We also discuss the possibility that the same mechanism could generate bacteria diversity in natural scenarios with a role in the evolution of species. Finally, we provide a new model on how asbestos promotes mutagenesis and cancer based on the genotoxicity that we observe in bacteria.

Keywords: sepiolite, mutagenesis, antibiotic resistance, double-strand break, microbiota evolution, genetic diversity, asbestos, asbestosis, carcinogenesis

Introduction

A significant concern arises from fibrous clays or industrial nanofibres which are responsible for severe human diseases such as asbestosis [1]. These materials are considered genotoxic and carcinogenic, likely due to their ability to damage DNA [2]. They have assayed in several experimental models including bacteria and cell in cultures, but they display a poor correlation with mutagenicity or carcinogenesis found *in vivo* [3,4]. Short or long periods of exposure to fibres have been failing to identify a molecular basis of DNA damage in different several genotoxicity tests [1]. Thus, nowadays the mechanisms underlying the genotoxicity and carcinogenicity of asbestos and other fibres remain obscure.

The transformation of bacteria by foreign DNA can be achieved if clay fibres are spread applying some friction or vibrations. This phenomenon is known as Yoshida effect [5]. This transformation relies on the ability of mineral nanofibres or nanoneedles to adsorb DNA and penetrate bacterial cells under sliding friction forces [6]. By its mechanical nature, the Yoshida effect can be used to transform diverse bacterial species [8–10]. The action of sepiolite and other clays fibres is not only capable of delivering DNA into the receptor bacteria but also able to promote the releasing of DNA by disrupting the cell envelope of the portion of the population by the abrasive action of clays [11]. Before Yoshida began his experiments with bacteria, the ability of asbestos to transform eukaryotic cells has been reported at the end of the eighties [12].

Clays have the potential to enhance antibiotic resistance in farming activities [13]. In natural sceneries, sediments and stones (gastroliths) are frequently swallowed by animals resulting unavoidably in the exposure of their microbiota to pebbles, sand, and clays. Soils and waters are a primary source of antimicrobials, either by natural microbial production or environmental antibiotic pollution, a major selective pressure that favours resistant strains [14,15]. Even, gut microbes can produce antibiotic compounds [16].

Here, we experimentally show how fibrous clays such as sepiolite and asbestos are not only able to transform bacteria, but also induce mutagenic DNA double-strand breaks (DSBs) when they are exposed to friction forces. Additionally, we propose a molecular mechanism of action for asbestos, that were strong inducers of DSBs in Escherichia coli if friction is present. The observed mutagenesis can contribute to antibiotic resistance via mutations in farming settings. Besides, it is a potential source of bacterial diversity in the natural context we hypothesise that this novel mutation supply has important contribution for the speciation processes of metazoa.

Results and Discussion

Different type of clays are able to transform bacteria penetrating the cell envelope. We resoned that this penetration could also interact with the DNA and promote mutations. We first investigated whether the treatment with sepiolite under friction forces (as in transformation) has an impact on the mutant frequency of *Escherichia coli* measured by plating in the antibiotic fosfomycin (figure 1). Simultaneously, we checked the effect of the treatment on cell viability (figure 2). We did not detect any significant differences in mutant frequencies when the cells were merely exposed to sepiolite without applying any friction on agar plates surface. In contrast, we found values around six-fold increase in mutant frequency when friction is present for two or three minutes (P=0.006) and a modest value when the treatment time lasted for one minute but not significant. Interestingly, we noted that only cells in the stationary phase displayed an increase in mutant frequency, while no significant mutagenesis was observed when bacteria came from exponential cultures (figure S1). We initially attributed this difference to a higher sensitivity to the treatment (figure 2).

Under friction forces on an hydro-gel, sepiolite and other nanosized acicular materials can penetrate bacterial cells [5]. Many minerals containing metals such as iron, aluminium or copper are toxic for bacteria and their toxicity is caused by the generation of reactive oxygen species (ROS) [17]. We reasoned that the release of metal ions inside the cell could elicit the Fenton reaction and promote mutagenesis. Despite the addition of 2-2′ bipyridyl shortly before treatment mutagenesis was still observed (figure S2). This result supports the idea that the mutagenic effect in this situation does not depend on the metals present in the fibres.

A second likely explanation is the physical interaction of individual clay fibres in motion during penetration directly damage the DNA, causing strand breaks (DSBs). Physical or mechanical stress on the DNA duplex is a relevant cause of DSBs [18]. To evaluate this possibility, we designed a simple experiment where *E. coli* DH5-alpha strain (*recA* deficient) carrying a plasmid (pET-19b) were subjected to treatment with sepiolite and sliding friction while the addition of sepiolite without friction and bacterial cells alone were used as controls. The extracted plasmid from the sepiolite group (under friction) presented a significantly high level of linearised molecules when compared to the control groups (P=0.00016). Thus, we infered that the joint action of sepiolite and friction are responsible for induction of double-strand breaks in the DNA. Interestingly, no increase in nicked DNA (single-strand break) was observed, indicating that if this type of lesion occurs, it happens at a non-detectable rate by this technique. Typically, during plasmid DNA extraction, three molecular

conformations are found: the supercoiled (which migrates very fast), nicked DNA (which is also closed circular but relaxed due to single strand breaks and it has an intermediate migration rate) and linear molecules (with a lower migration speed) [19]. These latter DNA molecules were especially abundant in the friction-sepiolite treated group at the time that they are present in a low level in control groups (figure 3).

The view of mutagenic DSBs by mechanical shearing is very consistent with the absence of mutagenic effect in exponentially growing bacteria. If the organism is diploid (even if the diploidy is only transient, as in replicating bacteria or replicating haploid yeast), then homology-directed repair can be used [18]. There is a pathway to deal with lethal double-strand breaks by non-homologous DNA end joining at the cost of introducing mutations [18]. Because *E. coli* lacks a pathway to join non-homologous ends, homologous recombination is the only means to salvage broken chromosomes [20]. But how can *E. coli* repair DSBs in stationary phase by homologous recombination? Stationary-phase cultures contained cells with several chromosome copies [21]. In exponentially growing *E. coli* DSB repair is non-mutagenic [22,23]. However, breaks repair becomes mutagenic during the stationary phase and requires the Sigma S factor (RpoS), the SOS response, and the error-prone DNA polymerase PolIV. The change from one situation to the other has been described as a switch from high-fidelity repair in the exponential phase to error-prone DNA double-strand breaks during the stationary phase [22,23]. Because DSBs are lethal unless repaired, and repair action requires RecA protein [22,23], we designed an experiment to confirm this notion. We repeated the experiment of sepiolite mutagenesis with a RecA deficient strain that is impaired in the SOS response triggering. We found that *recA* gene inactivation completely abolished sepiolite mutagenesis (figure 3). Thus, the lower level of mutant frequency in the *recA* deficient strain could be explained by the death of cells that suffered DSBs and were unable to repair them. Mutations introduced by DSB repair are considered a mechanism of diversity via mutagenic repair in bacteria [24,25].

Potentially, the mutagenicity of clay treatment is also enhanced in stationary phase cells due to DNA being more tightly compacted than in the exponential phase [26]. Indeed, in *Escherichia coli*, DNA goes to a co-crystallization state with the stress-induced protein Dps offering protection to several types of stress, normally chemical damage [27]. However, crystallization is often associated with less flexibility or added fragility to direct physical contact. In contrast, less compacted DNA of proliferating *E. coli* is elastic and soft [28], which may limit the number of DSBs. It is then possible that mineral fibres under friction can break DNA strands more easily in the stationary than in the

exponential phase.

To reunite more evidence that penetration and interaction of fibres with DNA cause DSBs inside the cell, we performed a direct observation of sepiolite-treated bacteria by scanning electromicroscopy. We noticed that fibres look compatible in dimensions able to penetrate bacteria without completely destroying the envelope. Additionally, we observed bacteria directly penetrated by fibres while those that were exposed to mineral without friction were not (figure 5). This observation joined to the failure of 2-2′ bipyridyl in suppressing the mutagenic effect of sepiolite point to the mechanical action as causing agent of the damage. The notion of mechanical breaks is in good agreement with the results in cell-free systems. In these experiments, breakage of plasmid DNA was not directly associated with the amount of iron released by asbestos fibres when they are incubated together [1].

Clays (e.g. sepiolite) are jointly used with antibiotics in farming as growth promoters. This practice improves growth and animal product quality, and these additives are common in feed for broiler chickens and pigs [29,30]. Sepiolite is considered to be safe, stable and chemical inert hence; it is also used in tablet formulation for human medicine [31]. Recently, we proposed that clays used as animal feed additive can increase the risk of horizontal gene transfer (HGT) among microbes, resulting possibly in a rise of antibiotic resistance [11,32].

Sepiolite also contains very short fibres (figure S3). In the case of asbestos, there is a certainty that long fibres are much more dangerous by their carcinogenic potential. We decided to investigate if, in bacteria, the length of sepiolite fibre is relevant for mutagenesis. We prepared a suspension of short fibres (less than 1 µm). The exposure of stationary phase bacteria to this preparation did not cause any significant DNA damage if compared with the control and in contrast with the long-fibre original mineral suspension (figure 6).

Bacterial genotoxicity experiments are considered an important step in the assessment of mutagenic properties of chemicals, drugs or materials in general [33]. Because asbestos fibres resemble sepiolite ones, we decided to test if asbestos fibres provoke an increase in mutagenesis. We employed for this analysis the crocidolite asbestos (figure S4), the most the most hazardous one. In our assay, the addition of asbestos to bacteria in the plates without friction did not increase the mutant frequency. In contracts, the application of friction when the fibres were present increases the mutant frequency even more than sepiolite (figure 7), probably by the same mode of action. Yoshida *et al.* have suggested that asbestos and other clays can be potentially mutagenic base on integrity

analysis of genomic DNA from treated bacteria [34].

Based on our results and by compiling the current knowledge about asbestos-induced carcinomas, we propose the following model. People exposed to asbestos fibres inhale them during prolonged periods and fibres accumulate in the respiratory tract. The movement of the fibres into the pleural cavity is well documented [35]. The cyclical mechanical movement between the parietal pleura (covering membrane of the inner surface of the thoracic cavity) and the visceral pleura (covering membrane of the lung surface) provokes the movement of asbestos, transpassing occasionally the epithelial cell membranes and fiscally interacting with the DNA. In eukaryotic cells, double strand breaks generate chromosome aberrations or fragmentations. If this physical interaction happens in the right time, with adequate intensity, could induce DSBs. With years of continued exposure and related with other symptoms due to direct toxicity, the higher frequency of double-strand breaks increases the probability of malignancy by finding the randomly the right mutations. The proposed model for eukaryote cells would need *in vitro* testing with appropriate cell lines but this is beyond the scope of the current study and left for future research directions.

What other implications has our finding that nanofibres induce DSB in bacteria? It has been suggested that gut microbes play a crucial role in keeping species apart or enhance the speciation [36]. It is tempting to speculate that animals that use gastroliths or sediment ingestion expose their microbiota to the abrasive action of stone derivative fibres. Therefore, the shaping of their own microbes is expected to contribute to their own speciation trajectories. Among animals that use or used gastroliths in their evolutionary trajectories, we find several branches of fishes, amphibians, reptiles (including dinosaurs) and birds. Gastroliths also regularly occur in several groups of invertebrates [37]. Wings (2007) recommends making a distinction between lithophagy and geophagy. Lithophagy (stones larger than 0.063 mm in diameter) is defined as the deliberate consumption of stones that turn into gastroliths after their ingestion. Geophagy is the consumption of soil and it is known for reptiles, birds, and mammals. These soils, rich in clays, salts or fat, serve mainly as a food supplement for the supply of specific minerals or for medical purposes [37]. Both concepts can contribute to getting together all the components that this mechanism needs to operate: gut microbiota, gut mucin mucoid layer (hydro-gel) and friction forces provided by the peristaltic pressure of digestive tract in animals, especially the gizzard and the stomach.

One interesting question is why sepiolite from limestone gastroliths does not damage the animal gut. A convincing explanation is that the mucoid layer in the gut protects it from the action of these

sharp fibres at the time that serve as a protective layer for gut epithelium. It is known that in mammals, this mucoid layer is around 200 µm thick and is under continuous renovation [38]. Sepiolite is a natural clay mineral characterised by a nanofibre structure with average dimensions less than or equal to 0.2 micrometers in diameter, and from 2 to 5 micrometers in length, although longer fibres can be present.

An implication of our study is that is important to take other factors in consideration during assessing of genotoxicity by certain materials. In our case, was essential to introduce the variable of friction. Until now, many studies associate clay-induced damage mostly with oxygen reactive species [1]. DNA damage can be produced by oxidoreduction processes generated by metal containing-fibres. Asbestos fibres are carcinogenic for both, humans and experimental animals [39]. Carbon nanotubes, a novel industrial material with many applications, are another example of a potentially dangerous material. The genetic alterations provoked by these nanotubes in rat malignant mesothelioma were similar to those induced by asbestos. The nanoscale size and needle-like rigid structure of CNTs appear to be associated with their pathogenicity in mammalian cells, where carbon atoms are major components in the backbone of many biomolecules [39]. Coincidentally, carbon nanotubes can be used to transform bacteria with plasmids [40] in a similar fashion that asbestos [12,41] and sepiolite do [5,42]. It would not be surprising that all these fibreus nanomaterials share their ability to mechanically induce DSBs.

It is worthy to note that in all studies related to the assessment of carcinogenesis by fibres (mainly in asbestos), there was a key element missing: the friction forces. It is known that asbestos produces DNA breaks leading to the formation of micronucleus (a type of chromosome aberration). This kind of damage seems to be caused more by mechanical action rather that ROS generation, which can worsen the situation but not necessarily has to be determinant. A good example is that in the lung cancer caused by asbestos, the most affected part of the organ is the pleural tissue. The pleura is an area in a continuous movement that creates a strong pressure and thus seems to be a good candidate to provide the friction force that mechanically can enhance the fibre penetration of the epithelial cells, being the size of the fibre is a determinant factor.

Based on our results and by compiling the current knowledge about asbestos-induced carcinomas, we propose the following model. People exposed to asbestos fibres inhale them during prolonged periods and fibres accumulate in the respiratory tract. It is frequent to find asbestos fibres into the pleural cavity, and maybe they increase the friction coefficient in the pleural space, a parameter that

in physiological conditions has a very small value [35]. The cyclical mechanical movement between the parietal pleura (covering membrane of the inner surface of the thoracic cavity) and the visceral pleura (covering membrane of the lung surface) provoke the movement of asbestos, transpassing the epithelial cells and promoting DSBs. In eucaryotic cells, double strand breaks generate chromosome aberrations or fragmentations. With years of continued exposure and related with other symptoms due to direct toxicity, the higher frequency of double-strand breaks increases the probability of lung cancer. The proposed model would need *in vitro* validation with epithelial cells but this is beyond the scope of the current study and left for future research directions. This model does not exclude other toxic and genotoxic mechanism of asbestosis such as reactive species arising from metal action or inflammatory response. It is also possible that irruption of fibres can break some microtubules and disrupt the spindle in inside mitotic cells, something that may cause chromosome aberrations and led to carcinogenesis [43]. Its is possible that the whole pathological process is limited to a threshold of fibres in the target tissues, the low probability of introducing the right mutations and some additional factors that are in agreement with the association to chronicle exposure. Hence, it can take from 10 to 40 years for the development of symptoms or tumours after asbestos exposure. This model does not exclude other toxic and genotoxic mechanism of asbestosis such as reactive species arising from metal action contained in the fibres or inflammatory response. Indeed, if ROS is taking part, the introduction of fibres directly into to cytoplasmic compartment would be a good approach to study these interactions.

If our hypothesis is certain, the poor correlation between DNA damage *in vivo* and *in vitro* [3] may be explained by the limited or lack of penetration of asbestos in experimental designs. Thus, the introduction of some friction can be a cornerstone in determining a molecular mechanism of carcinogenic fibres. The mechanism(s) underlying asbestos toxicity associated with the pathogenesis of mesothelioma has been a challenge to unravel for more than seven decades [44]. Based on bacterial DNA damage induced by sepiolite and asbestos fibres that we observe in this study, we propose that the coelomic movement (more prominent in pleural space), with the participation of clay fibres, may account to generate sliding friction forces to allow penetration by fibres. Thus, this factor deserves to be investigated as an important parameter in fibre carcinogenesis studies *in vitro*, including the tests of genotoxicity with bacteria and human cells in culture. Although Yoshida described the ability of nanoclays to penetrate bacteria, there was a clear antecedent indicating that chrysotile (a type of asbestos) were able to transform monkey cells in culture by exogenous plasmid DNA [12]. Unfortunately, the authors did not describe in details how the incubation steps were carried out. It is logic to think that this transformation requires penetration

of the plasmatic membrane. One could believe that the penetration can account for pore generation and make the cells to burst, but not necessarily. A good example is the microinjection technique, implying necessarily membrane transpassing by microneedles or micropipettes [45], often of bigger diameter than asbestos fibres and the cells resist it.

The most important limitation of our study is the lack of an animal model to test if our finding of mutagenicity in bacteria by clays occurs *in vivo*. We theoretically anticipated that clays present in livestock feed could promote antibiotic resistance and virulence in pathogenic bacteria by their transformation ability, we extend now this possibility to the antibiotic resistance via mutations. However, testing conditions are hindered by the fact that experiments would require at least S1 security level, and this is difficult to achieve with livestock animals [32]. Another important issue is the need for a co-action of several parameters responsible for a successful introduction of mutations. Because of labour-intensity of this type of experiments, we fix only one concentration for sepiolite and asbestos as proof of principle to match the one that is described in the literature as the optimal for transformation value. Nevertheless, the transformation of plasmid DNA requires penetration and sepiolite and other clays have shown this capacity in a wide range of concentrations although it diminishes at high concentration due to the killing of bacteria [10,46–48]. We discussed in a previous article that the values of pressure in the gut of many animal species, meeting the criteria very well [32]. The presence of an hydrogel does not seem to be a problem since both mucin layer of the gut or mucoid secretion in the respiratory tract can play that role, particularly if fibres have the capacity to change viscosity locally or gradients of viscosity exist across these body compartments.

There is the recent debate about a possible link between talcum powder and ovarian cancer risk associated with asbestos contamination in talc. Although the risk is small, some studies are indicating a low or moderate but significant chance of cancer, while other consider that there is not [49–51]. This debate points that is necessary to advance the understanding of molecular base of DNA damage by asbestos and other industrial fibres. If other studies confirm our proposal of mechanical/physical DNA breaks, it is possible to suggest that some genotoxicity assays intended to unveil mutagenic properties of materials, such as the test of Ames, should be modified accordingly to include a standardised procedure of friction or promoting some sort of shaking during incubation steps. Similarly, several *in vitro* test, with both bacteria and eukaryotic cells, were modified by researchers and regulatory agencies where introduced the metabolic activation by fraction S9 of liver homogenate [52].

Overall, one of the most significant contributions of this article is that provide for the first time a bacterial model to test genotoxicity of nanofibers and uncover a new mechanism of action for asbestos that correlates better with *in vivo* observations. Asbestosis is a world health and environmental problem, which molecular basis has been a challenge during several decades [44]. One more observation in favour of our mechanism is that although asbestos fibres are widely distributed in the anatomy of patients [44,53], the most common cancers caused by asbestos originate in lungs (mostly mesothelioma). If the most explored mechanism of action is based on reactive radicals (chemical damage), why is not there big differences in the frequencies of other types of carcinoma such us leukaemia, lymphoma or digestive track system among exposed populations? In the last place, and not less important, is the tighter contact of slippery membranes (a monolayer of flattened epithelial-like cells) of the mesothelium. This area, the pleural space, is in continuous movement and constitute preferential target of asbestos-induced carcinogenesis. Of particular interest are free-floating mesothelial cells of the cavity, that even proliferate under damaging conditions [54]. The free-floating cells are the ideal candidates to be penetrated by asbestos in the pleural space. They may be more sensitive to suffer direct (physical) or indirect (chemical) DNA damage and become into a mesothelioma. If the mechanical penetration due to coelemic movement is going to be tested *in vitro*; a cell tissue culture system should be designed in order to reflect closely the situation of pleural space including floating cells. We suggest an experimental design as starting point for this type of experiment (supplementary figure S5). It is clear that the current systems to assess carcinogenicity risk with nonofibres (asbestos, carbon nan-tubes, silicates, glass fibres etc) has to be modified because the current set-ups fail most of the time in detection of damage [4].

Finally, it did not escape to our attention that sepiolite transformation technique gained some popularity in the last years because there is no need to prepare competence cells [7,10,42,55], diverse bacteria can be transformed [32] in both stationary and exponentially growing bacteria are equally transformable. However, to prevent undesired mutations in both, plasmid and genomic DNA, we recommend using exponential phase bacteria, where mutagenesis is not significant.

Methods

Bacteria and growth conditions. The *E. coli* MG1655 wild-type strain and its derivative mutants were cultured in Lysogenic Broth (LB). All experiments were performed at 37°C, with shaking in liquid culture. All solid cultures were grown in LB agar 1.5% for standard procedures and 2% for the sepiolite treatment. All cultures were supplemented with antibiotic when appropriate.

Mutant frequency estimation of sepiolite treated cells. Approximately 2×10^9 bacterial cells per ml of *E. coli* MG1655 and its derivative mutants from overnight or mid-exponential growing cultures were centrifuged and resuspended in 100 µl of sterilized transformation mixture, consisting of sepiolite (Kremer Pigmente, Germany) suspended in aqueous solution at a final concentration of 0.1 mg/ml. Resuspended cells were spread on plates containing fresh Müller-Hinton-Agar (Sigma-Aldrich, Germany) medium solidified with 2% agar, and Petri dishes were pre-dried in a biological safety flow cabinet for 20 minutes before use. Friction force was provided by streaking bacterial cultures plus sepiolite with sterile glass stir sticks gently pressed onto the medium surface for 1, 2 and 3 minutes, applying as much pressure as possible without breaking the agar gel. Petri dishes were incubated at 37°C for 2 hours to allow DNA repair if any damage occurred. The plates were washed gently four times with 5 ml of 0.9% sodium chloride solution using a 5 ml pipette and the bacterial suspensions were transferred to 10 ml tubes to recover the cells by centrifugation at 3000 g for 10 minutes. The resulting pellets were resuspended in a final volume of 1 ml of fresh LB an incubate during 1 hour at 37°C to allow the cells to recover. Appropriate dilutions were plated onto LB plates to estimate bacterial viability and in LB plus fosfomycin (50 µg/ml) to estimate the number of resistant mutants. Plates were incubated overnight at 37°C. Each experiment consisted of 5 replicates and was repeated at twice. Mutant frequencies were calculated by using the on-line web-tool Falcor [56].

Mutant frequency estimation of asbestos treated cells. The procedure was carried out identically that the one described for sepiolite in this section. The time was set to 2 minutes and the same concentration that was used, 0.1 mg/ml. We used the crocidolite asbestos analytical standard (SPI Supplies, USA). The asbestos fibres in distilled water were autoclaved and sonicated in bath during 10 minutes before use to render an homogeneous suspension.

Long fibre-depleted sepiolite. To assess the role of long fibre of sepiolite in mutagenesis, we obtained a sepiolite preparation depleted of fibres longer than 1 µm. A 100 ml sepiolite suspension

(1 mg/ml) in distilled water was passed though Pall® Acrodisc® glass fibre syringe filters (Sigma, USA) several times. The resulting suspension was desiccated by evaporation at 70°C overnight. A non-filtered solution was used as a control. From the obtained powder, two suspensions were prepared to a final proportion of 0.1 mg/ml. These two solutions were used for a mutagenesis experiment plating in fosfomycin as indicated previously, using a friction time of two minutes.

Influence of 2-2′ bipyridyl on sepiolite mutagenesis. The effect of 2-2′ bipyridyl, a metal chelating agent [57], on sepiolite mutagenesis was determined by measuring its influence on the mutant frequency for a selected concentration of sepiolite, where mutagenesis was observed. The experiment consisted of adding a titrating concentration of 2-2′ bipyridyl (200 µM) to chelate metals 5 minutes before the treatment. Cultures treated with sepiolite and friction without the addition of 2-2′ bipyridyl and bacteria alone without sepiolite were used as a control. The mutant frequencies for these groups were determined as described elsewhere in this section.

Assessing double-strand breaks with a plasmid system. To evaluate if sepiolite under friction treatment induces double-strand breaks in plasmid DNA, the strain *Escherichia coli* DH5 alpha (fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17) carrying the plasmid pET-19b (Novagen, Germany) was treated with sepiolite and sliding friction forces during one minute. Several samples were recovered from the plates and pooled to compensate viability losses due to friction. The recovery was done by washing the surface with 5 ml 0.9 % NaCl saline solution four times as described for mutagenesis experiments. The recovered pellets were washed with 1 ml of TE buffer and the OD_{600} adjusted to 1 for each type of sample. Plasmid DNA samples were extracted using a Qiagen mini plasmid extraction kit (Qiagen, Germany). Added sepiolite with or without friction and no sepiolite groups were used as a control group. Each experiment consisted of five replicates. The same amount of plasmid DNA per replicate was applied per well to an agarose gel that was stained with SYBR® Gold Nucleic Acid Gel Stain kit (Molecular Probes, USA). A NdeI (Promega, USA) digested aliquot of pET-19b was used as control of linear migration rate. The proportion of linear molecules of the plasmid were compared among groups using a densitometry analysis by ImageJ [58].

RecA deficient strain construction. The recA null mutant was constructed following the protocol previously described [59]. Briefly, the following primers were used to amplify the chloramphenicol cassette: 5'-CAGAACATATTGACTATCCGGTATTACCCGGCATGACAGGAGTAAAAATGGT-GTAGGCTGGAGCTGCTTC-3' and 5´-ATGCGACCCTTGTGTATCAAACAAGACGATTAA-

AAATCTTCGTTAGTTTCATGGGAATTAGCCATGGTCC-3' (forward and reverse respectively) from pKD3 donor plasmid. Underlined sequences represent the pairing sequence to the plasmid, while the remaining parts belong to the homologous upstream and downstream regions of the *recA* gene. The the mutant was checked by PCR amplification using the primers c1 5'-TTATACGCAAGGCGACAAGG-3' and c2 5'-GATCTTCCGTCACAGGTAGG-3' in combination with specific primers for upstream and downstream regions of *recA* gene: 5'-ATTGCAGACCTTGTGGCAAC-3' and 5'-CGATCCAACAGGCGAGCATAT-3' respectively. Additionally, we tested phenotypically the increased susceptibility to UV light and mitomycin C in comparison to the parental strain. The antibiotic resistance gene was eliminated using the pCP20 plasmid as described previously [59].

Scanning electron microscopy of *E. coli* treated with sepiolite. Approximately $2x10^9$ cfu of stationary phase *E. coli* MG1655 were treated with sepiolite and friction force was applied for one minute as described for the mutagenesis experiment. Circular agar blocks were taken from agar plates with a sterile cork borer (1 cm of diameter). Then, a thin surface layer was cut off, placed on a circular glass coverslip (1.5 cm of diameter) and incubated for 45 minutes at room temperature in a laminar flow cabinet to allow air drying of the samples. The cover glasses with dehydrated agar sections were mounted on aluminium stubs using double-sided adhesive tape and coated with gold in a sputter coater (SCD-040; Balzers, Union, Liechtenstein). The specimens were examined with a FEI Quanta 200 scanning electron microscope (FEI Co., Hillsboro, OR) operating at an accelerating voltage of 15 kV under high vacuum mode at different magnifications. At least 5 sections from independent plates were observed to check physical penetration by the mineral. Some samples of sepiolite or asbestos (crocidotiles) alone were processed and observed in the same way.

Statistical analysis. To compare experimental groups, Kuskal-Wallis test or One-way ANOVA test. In case of case of significance were used followed by Bonferroni-corrected one-tailed Mann-Whitney U test or Tukey HSD Test respectively. P values less than or equal to 0.05, after correction if needed, were considered statistically significant. All tests were performed with the statistic software R [60].

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Figure Legends

Figure 1. Changes in mutant frequency induced by sepiolite treatment in *E. coli* MG1655 stationary phase cells. The friction force is exerted by a glass spatula over agar surface spreading the mixture of sepiolite and bacteria during 1, 2 and 3 minutes. The increase of mutant frequency after friction treatment with sepiolite supports the notion that clays can be mutagenic for bacteria in this situation. Plotted values correspond to the median of five replications while error bars represent the interquartile ranges.

Figure 2. Box plotting of the survival of *E. coli* MG1655 to the action of friction with sepiolite during 1, 2 and 3 minutes of treatment as described in Material and Methods section. Groups with and without sepiolite gently spread with glass beads onto agar plates were used as controls. Note how as spreading time (time of friction) is increased, viability decreases. Stationary phase cells seem to be more resistant to the killing.

Figure 3. Plasmid pET-19b extracted from sepiolite-treated E. coli DH5α, a recA deficient strain, during one minute (five extractions per treatment). Note the enrichment in linearised plasmid DNA molecules from bacteria treated with sepiolite under two minutes of friction applied in 1% agarose gel (A). The plasmid pET-19b digested with a single cut site enzyme NdeI was used as a control for the linear molecule migration rate and as a reference to calculate relative intensities using a densitometry analysis with the gel tool of ImageJ (B).

Figure 4. Inactivation of the *recA* gene suppresses the mutagenic effect of sepiolite under friction in E. coli MG1655. This indicates that the mutagenic effect is due to double-strand break repair of DNA that depends on RecA protein. Sepio 2' WT and Sepio 2' *recA* represent a treatment with sepiolite during two minutes for the WT and its derivative recA mutant respectively. Plotted values correspond to the median while error bars represent the interquartile ranges.

Figure 5. Samples of stationary phase *E. coli* MG1655 treated with sepiolite were prepared for a scanning electro microscopy examination. Note the dimensions of bacteria compared to the fibres and the penetration by sepiolite fibres when friction is applied. Red arrows represent potential sites of sepiolite fibre penetration. I contrast, this kind of interaction were not observed in samples that were friction was not applied.

Figure 6. Removal of sepiolite fibres longer than 1 µm decreases fibre-induced mutagenesis to the level of the control. Filtered, dry and reconstituted sepiolite (in the graph long fibre-depleted sepiolite, lf-depleted sepiolite) was used in a mutagenesis experiment. Dry and reconstituted sepiolite (normal sepiolite) and bacterial cells (labelled as control) with no sepiolite were used to compare the effects of long fibre removal. The application of friction force was set in two minutes. Plotted values correspond to the median while error bars represent the interquartile ranges.

Figure 7. Asbestos (crocidolite fibres) show similar mutagenenic properties that sepiolite, with an increase in mutant frequency around one order of magnitude bigger than control or asbestos without friction. Crosidolites, one of the most hazardous asbestos, contains high level of iron but the addition the chelating agent as 2-2' bipyridyl did not significantly decrease the mutagenesis indicating that at least in stationary phase bacteria, metals are not responsible for mutations. Values correspond to the median of five replications while error bars represent the interquartile ranges.

Figure S1. Treatment sepiolite and friction do not increase mutant frequency when E. coli MG1655 growths exponentially. The lack of mutagenesis can be explained because double strand breaks are not mutagenic because in this phase [61]. Plotted values correspond to the median of five replications while error bars represent the interquartile ranges.

Figure S2. Although sepiolite and other minerals contain metals such as iron or aluminum, the addition of a chelating agent as 2-2' bipyridyl does not significantly suppress or diminish the mutagenic effect of sepiolite. Note also that in stationary phase bacteria, there is a high level of proteins such as DPS intended to control free iron inside the cell.

Figure S3. Direct observation of sepiolite fibres by scanning electro microscopy with two different magnifications. Note the heterogeneity in sizes, the fibrous nature and the small diameter compatible with the penetration capability.

Figure S4. Scanning electro microscopy of asbestos fibres used in mutagenesis experiment with two different magnifications. In comparison with sepiolite, asbestos (crocidotile in this case) show longer and sharper fibres.

Figure S5. A proposed approach to testing genotoxicity of asbestos or any industrial nano-fibres. The design is intended to resemble physiological conditions of the pleural cavity, one of the most frequently affected tissue by asbestosis. PDMS sheets can be colonised by human or animal cell lines (ideally mesothelial or epithelial cells) in appropriate conditions. For the experiments, two sheets of colonised PDMS pieces can be attached with both cell monolayers facing each other forming a 'sandwich', place it in a tissue culture dish (containing culture medium or ideally pleural liquid) and incubate with the desired amount of fibres. The same procedure without fibres can be used to generate a control group. The PDMS sheets can be removed at a given time, and both, attached and free cells can be analysed using different genotoxicity tests such as the formation micronucleus or other types of chromosome aberration. PDSM offer the advantage that is chemically inert, gas permeable and the surface can be easily modify or treated to meet the required properties for cell adhesion and growth. This system could be useful to study cytotoxicity of fibres in vitro.

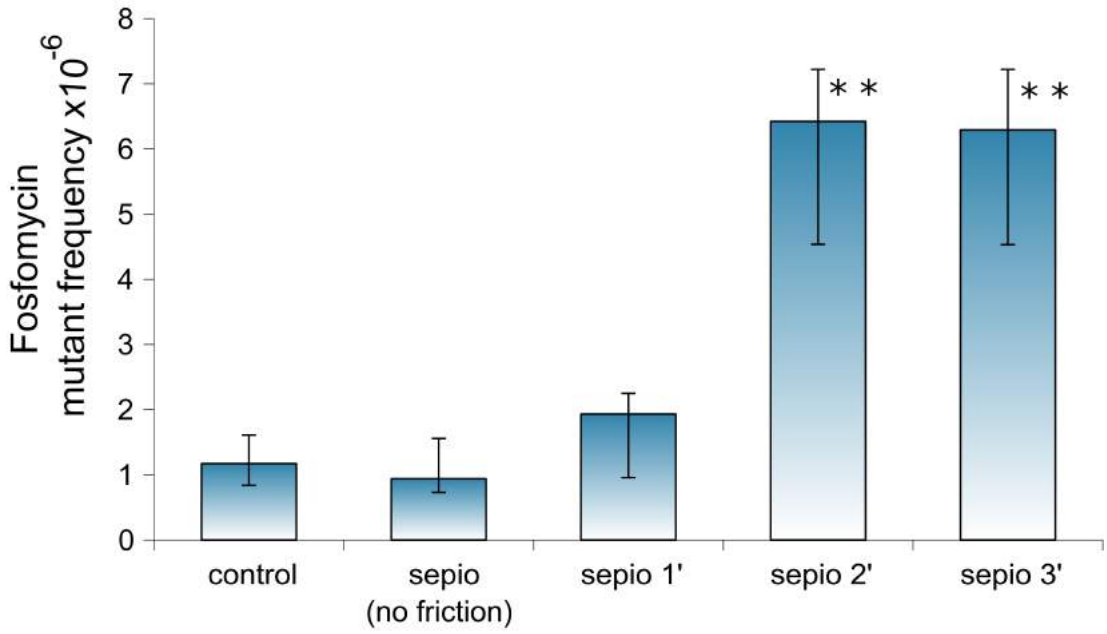


Figure 1.

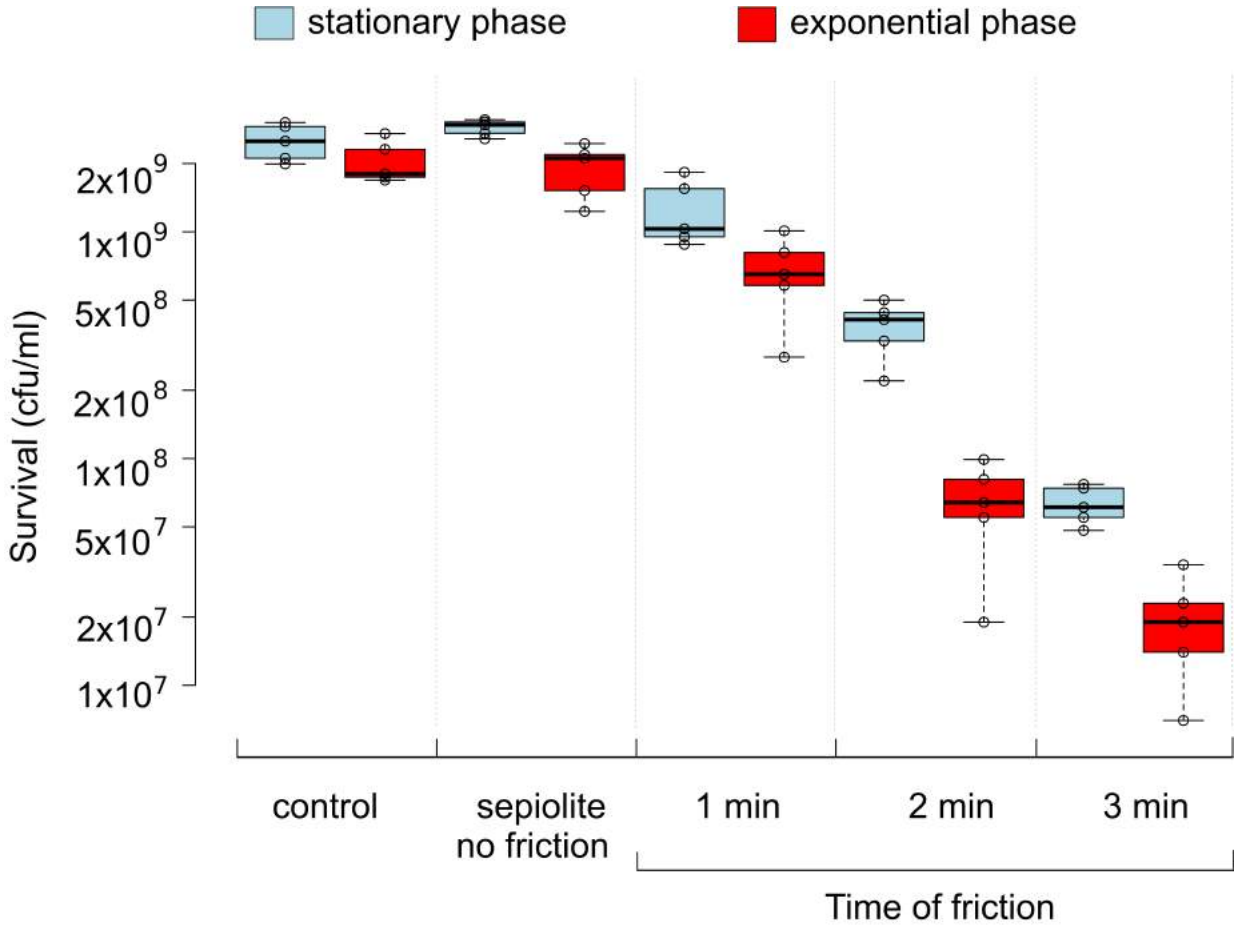


Figure 2.

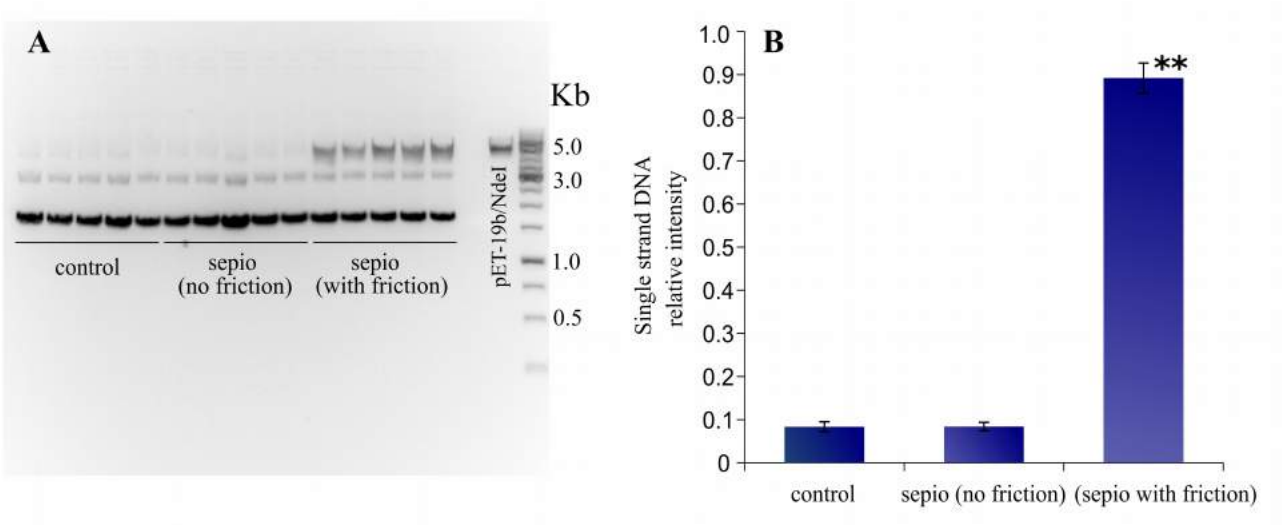


Figure 3.

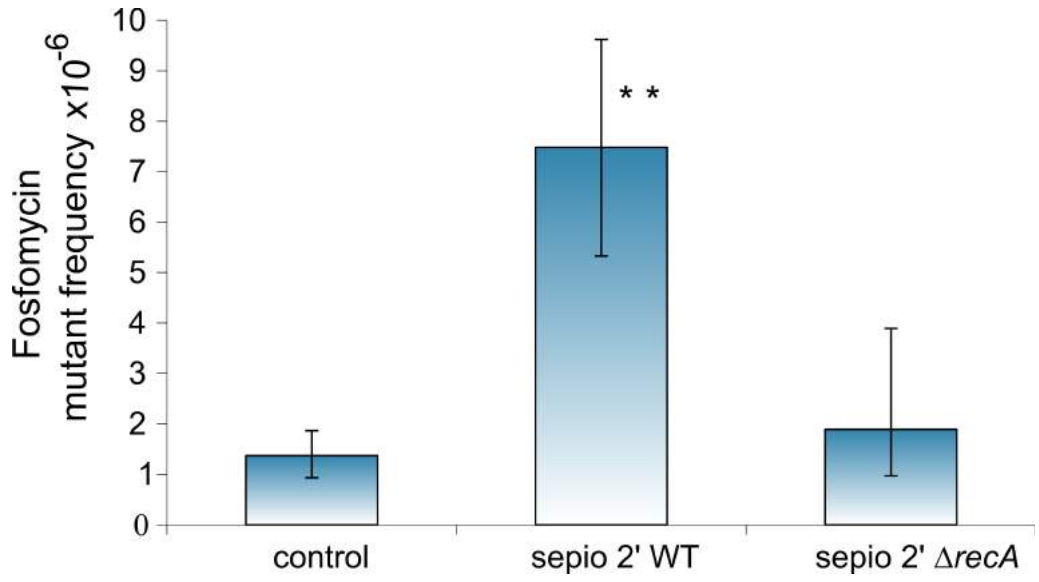


Figure 4.

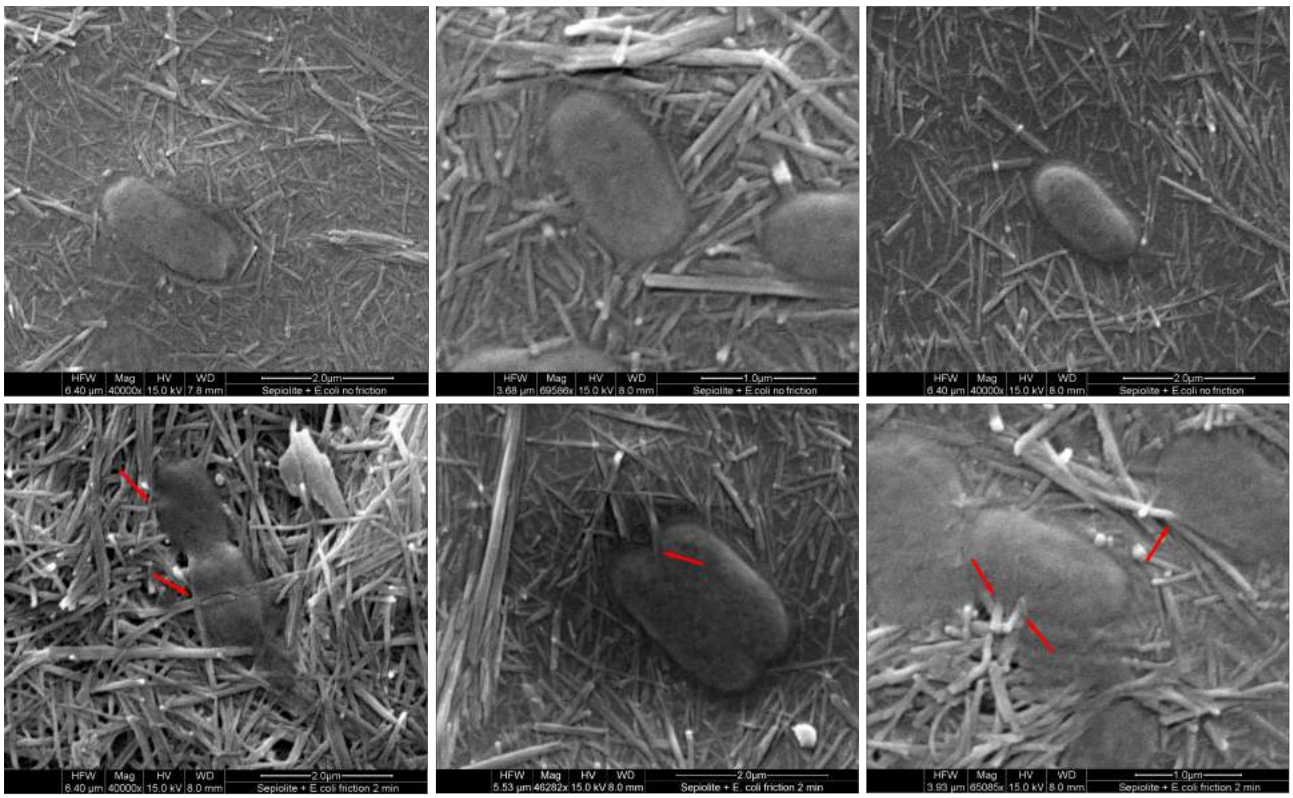


Figure 5.

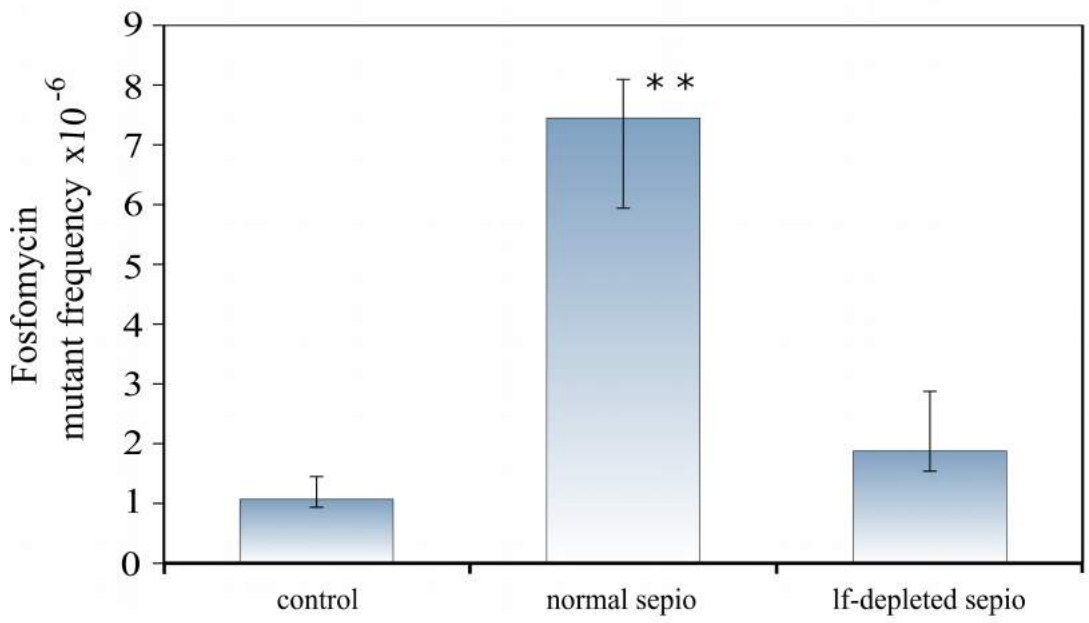


Figure 6.

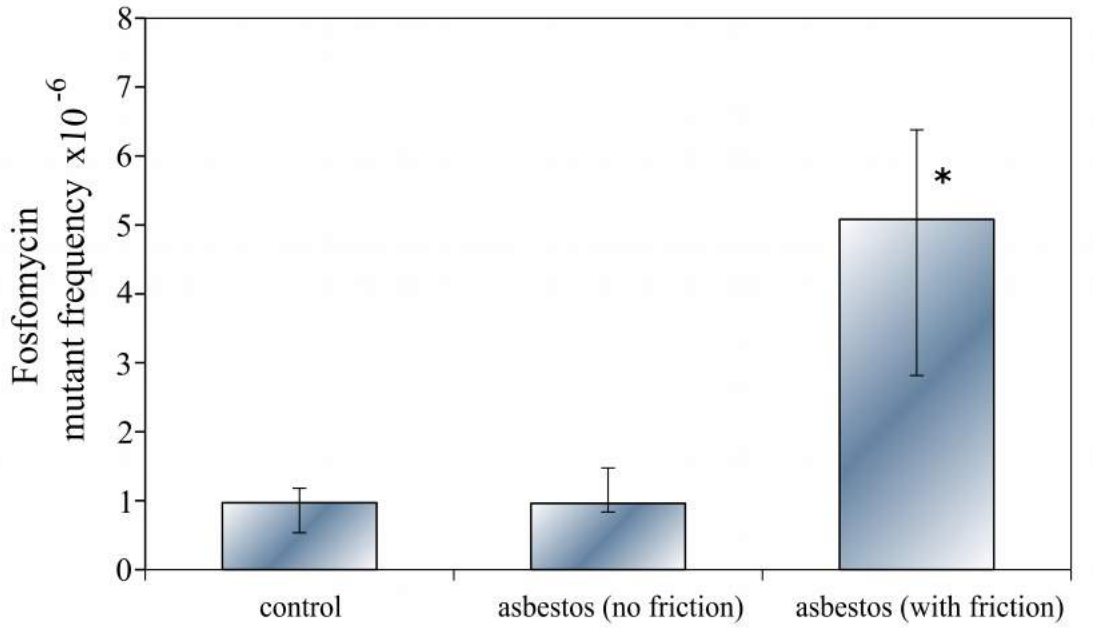


Figure 7.

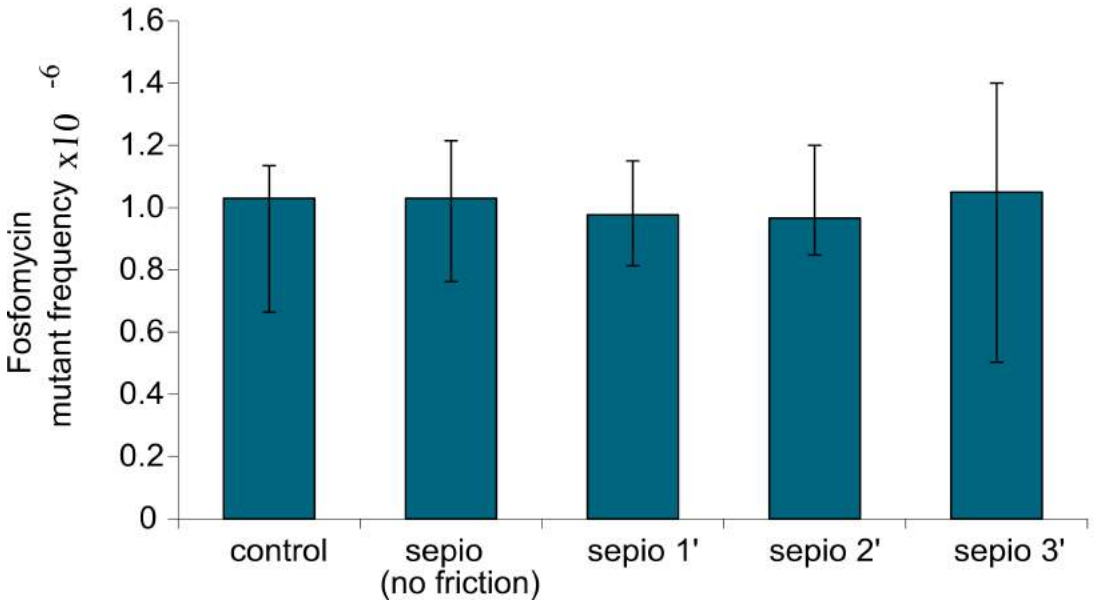
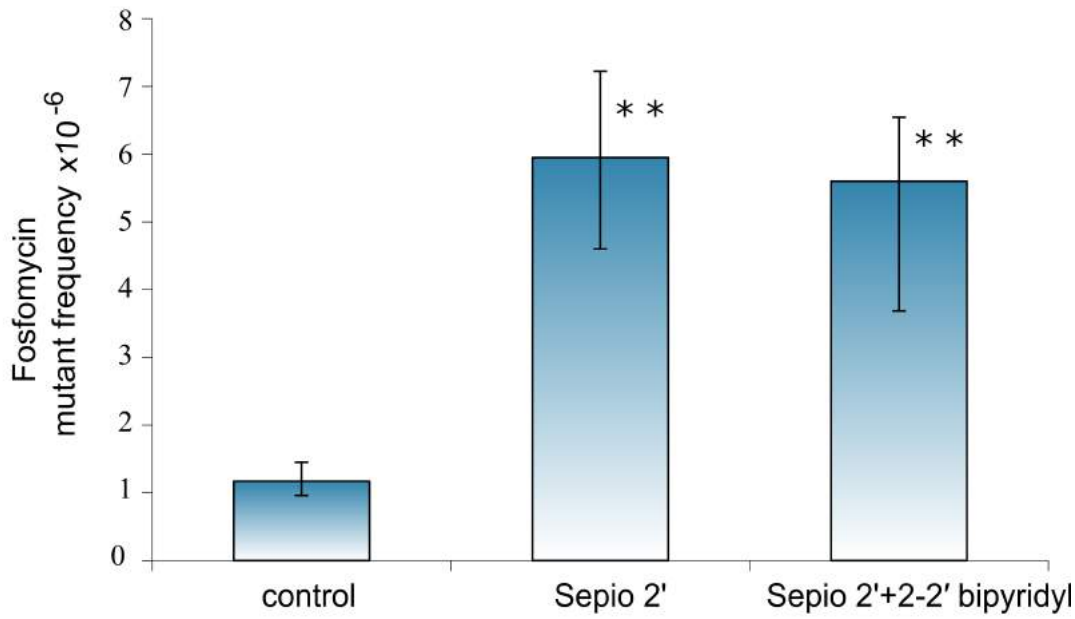


Figure S1.



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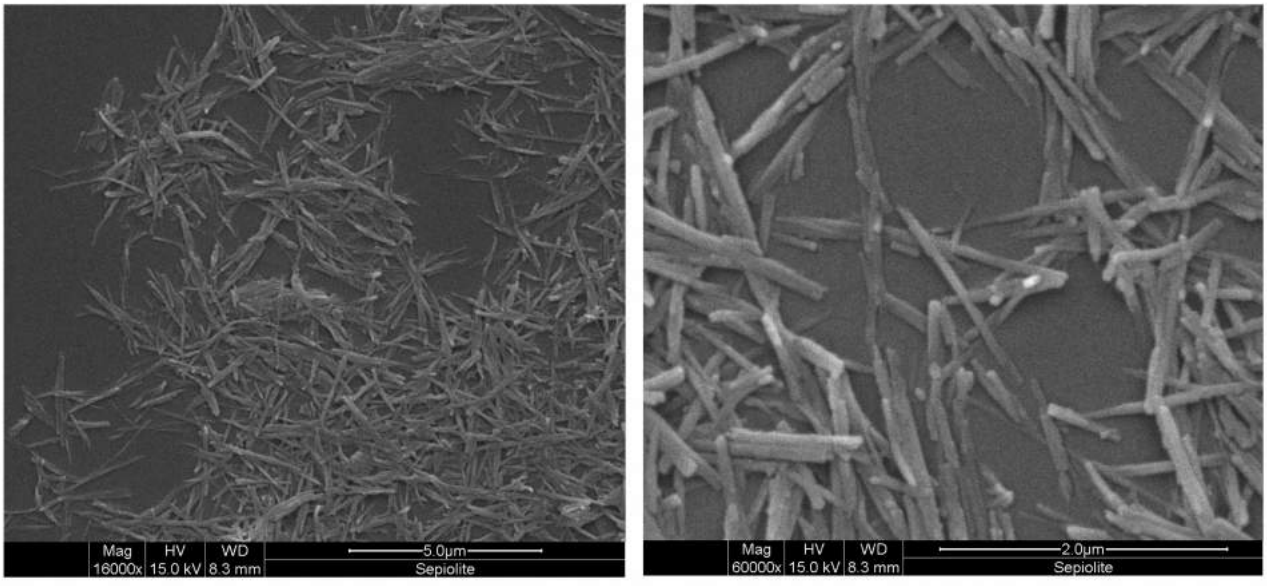

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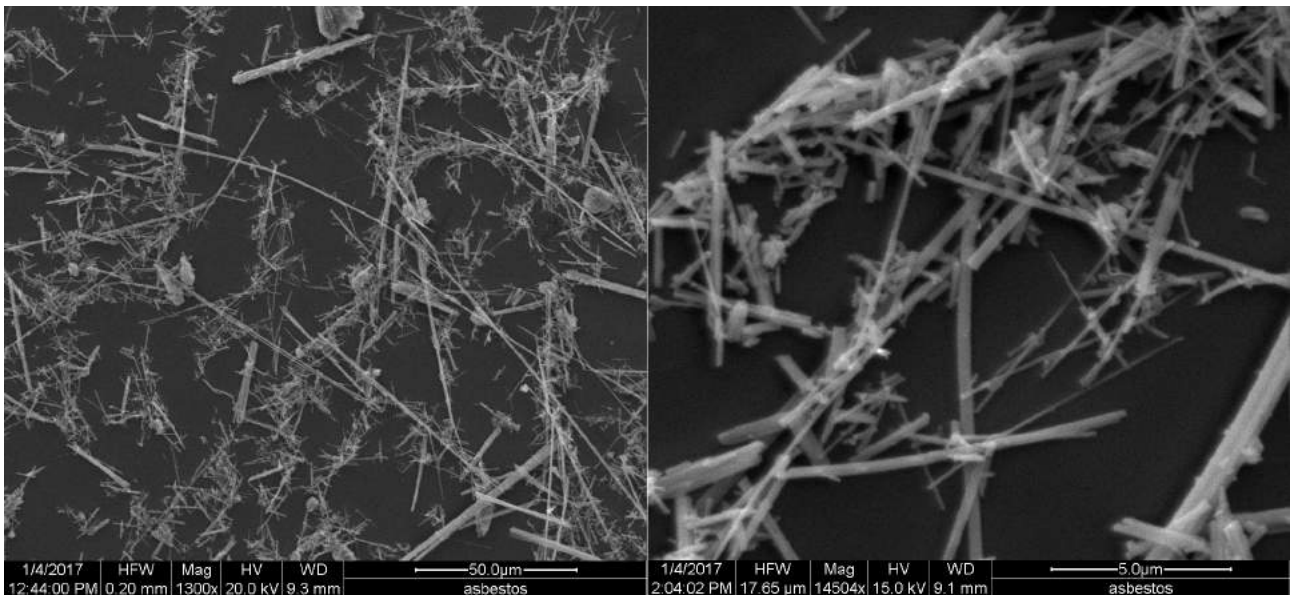

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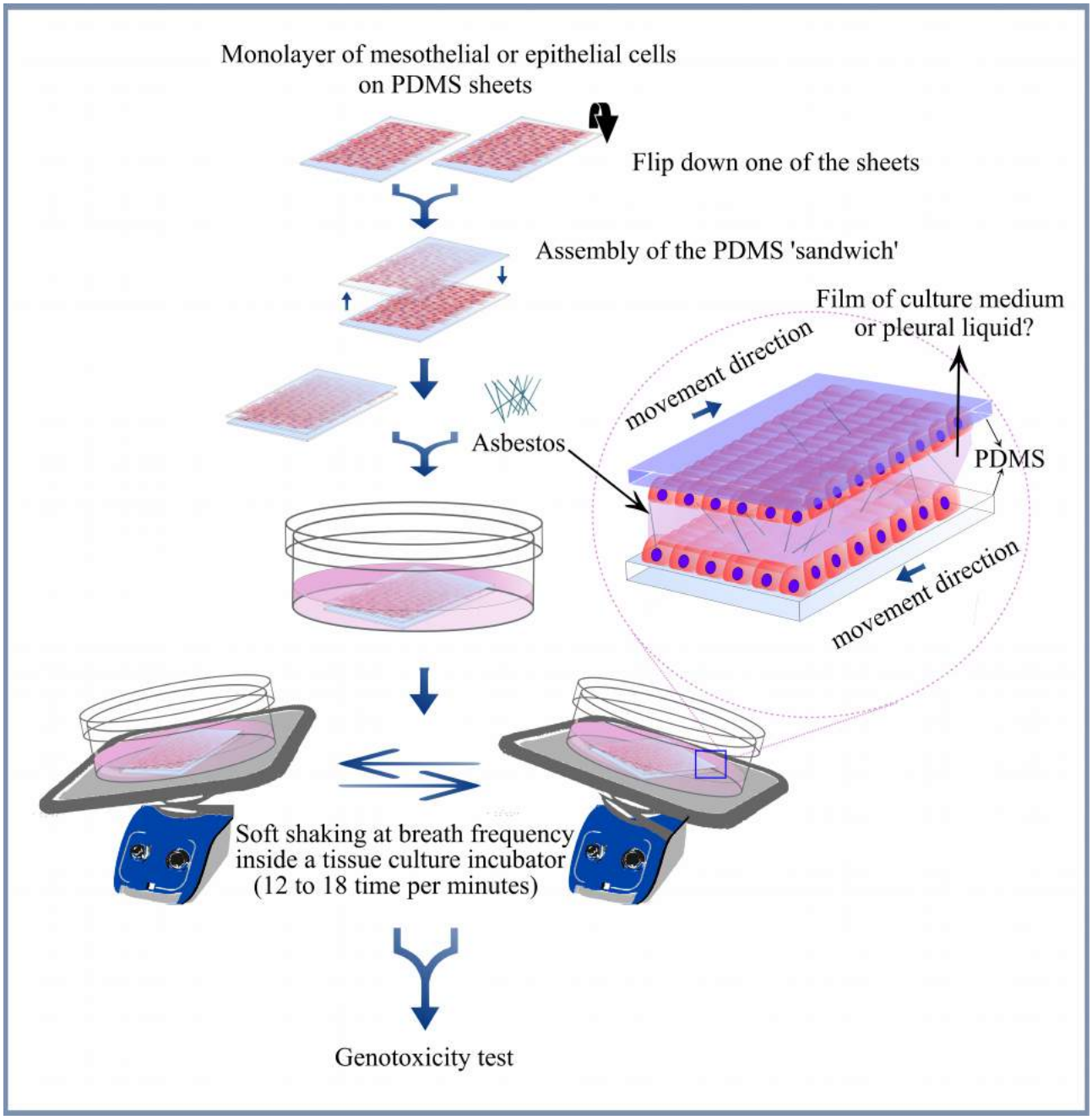

Figure S5.