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1 **Clay-induced DNA double-strand breaks underlay genetic diversity, antibiotic resistance and**
2 **could be a molecular basis for asbestos-induced cancer**
3
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16
17 **Abstract**
18
19 Some natural clays and synthetic nanofibres present in the environment have a severe impact on
20 human health. After several decades of research, the molecular mechanism of how asbestos induce
21 cancers is not well understood. Different fibres, including asbestos, can penetrate the membrane and
22 introduce DNA in both, bacterial and eukaryotic cells. Incubating Escherichia coli with sepiolite, a
23 clayey material, and asbestos under friction forces, both fibres cause double-strand breaks in
24 bacteria. Since antibiotics and clays are used together in animal husbandry, the mutagenic effect of
25 these fibres might constitute a pathway to antibiotic resistance due to the friction provided by
26 peristalsis of the gut from farm animals in addition to the previously proposed horizontal gene
27 transfer. Moreover, we raise the possibility that the same mechanism could generate bacteria
28 diversity in natural scenarios with a role in the evolution of species. Finally, we provide a new
29 model on how asbestos may promote mutagenesis and cancer based on the observed mechanical
30 genotoxicity.
31
32 **Keywords**: sepiolite, mutagenesis, antibiotic resistance, double-strand break, microbiota evolution,
33 genetic diversity, asbestos, asbestosis, carcinogenesis
34

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35 **Introduction**

36

37 Clays such as sepiolite are jointly used with antibiotics in farming as growth promoters. This
38 practice improves growth and animal product quality, and these additives are common in feed for
39 broiler chickens and pigs [1,2]. Sepiolite is considered to be safe, stable and chemical inert hence
40 being also used in tablet formulation for human medicine [3]. However, in a recent study clays used
41 as animal feed additive can increase the risk of horizontal gene transfer (HGT) among microbes,
42 resulting possibly in a rise of antibiotic resistance [4,5].

43

44 In this case, the transformation of bacteria by foreign DNA can be achieved when clay fibres are
45 spread by friction or vibrations. This phenomenon is known as Yoshida effect [6] and relies on the
46 ability of mineral nanofibres or nano-needles to adsorb DNA and to penetrate bacterial cells under
47 sliding friction forces [7]. By its mechanical nature, the Yoshida effect can be used to transform
48 diverse bacterial species [5,8,9]. The action of sepiolite and other clays fibres is not only capable of
49 delivering DNA into the receptor bacteria but also able to promote the releasing of DNA by
50 disrupting the cell envelope of the portion of the population by the abrasive action of clays [5].

51

52 Before Yoshida began his experiments with bacteria, the ability of asbestos to transform eukaryotic
53 cells was reported at the end of the eighties [10]. In fact, fibrous clays and industrial nanofibres are
54 considered genotoxic and carcinogenic, likely due to their ability to damage DNA [11]. They have
55 assayed in several experimental models including bacteria and cell in cultures, but they display a
56 poor correlation with mutagenicity or carcinogenesis found *in vivo* [12,13]. According to these
57 observations, a significant concern arises from fibrous clays or industrial nanofibres which are
58 responsible for severe human diseases such as asbestosis [14]. However, short or long periods of
59 exposure to fibres have been failing to identify a molecular basis of DNA damage in different
60 several genotoxicity tests [14]. Thus, nowadays the mechanisms underlying the genotoxicity and
61 carcinogenicity of asbestos and other fibres remain obscure.

62

63 Additionally, clays may have the potential to enhance antibiotic resistance in farming activities [4].
64 In natural scenarios, sediments and stones (gastroliths) are frequently swallowed by animals
65 resulting unavoidably in the exposure of their microbiota to pebbles, sand, and clays. Soils and
66 waters are a primary source of antimicrobials, either by natural microbial production or
67 environmental antibiotic pollution, a major selective pressure that favours resistant strains [15,16].
68 Even, gut microbes can produce antibiotic compounds [17].

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69 In this study, the ability of fibrous clays such as sepiolite and asbestos to transform bacteria and to
70 induce mutagenic DNA double-strand breaks (DSBs) when they are exposed to friction forces was
71 experimentally shown. Additionally, a molecular mechanism of action for asbestos, which was a
72 strong inducer of DSBs in *Escherichia coli* when friction is present, was proposed. Finally, the
73 importance of this mechanism is discussed for the speciation processes of animals that use
74 gastroliths.

75

76 **Results and Discussion**

77

78 **Mutant frequency in sepiolite-treated cells is higher than in non sepiolite-treated cells.**
79 Different types of clays can transform bacteria by absorbing DNA and penetrating the cell
80 envelope. In that case, the penetration could allow the clays to interact with the intracellular DNA
81 and promote mutations. To test whether sepiolite under friction forces (as in transformation) has an
82 impact on bacterial mutation rate, the mutant frequency of *Escherichia coli* was measured by
83 plating in the antibiotic fosfomycin and enumerating spontaneous mutants (fig 1). When the cells
84 were merely exposed to sepiolite without any friction on agar plates surface, no significant
85 differences in mutant frequencies were detected (Mann-Whitney U test; $P=0.999$). In contrast, a six-
86 fold increase in mutant frequency was found when friction was present for two or three minutes
87 (Mann-Whitney U test; $P=0.008$) and a modest increase—but not significant—when the treatment
88 time lasted for one minute (Mann-Whitney U test; $P=0.421$). Interestingly, only cells in the
89 stationary phase displayed an increase in mutant frequency (Kruskal-Wallis test; $P=0.001$), while no
90 significant mutagenesis was found when bacteria came from exponential cultures (Kruskal-Wallis
91 test; $P=0.954$; fig 1). Along with the mutant frequency experiments, the effect of the treatments on
92 cell viability was checked. Sensitivity to the treatment is higher in exponential phase than in
93 stationary phase cultures (fig 2). The observed mutant frequency differences was initially attributed
94 to a higher sensitivity to the treatment. However, several hypotheses can explain the observed
95 increase in mutant frequency and reduced-sensitivity in stationary phase cultures.

96

97 **Heavy metals are non relevant on sepiolite mutagenesis.** Many minerals containing metals such
98 as iron, aluminium or copper are toxic for bacteria because of the generation of reactive oxygen
99 species (ROS) via the Fenton reaction [18]. The release of metal ions inside the cell could therefore
100 be the reason of the increase in mutagenesis. In fact, despite the addition of 2-2′ bipyridyl, a
101 chelating agent, shortly before treatment, mutagenesis was still observed (Kruskal-Wallis test;

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102 P=0.008; fig S1). This result indicates that the mutagenic effect does not depend on the metals
103 present in the fibres.
104
105 **Sepiolite interacts with the DNA by causing double-strand breaks.** A second likely explanation
106 is the physical interaction of individual clay fibres in motion directly damaging DNA by creating
107 DSBs. The ability of sepiolite fibres to penetrate and interact with DNA has already been stated
108 [6,19]. Physical or mechanical stress on the DNA duplex is a relevant cause of DSBs [20]. To
109 evaluate this possibility, *E. coli* DH5α strain (*recA* deficient) carrying the plasmid (pET-19b) was
110 subjected to treatment with sepiolite and sliding friction. Sepiolite without friction and bacterial
111 cells alone were used as controls. The plasmid content was extracted, and its integrity was evaluated
112 by gel migration (fig 3A). Typically, during plasmid DNA extraction, three molecular
113 conformations are found: the supercoiled (which migrates very fast), nicked DNA (which is also
114 closed circular but relaxed due to single strand breaks and it has an intermediate migration rate) and
115 linear molecules (with a lower migration speed) [21]. These latter DNA molecules were especially
116 abundant in the friction-sepiolite treated group at the time that they are present in a low level in
117 control groups. In fact, plasmids from the sepiolite group (under friction) presented a significantly
118 high level of linearised molecules when compared to the control groups (One-Way ANOVA test;
119 $P=3.33\times10^{-16}$). According to these results, the joint action of sepiolite and friction are responsible
120 for induction of DSBs in the DNA. Interestingly, no increase in nicked DNA (single-strand break)
121 was observed, indicating that if this type of lesion occurs, it happens at a non-detectable rate by this
122 technique (fig 3B).
123
124 The view of mutagenic DSBs by mechanical shearing is very consistent with the absence of
125 mutagenic effect in exponentially growing bacteria. If the organism is diploid (even if the diploidy
126 is only transient, as in replicating bacteria or replicating haploid yeast), then homology-directed
127 repair can be used [20]. Because *E. coli* lacks a pathway to join non-homologous ends, homologous
128 recombination is the only mechanism to salvage broken chromosomes [22]. But how can *E. coli*
129 repair DSBs in stationary phase by homologous recombination? Stationary-phase cultures contain
130 cells with several chromosome copies [23]. In exponentially growing *E. coli* DSB repair is non-
131 mutagenic [24,25]. However, break repair becomes mutagenic during the stationary phase and
132 requires the Sigma S factor (RpoS), the SOS response, and the error-prone DNA polymerase PolIV.
133 The change from one situation to the other has been described as a switch from high-fidelity repair
134 in the exponential phase to error-prone DNA double-strand breaks during the stationary phase
135 [24,25]. Because DSBs are lethal unless repaired, and repair action requires RecA protein [24,25],

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136 the experiment of sepiolite mutagenesis was repeated with *E. coli* DH5α that is impaired in the SOS
137 response triggering to confirm this notion. In such analysis, sepiolite mutagenesis was completely
138 abolished by *recA* gene inactivation in stationary phase (Kruskal-Wallis test; P=0.011; fig 4). Thus,
139 the lower level of mutant frequency in the *recA* deficient strain could be explained by the death of
140 cells that suffered DSBs and were unable to repair them. Mutations introduced by DSB repair are
141 considered a mechanism of diversity via mutagenic repair in bacteria [26,27].
142
143 Potentially, the mutagenicity of clay treatment is also enhanced in stationary phase cells due to
144 DNA being more tightly compacted than in the exponential phase [28]. Indeed, in *Escherichia coli*,
145 DNA goes to a co-crystallization state with the stress-induced protein Dps offering protection to
146 several types of stress, ordinarily chemical damage [29]. However, while crystallization is often
147 associated with less flexibility or added fragility to direct physical contact, less compacted DNA of
148 proliferating *E. coli* is elastic and soft [30], which may limit the number of DSBs. It is then possible
149 that mineral fibres under friction can break DNA strands more easily in the stationary than in the
150 exponential phase.
151
152 **Sepiolite fibres can penetrate bacteria when friction forces are present.** To reunite more
153 evidence that penetration and interaction of fibres with DNA cause DSBs inside the cell, a direct
154 observation of sepiolite-treated bacteria by scanning electron microscopy (SEM) was performed.
155 Fibres look compatible in dimensions able to penetrate bacteria without completely destroying the
156 envelope. Additionally, bacteria were directly penetrated by fibres while those that were exposed to
157 mineral without friction were not (fig 5). This observation is in concordance with previous studies,
158 whereas sepiolite and other nano-sized acicular materials can penetrate bacterial cells under friction
159 forces on a hydrogel [6]. In fact, the partial destruction of the cell wall and the presence of mutants
160 after adding 2-2′ bipyridyl point to the mechanical action as causing agent of the damage. The
161 notion of mechanical breaks is in good agreement with the results in cell-free systems. In these
162 experiments, breakage of plasmid DNA was not directly associated with the amount of iron released
163 by asbestos fibres when they are incubated together [14].
164
165 **Sepiolite fibre length matters to cause significant DNA damage in the cell.** Sepiolite also
166 contains very short fibres (fig S2). In the case of asbestos, there is a certainty that long fibres are
167 much more dangerous by their carcinogenic potential. We designed an experiment to test the
168 influence sepiolite fibre length for mutagenesis in bacteria. The exposure of stationary phase
169 bacteria to a suspension of short fibres (less than 1 μm) did not cause any significant DNA damage

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170 when compared with the control and in contrast with the long-fibre original mineral suspension
171 (Kruskal-Wallis test; P=0.005; fig 6).
172
173 **Asbestos fibres increase the mutant frequency in the same way as sepiolite do.** Bacterial
174 genotoxicity experiments are considered a key step in the assessment of mutagenic properties of
175 chemicals, drugs or materials in general [31]. Because asbestos fibres resemble sepiolite ones, an
176 experiment to test if asbestos fibres provoke an increase in mutagenesis was designed using
177 crocidolite asbestos (fig S2). In our assay, the addition of asbestos to bacteria in the plates without
178 friction did not increase the mutant frequency. In contrast, the application of friction when the fibres
179 were present increased the mutant frequency even more than sepiolite alone (Kruskal-Wallis test;
180 P=0.002; fig 7), probably by the same mode of action. Yoshida *et al.* have suggested that asbestos
181 and other clays can be potentially mutagenic based on integrity analysis of genomic DNA from
182 treated bacteria [32]. A clear antecedent of the ability of fibrous nanoclays to penetrate bacteria was
183 the transformation of monkey cells in culture by exogenous plasmid DNA using chrysotile (a type
184 of asbestos) [10]. Although procedures are not described in details, we think that this transformation
185 requires penetration of the cell membrane.
186
187 **Further discussion.** The poor correlation between DNA damage *in vivo* and *in vitro* described in
188 previous studies [12] may be explained by the limited or lack of penetration of asbestos in
189 experimental designs. Thus, the introduction of some friction or shaking can in determining if
190 penetration of cells by asbestos and other fibres underly a molecular mechanism of carcinogenesis.
191 The mechanism(s) underlying asbestos toxicity associated with the pathogenesis of mesothelioma
192 has been a challenge to unravel for more than six decades [33]. According to our results and the
193 current knowledge about asbestos-induced carcinomas, we speculate about a model that explains a
194 potential path leading to carcinomas. Briefly, we think that people exposed to asbestos fibres during
195 prolonged periods accumulate them in the respiratory tract. It is frequent to find asbestos fibres into
196 the pleural cavity, and maybe they increase the friction coefficient in the pleural space, a parameter
197 with a very small value in in physiological conditions [34]. The coelomic movement (a cyclical
198 mechanical movement between the parietal pleura—covering membrane of the inner surface of the
199 thoracic cavity—and the visceral pleura—covering membrane of the lung surface—) provokes the
200 movement of asbestos, trespassing occasionally the mesothelial cell membranes or floating
201 mesothelial cells, physically interacting and disrupting the DNA or spindle. This physical
202 interaction, with adequate intensity, could induce DSBs, which generate chromosome aberrations or
203 fragmentations in eukaryotic cells as we found here for bacteria. After years of exposure, DSBs or

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204 spindle disruption can cause chromosome damages or losses or aneuploidy that increase the
205 probability of malignancy. The proposed model for eukaryote cells would need *in vitro* validation
206 with epithelial cells but this is beyond the scope of the current study and left for future research.
207 Moreover, this model does not exclude other toxic and genotoxic mechanisms of asbestosis such as
208 reactive species arising from metal action or inflammatory response.
209
210 One of the most important limitations of our study is the lack of an animal model to test if our
211 finding of mutagenicity in bacteria by clays occurs *in vivo*. In theory, clays present in livestock feed
212 could promote antibiotic resistance and virulence in pathogenic bacteria by not only their
213 transformation ability but also via mutations. However, testing conditions are hindered by the fact
214 that experiments would require at least S1 security level, and this is difficult to achieve with
215 livestock animals [4]. Transformation of plasmid DNA requires penetration and sepiolite and other
216 clays have shown this capacity in a wide range of concentrations although it diminishes at high
217 concentration due to the killing of bacteria [9,35–37]. In a previous study, the values of pressure in
218 the gut of many animal species were discussed, meeting the criteria very well [4]. The presence of a
219 hydrogel does not seem to be a problem since both mucin layer of the gut or mucoid secretion in the
220 respiratory tract can play that role, particularly if fibres have the capacity to change viscosity locally
221 or gradients of viscosity exist across these body compartments.
222
223 An implication of our study is the consideration of other factors (such the friction forces) in
224 assessing of genotoxicity and carcinogenesis by certain fibrous materials. Until now, many studies
225 associate clay-induced damage mostly with ROS [14]. DNA damage can be produced by oxido-
226 reduction processes generated by metal containing-fibres. Asbestos fibres are carcinogenic for both,
227 humans and experimental animals, because asbestos produce DNA breaks leading to the formation
228 of micronucleus (a type of chromosomal aberration) [38]. This kind of damage seems to be caused
229 more by mechanical action rather than ROS generation, which can worsen the situation but not
230 necessarily has to be determinant. In other words, we think that ROS is more a symptom than a
231 cause. Another example of a potentially dangerous material are the carbon nanotubes (CNTs), a
232 novel industrial material with many applications. The genetic alterations provoked by these
233 nanotubes in rat malignant mesothelioma were similar to those induced by asbestos [39].
234 Interestingly, CNTs lack heavy metals in their composition. The nanoscale size and needle-like
235 rigid structure of CNTs appear to be associated with their pathogenicity in mammalian cells [38].
236 Coincidentally, CNTs can be used to transform bacteria with plasmids [40] in a similar fashion that

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237 asbestos [10,41] and sepiolite do [6,42]. It would not be surprising that all these fibrous
238 nanomaterials share their ability to mechanically induce DSBs.
239 Recently, a possible link between talcum powder and ovarian cancer risk associated with asbestos
240 contamination in talc is under discussion. Although the risk is small, some studies suggested a low
241 or moderate but significant chance of cancer, while other rejected/discarded this correlation [43–
242 45]. It is necessary to advance the understanding of molecular base of DNA damage by asbestos
243 and other industrial fibres. If the proposed model of mechanical/physical DNA breaks is validated
244 in future studies, some genotoxicity assays intended to unveil mutagenic properties of materials
245 (e.g. the test of Ames) should be modified accordingly to include a standardised procedure of
246 friction or promoting some sort of shaking during incubation steps. Similarly, several *in vitro* test,
247 with both bacteria and eukaryotic cells, were modified by researchers and regulatory agencies
248 where introduced the metabolic activation by fraction S9 of liver homogenate [46].
249
250 Other implications of the induction of DSBs by nanofibres in bacteria could be related with the
251 microbiota of animals that use gastroliths. It has been suggested that gut microbes play a crucial
252 role in keeping species apart or enhance the speciation [47]. It is tempting to speculate that animals
253 that use gastroliths or sediment ingestion expose their microbiota to the abrasive action of stone
254 derivative fibres. Therefore, the shaping of their own microbes is expected to contribute to their
255 own speciation trajectories. Among animals that use or used gastroliths in their evolutionary
256 trajectories, we find several branches of fishes, amphibians, reptiles (including dinosaurs) and birds.
257 Gastroliths also regularly occur in several groups of invertebrates [48]. Wings (2007) recommends
258 making a distinction between lithophagy and geophagy. Lithophagy (stones larger than 0.063 mm
259 in diameter) is defined as the deliberate consumption of stones that turn into gastroliths after their
260 ingestion. Geophagy is the consumption of soil and it is known for reptiles, birds, and mammals.
261 These soils, rich in clays, salts or fat, serve mainly as a food supplement for the supply of specific
262 minerals or for medical purposes [48]. Both concepts can contribute to getting together all the
263 components that this mechanism needs to operate: gut microbiota, gut mucin mucoid layer
264 (hydrogel) and friction forces provided by the peristaltic pressure of digestive tract in animals,
265 especially the gizzard and the stomach. An interesting question is why sepiolite from limestone
266 gastroliths does not damage the animal gut. A convincing explanation is that the mucoid layer in the
267 gut protects it from the action of these sharp fibres at the time that serve as a protective layer for gut
268 epithelium. In mammals, this mucoid layer is around 200 μm thick and is under continuous
269 renovation [49]. Sepiolite is a natural clay mineral characterised by a nanofibre structure with

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270 average dimensions less than or equal to 0.2 micrometers in diameter, and from 2 to 5 micrometers
271 in length, although longer fibres can be present.
272
273 **Concluding remarks.** Overall, one of the most significant contributions of this article is the
274 proposition for the first time of a bacterial model to test genotoxicity of nanofibres and uncover a
275 new mechanism of action for asbestos that correlates better with *in vivo* observations. Asbestosis is
276 a global health and environmental problem, which molecular basis has been a challenge for several
277 decades [33]. Although asbestos fibres are widely distributed in the anatomy of patients [33,50], the
278 most common cancers caused by asbestos originate in lungs (mostly mesothelioma). If the most
279 explored mechanism of action is based on reactive radicals (chemical damage), why is not there
280 significant differences in the frequencies of other types of carcinoma such as leukaemia, lymphoma,
281 liver or kidney cancer among exposed populations? In the last place, and not less important, is the
282 tighter contact of slippery membranes (a monolayer of flattened epithelial-like cells) of the
283 mesothelium. The pleural space is in continuous movement and constitute preferential target of
284 asbestos-induced carcinogenesis. Of particular interest are free-floating mesothelial cells of the
285 cavity, that even proliferate under damaging conditions [51]. The free-floating cells are the ideal
286 candidates to be penetrated by asbestos in the pleural space. They may be more sensitive to suffer
287 direct (physical) or indirect (chemical) DNA damage and become into a mesothelioma. Finally,
288 sepiolite transformation technique gained some popularity in the last years because there is no need
289 to prepare competence cells [9,19,42,52]. In that case, diverse bacteria can be transformed [4] in
290 both stationary and exponentially growing phases. However, to prevent undesired mutations in
291 both, plasmid and genomic DNA, it is highly recommendable to use exponential phase bacteria,
292 where mutagenesis is not significant, at least in *E. coli*.
293
294 **Methods**
295
296 **Bacteria and growth conditions.** The *E. coli* MG1655 wild-type strain and its derivative mutants
297 were cultured in Lysogenic Broth (LB). All experiments were performed at 37°C, with shaking in
298 liquid culture. All solid cultures were grown in LB agar 1.5% for standard procedures and 2% for
299 the sepiolite treatment. All cultures were supplemented with antibiotics when appropriate.
300
301 **Mutant frequency estimation of sepiolite treated cells.** Approximately 2×10^9 bacterial cells per
302 ml of *E. coli* MG1655 and its derivative mutants from overnight or mid-exponential growing
303 cultures were centrifuged and resuspended in 100 μl of sterilised transformation mixture, consisting

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304 of sepiolite (Kremer Pigmente, Germany) suspended in aqueous solution at a final concentration of
305 0.1 mg/ml. Resuspended cells were spread on plates containing fresh Müller-Hinton-Agar (Sigma-
306 Aldrich, Germany) medium solidified with 2% agar, and Petri dishes were pre-dried in a biological
307 safety flow cabinet for 20 minutes before use. Friction force was provided by streaking bacterial
308 cultures plus sepiolite with sterile glass stir sticks gently pressed onto the medium surface for one,
309 two and three minutes, applying as much pressure as possible without breaking the agar gel. Petri
310 dishes were incubated at 37°C for 2 hours to allow DNA repair if any damage occurred. The plates
311 were gently washed four times with 5 ml of 0.9% sodium chloride solution using a 5 ml pipette. The
312 bacterial suspensions were transferred to 10 ml tubes to recover the cells by centrifugation at 3000 g
313 for 10 minutes. The resulting pellets were resuspended in a final volume of 1 ml of fresh LB an
314 incubate during 1 hour at 37°C to allow the cells to recover. Appropriate dilutions were plated onto
315 LB plates to estimate bacterial viability and in LB plus fosfomycin (50 µg/ml) to estimate the
316 number of resistant mutants. Plates were incubated overnight at 37°C. Each experiment consisted of
317 5 replicates and was repeated at twice. Mutant frequencies were calculated by using the FALCOR
318 web-tool [53].
319
320 **Influence of 2-2′ bipyridyl on sepiolite mutagenesis.** The effect of 2-2′ bipyridyl, a metal
321 chelating agent [54], on sepiolite mutagenesis was determined by measuring its influence on the
322 mutant frequency for a selected concentration of sepiolite, where mutagenesis was observed. The
323 experiment consisted of adding a titrating concentration of 2-2′ bipyridyl (200 µM) to chelate
324 metals five minutes before the treatment. Cultures treated with sepiolite and friction without the
325 addition of 2-2′ bipyridyl and bacteria alone without sepiolite were used as a control. The mutant
326 frequencies for these groups were determined as described elsewhere in this section.
327
328 **Assessing double-strand breaks with a plasmid system.** To evaluate if sepiolite under friction
329 treatment induces double-strand breaks in plasmid DNA, the strain *Escherichia coli* DH5α (fhuA2
330 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17) carrying
331 the plasmid pET-19b (Novagen, Germany) was treated with sepiolite and sliding friction forces
332 during one minute. Several samples were recovered from the plates and pooled to compensate
333 viability losses due to friction. The recovery was done by washing the surface with 5 ml 0.9 % NaCl
334 saline solution four times as described for mutagenesis experiments. The recovered pellets were
335 washed with 1 ml of TE buffer and the OD_{600} adjusted to 1 for each type of sample. Plasmid DNA
336 samples were extracted using a Qiagen mini plasmid extraction kit (Qiagen, Germany). Added
337 sepiolite with or without friction and no sepiolite groups were used as a control group. Each

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338 experiment consisted of five replicates. The same amount of plasmid DNA per replicate was
339 applied per well to an agarose gel that was stained with SYBR® Gold Nucleic Acid Gel Stain kit
340 (Molecular Probes, USA). A NdeI (Promega, USA) digested aliquot of pET-19b was used as
341 control of linear migration rate. The proportion of linear molecules of the plasmid were compared
342 among groups using a densitometry analysis by ImageJ [55].
343
344 **RecA deficient strain construction.** The *recA* null mutant was constructed following a previously
345 described methodology [56] with the primers 5'-CAGAACATATTGACTATCCGGTATTACCCG-
346 GCATGACAGGAGTAAAAATGGT-GTAGGCTGGAGCTGCTTC-3' and 5′-
347 ATGCGACCCTTGTGTATCAAACAAGACGATTAAAAATCTTCGTTAGTTTCATGGGAAT-
348 TAGCCATGGTCC-3' (forward and reverse respectively) using the pKD3 plasmid as template.
349 The mutant was checked by PCR amplification using the primers c1 5'-
350 TTATACGCAAGGCGACAAGG-3' and c2 5'-GATCTTCCGTCACAGGTAGG-3' in
351 combination with specific primers for upstream and downstream regions of *recA* gene: 5'-
352 ATTGCAGACCTTGTGGCAAC-3' and 5'-CGATCCAACAGGCGAGCATAT-3' respectively.
353 Additionally, the increased susceptibility to UV light and mitomycin C was tested phenotypically in
354 comparison to the parental strain. The antibiotic resistance gene was eliminated using the pCP20
355 plasmid as described previously [56].
356
357 **SEM of *E. coli* treated with sepiolite.** Approximately 2×10^9 CFU of stationary phase *E. coli*
358 MG1655 were treated with sepiolite and friction force was applied for one minute as described for
359 the mutagenesis experiment. Circular agar blocks were taken from agar plates with a sterile cork
360 borer (1 cm of diameter). Then, a thin surface layer was cut off, placed on a circular glass cover slip
361 (1.5 cm of diameter) and incubated for 45 minutes at room temperature in a laminar flow cabinet to
362 allow air drying of the samples. The cover glasses with dehydrated agar sections were mounted on
363 aluminium stubs using double-sided adhesive tape and coated with gold in a sputter coater (SCD-
364 040; Balzers, Union, Liechtenstein). The specimens were examined with a FEI Quanta 200 SEM
365 (FEI Co., Hillsboro, OR) operating at an accelerating voltage of 15 kV under high vacuum mode at
366 different magnifications. At least 5 sections from independent plates were observed to check
367 physical penetration by the mineral. Some samples of sepiolite or asbestos (crocidotiles) alone were
368 processed and observed in the same way.
369 **Long fibre-depleted sepiolite mutagenesis experiment.** To assess the role of long fibre of
370 sepiolite in mutagenesis, a sepiolite preparation depleted of fibres longer than 1 µm was obtained. A
371 100 ml sepiolite suspension (1 mg/ml) in distilled water was passed though Pall® Acrodisc® glass

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372 fibre syringe filters (Sigma, USA) several times. The resulting suspension was desiccated by
373 evaporation at 70°C overnight. A non-filtered solution was used as a control. From the obtained
374 powder, two suspensions were prepared to a final proportion of 0.1 mg/ml. These two solutions
375 were used for a mutagenesis experiment plating in fosfomycin as indicated previously, using a
376 friction time of two minutes.
377
378 **Mutant frequency estimation of asbestos treated cells.** The procedure was carried out identically
379 that the one described for sepiolite in this section. The time was set to two minutes and the same
380 concentration that was used, 0.1 mg/ml. We used the crocidolite asbestos analytical standard (SPI
381 Supplies, USA). The asbestos fibres were resuspended in distilled water, autoclaved and sonicated
382 in bath during 10 minutes before use to render a homogeneous suspension.
383
384 **Statistical analysis.** To compare experimental groups, Kruskal-Wallis test or One-way ANOVA
385 test were performed. In case of significance, Bonferroni-corrected one-tailed Mann-Whitney U test
386 or Tukey HSD Test were used respectively. P values less than or equal to 0.05, after correction if
387 needed, were considered statistically significant. All tests were performed with the statistic software
388 R v. 3.4.2 [57].
389

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391
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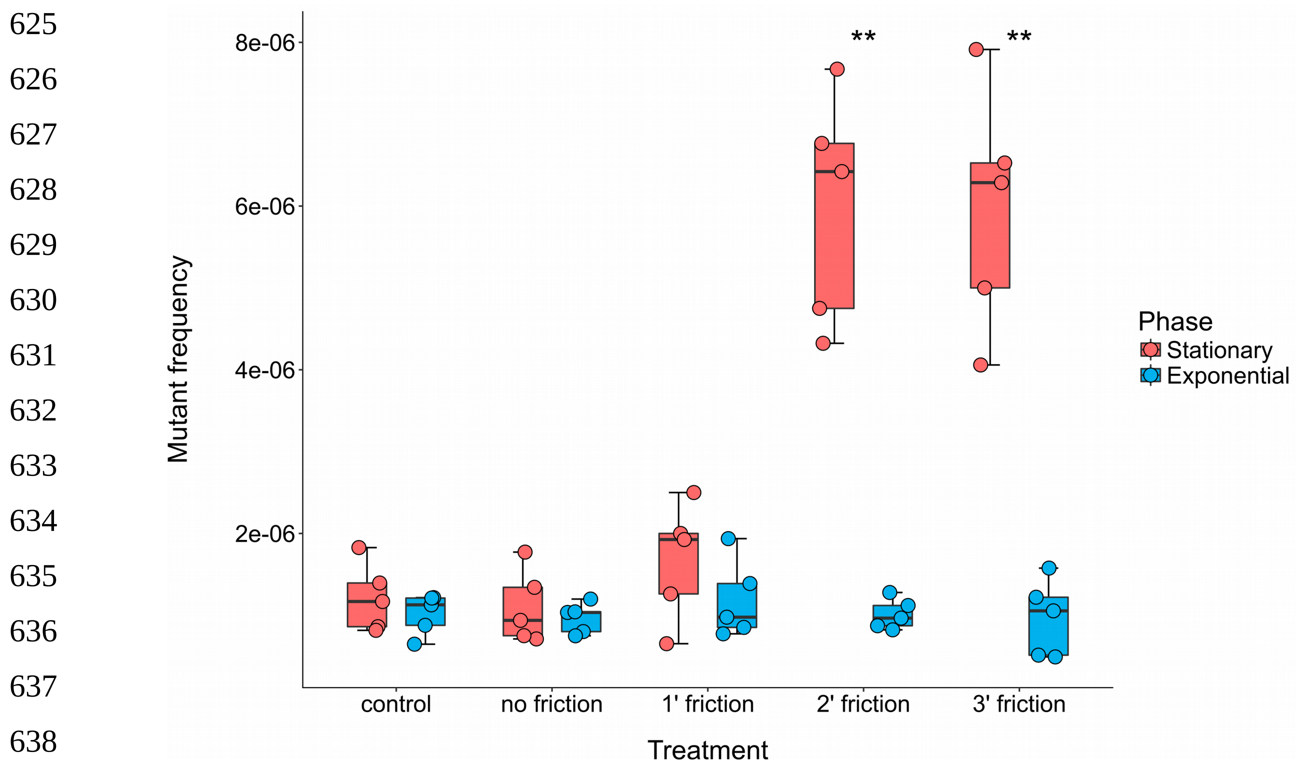

639
640 **Fig 1. Sepiolite can be mutagenic after friction treatment only in stationary phase.** Box-plot of the mutant
641 frequency induced by sepiolite treatment in *E. coli* MG1655 (A) stationary and (B) exponential phase cells. The x-axis
642 indicates the experimental treatment (control, mixture of bacteria and sepiolite without friction force, and with friction
643 force during one, two and three minutes). Asterisks represents significant difference; Mann-Whitney *U*: $P < 0.01$.
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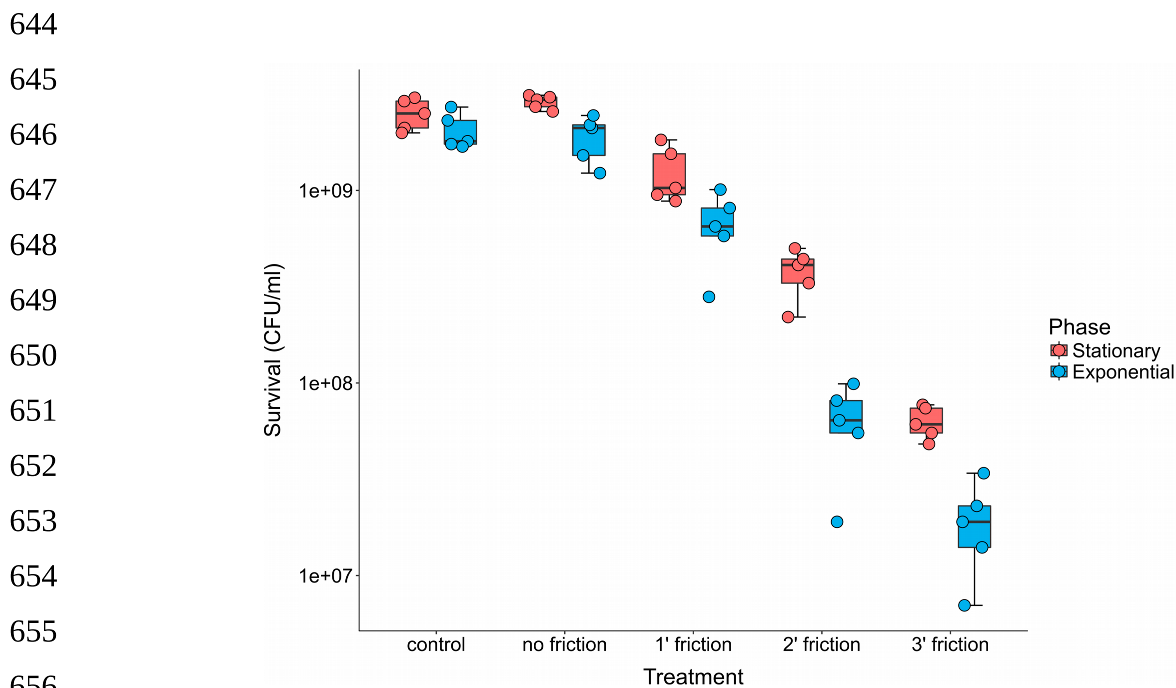

657 **Fig 2. The higher the time of friction, the smaller the cell viability.** Box-plot of the survival of *E. coli* MG1655 to
658 the action of friction with sepiolite during one, two and three minutes of treatment. Groups with and without sepiolite
659 gently spread with glass beads onto agar plates were used as controls.

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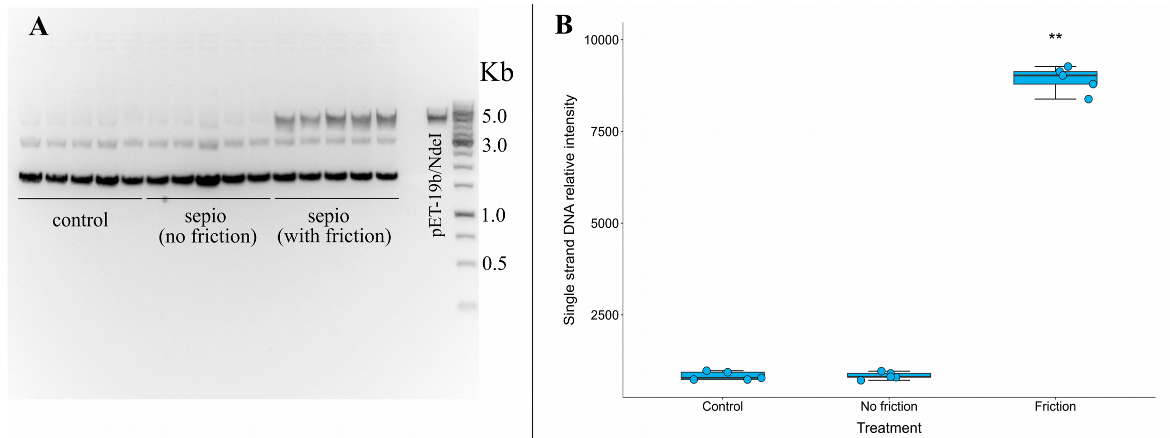

Fig 3. Linear plasmid DNA molecules abundance is higher when friction forces are applied. (A) Extraction of the plasmid pET-19b from sepiolite-treated *E. coli* DH5α, a *recA* deficient strain, during one minute (five extractions per treatment). Note the enrichment in linearised plasmid DNA molecules from bacteria treated with sepiolite under two minutes of friction applied in 1% agarose gel. (B) Box-plot of the abundance of single strand DNA molecules under different experimental treatments (control, sepiolite without friction and sepiolite with friction). 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. Asterisks represents significant difference; Tukey HSD test: $P < 0.01$.

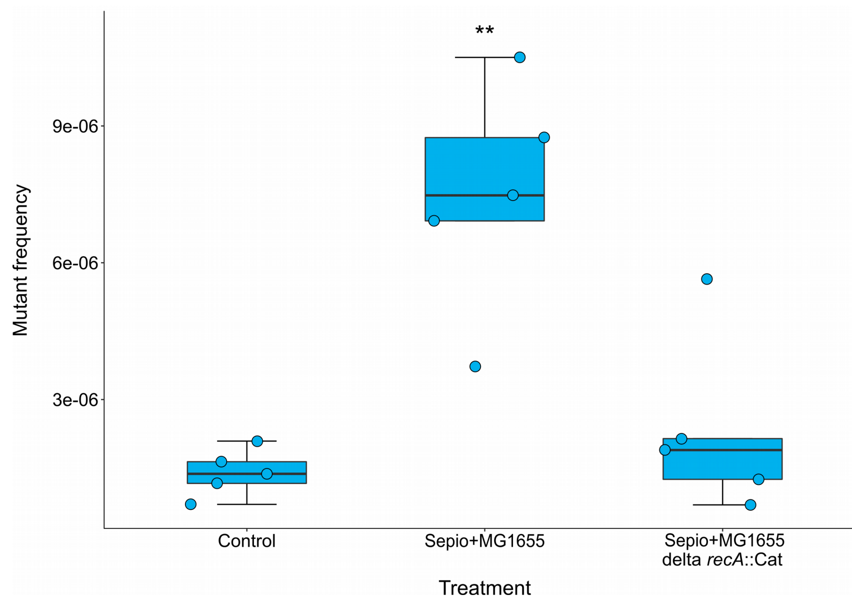

Fig 4. Inactivation of the *recA* gene suppresses the mutagenic effect of sepiolite under friction in *E. coli* MG1655. Box-plot of the mutant frequency of *E. coli* MG1655 and DH5α (derivative *recA* mutant) when treated with sepiolite during two minutes. Asterisks represents significant difference; Mann-Whitney U: $P < 0.01$.

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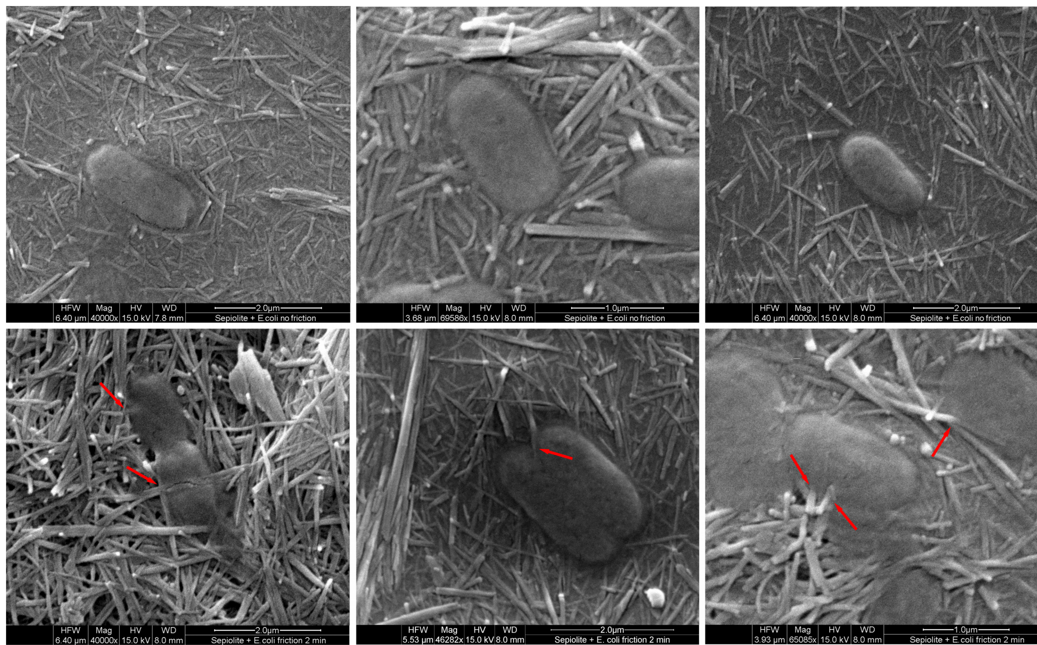

709 **Fig 5. Sepiolite can penetrate bacterial cells when friction forces are applied.** SEM of stationary phase *E. coli*
710 MG1655 treated with sepiolite. Red arrows represent potential sites of sepiolite fibre penetration. Bacteria were
711 observed with different magnifications ranging from 40 000X to 70 000X.
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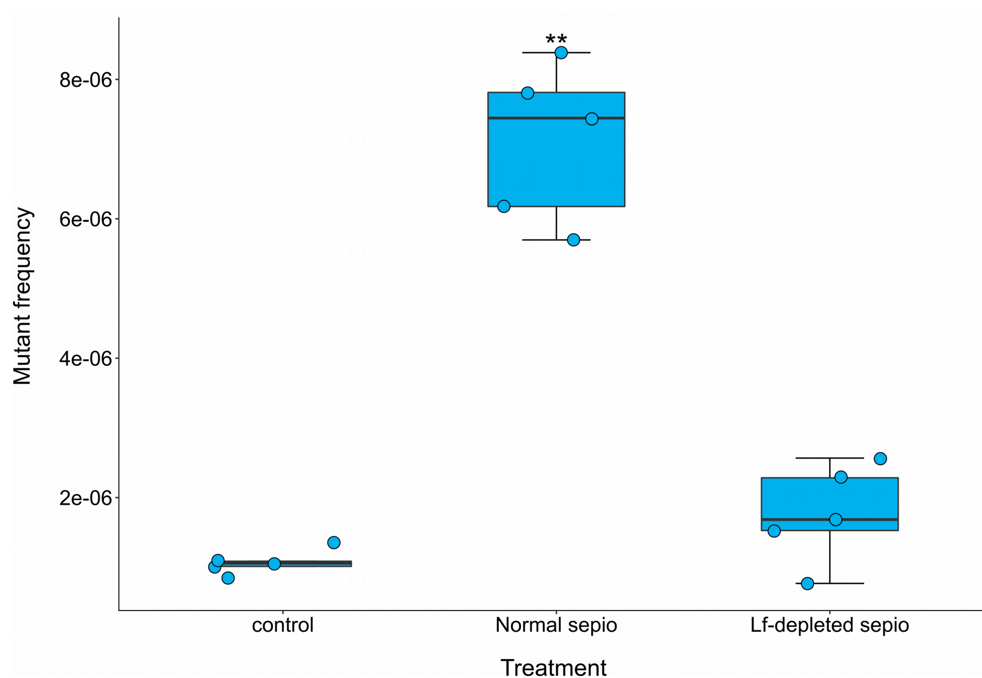

727 **Fig 6. Removal of sepiolite fibres longer than 1 μm decreases fibre-induced mutagenesis to the level of the**
728 **control.** Box-plot of the mutant frequency of *E. coli* MG1655 when sepiolite fibres longer than 1 μm were removed in
729 mutagenesis experiments (lf-depleted sepiolite). Dry and reconstituted sepiolite (normal sepiolite) and bacterial cells

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730 (labelled as control) with no sepiolite were used to compare the effects of long fibre removal. Asterisks represents
731 significant difference; Mann-Whitney *U*: P < 0.01.
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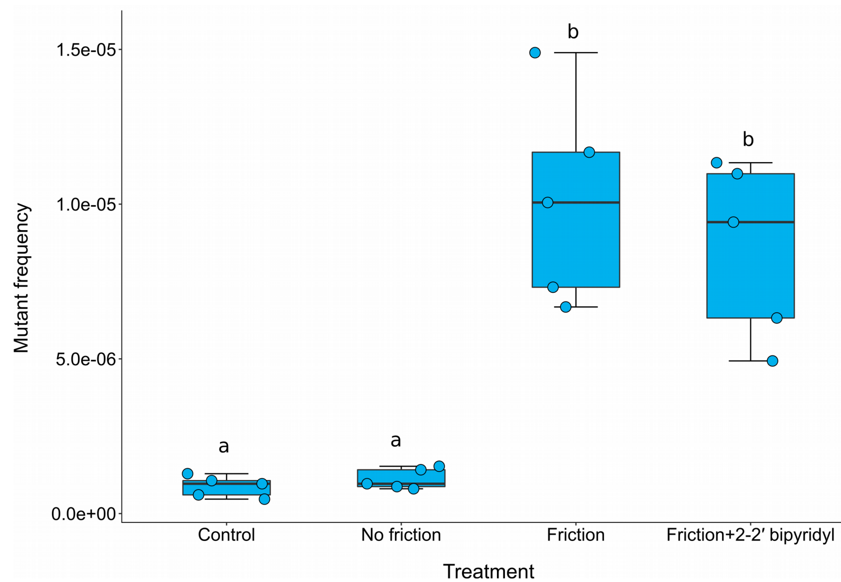

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744 **Fig 7. Asbestos can increase the mutant frequency of *E. coli* after friction around one order of magnitude.** Box-
745 plot of mutant frequency induced by asbestos (crocidolite fibres) treatment in *E. coli* MG1655. Asterisk represents
746 significant difference; Mann-Whitney *U*: P < 0.05. Equal letters represent no differences while different ones represent
747 significant differences.
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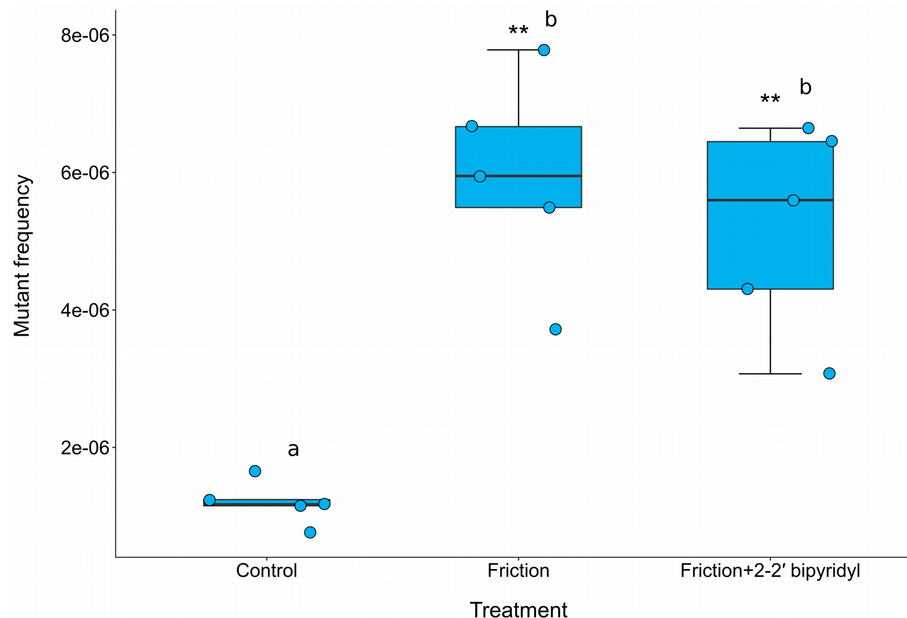

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761 **Fig S1. Addition of a chelating agent (2-2' bipyridyl) does not significantly suppress or diminish the mutagenic**
762 **effect of sepiolite.** Box-plot of mutant frequency of *E. coli* MG1655 when added 2-2' bipyridyl as chelating agent.
763 Asterisks represents significant difference; Mann-Whitney *U*: P < 0.01. Equal letters represent no differences while
764 different ones represent significant differences.

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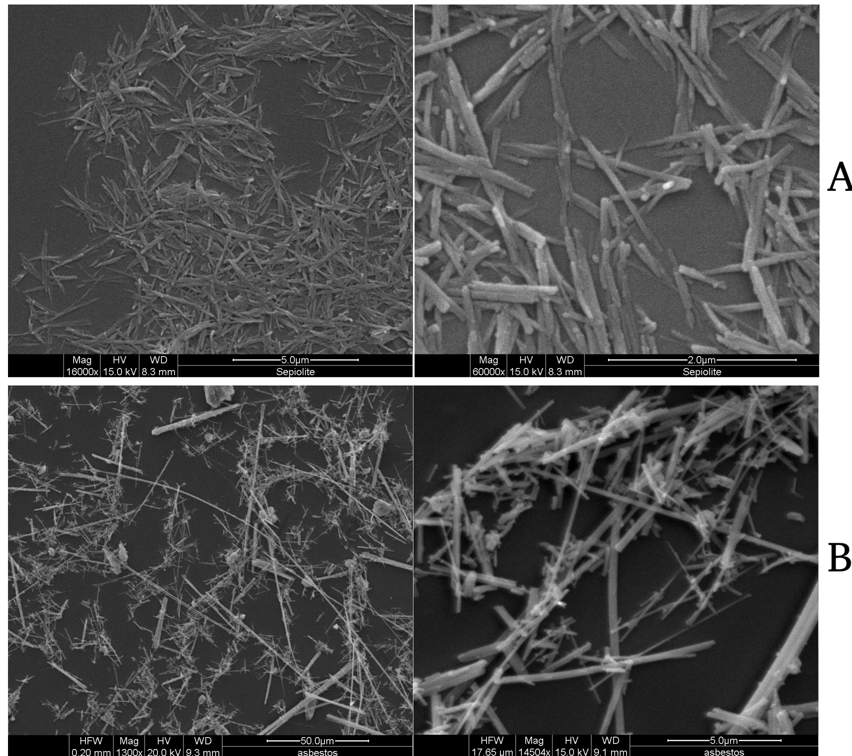

780 **Fig S2. Visualisation of sepiolite and asbestos fibres under SEM.** SEM examination of (A) sepiolite fibres and (B)
781 asbestos. Fibres were observed at different magnifications as indicated in the pictures.