

Original Paper

Evaluation of the antibacterial properties of commonly used clays from deposits in central and southern Asia

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Abstract

One potential solution to the rising threat of antibacterial drug resistance is the application of therapeutic clays to treat wound infections. Clays with antibacterial activity have been identified from a range of sources with their antibacterial properties often attributed to the release of toxic metal ions such as Fe(II) and Al(III). Here, clays from Afghanistan, Azerbaijan and Bangladesh that are utilized for washing and healing purposes were examined. Their antibacterial activities were assessed in suspension and as aqueous leachates against representative Gram-negative, *Escherichia coli*, and Gram-positive, *Bacillus subtilis*, bacteria. The majority of the clays conferred no deleterious effect and, in fact, tended to promote bacterial growth, likely as a result of released organic and inorganic nutrients. However, one of the clays, obtained from the Dhaka region of Bangladesh, displayed significant bactericidal activity against *E. coli* and *B. subtilis* as a clay suspension but not as an aqueous leachate. Further experiments confirmed that contact between clay and the bacteria was necessary for most of the antibacterial effects. Detailed analysis of bulk and <2 µm clay fraction mineralogy and geochemistry revealed no single defining parameter or mineral component that could be used to easily distinguish natural clays with antibacterial properties from those without. Overall, the results suggest a mechanism of antibacterial action of the Dhaka clay that arises from acidic conditions, likely enabled by the absence of calcite in the bulk clay, metal release, the presence of interstratified chlorite-smectite, and direct clay–bacteria interactions.

Keywords: antibacterial clay; chlorite-smectite; Gram-negative bacteria; Gram-positive bacteria; metal analysis

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Introduction

Clay deposits (hereafter termed ‘clay’ or ‘clays’) and clay minerals arise predominantly through the weathering of silicate rocks and are hence ubiquitous on the Earth’s surface. In sediments, clay minerals mainly consist of smectite, illite, chlorite, kaolinite and mixed layers (e.g. illite-smectite) of these minerals (Chamley, 1989). Clay minerals are typically defined by a <2 µm spherical diameter particle size (Moore and Reynolds, 1997), although the term ‘clays’ can also be applied to size fractions over 4 µm (Weaver, 1989). Clays frequently contain non-clay minerals, such as quartz, K-feldspars, plagioclase and carbonates (e.g. calcium carbonate (Weaver, 1989). The unique chemical and physical properties of clay minerals make them suitable for diverse applications, including paper coatings,

ceramics, storage of hazardous radioactive material, rheology modifiers and thickeners, adsorbents, and catalysts (Harvey and Lagaly, 2013; Wesley, 2014). In medicine, clay minerals have found use as adsorbents for toxins and as hosts for the delivery of bioactive molecules (Carretero *et al.*, 2006; Ghadiri *et al.*, 2015; Aguzzi *et al.*, 2016). Redox active clay minerals can be harnessed as antibacterial agents (Williams and Haydel, 2010; Dong, 2012; Morrison *et al.*, 2016; Williams, 2017; Gomes *et al.*, 2020; Guo *et al.*, 2021; Huang *et al.*, 2021; Dong *et al.*, 2022; Xia *et al.*, 2023) and for remediating soil and water from bacterial contamination, heavy metals, and other pollutants (Dong, 2012; Ismadji *et al.*, 2015; Unuabonah *et al.*, 2018; Dong *et al.*, 2022).

Since prehistoric times, clay minerals have been extracted from their deposits and employed for therapeutic processes. In fact, studies suggest that clays were used for curing wounds, cleaning skin and soothing indigestion by *Homo erectus* and *Homo neanderthalensis* (Carretero, 2002). The first recorded use of medicinal clay was in ancient Mesopotamia (Gomes and Silva, 2007; Williams and Hillier, 2014). Indigenous peoples around the world still use natural clays in traditional medicine but, apart from

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geophagy (Wilson, 2003; Henry and Cring, 2013), many of these clays and their deposits have not been widely studied from mineralogical, microbiological or anthropological perspectives. Interest in clay minerals for healing purposes in the Global North was revitalized by the work of a French humanitarian, Line Brunet de Coursou, who employed French Green clays to treat Buruli ulcer infections in the Ivory Coast, West Africa. As reported by Williams *et al.* (2004), daily application of a clay poultice to the area of skin infected with *Mycobacterium ulcerans* produced a dramatic recovery over a number of weeks of treatment.

Clay minerals have been hypothesized to damage bacteria and inhibit their proliferation by either physical or chemical means (Williams *et al.*, 2008; Wei *et al.*, 2011; Williams, 2017; Gomes *et al.*, 2020). In terms of physical interactions, clay platelets are attracted to the surfaces of bacteria, potentially restricting their capacity to acquire fresh nutrients and disperse waste products (Ferris *et al.*, 1987; Konhauser and Urrutia, 1999; Wei *et al.*, 2011; Williams and Hillier, 2014). Metal precipitation and mineralization at the bacterial surface may exacerbate this effect (Cuadros, 2017; Dong *et al.*, 2022). The needle-like structure of certain minerals themselves may cause physical damage to cell membranes (Williams *et al.*, 2008; Williams, 2019). For example, organically modified antibacterial clay minerals can apparently puncture bacterial cell envelopes (Hong and Rhim, 2008). The typically <2 µm particle size of clay minerals appears important for their antibacterial efficacy in some cases, further supporting the importance of surface contact with bacterial cells (Williams *et al.*, 2008; Williams *et al.*, 2011; Xia *et al.*, 2023). A natural clay from the Columbian Amazon was implicated in starving bacteria of essential nutrients such as magnesium and phosphorous (Londoño and Williams, 2016), while the bacteriostatic effects of a German clay on Gram-negative bacteria was attributed to lipopolysaccharide-siloxane clay surface interactions (Zarate-Reyes *et al.*, 2018). These physical effects are in keeping with the absorptive properties of clays in wound care settings, to stem bleeding, extract fluids and limit spread of infection (Williams, 2017).

In contrast, studies on the antibacterial properties of Kisameet (British Columbia, Canada), French Green (Massif Central, France) and Oregon Blue (Cascades, Oregon, USA) clays and clay leachates support chemical attack by the release of toxic metals as the primary mechanism of antibacterial activity (Williams *et al.*, 2008; Cunningham *et al.*, 2010; Williams *et al.*, 2011; Behroozian *et al.*, 2016; Morrison *et al.*, 2016; Williams, 2017; Behroozian *et al.*, 2020). It has been proposed that hydration of the clays results in mineral oxidation, dissolution and hydrolysis, and leads to increased acidity. At this lower pH, soluble metal ions can be released, for example Al^{3+} , Ca^{2+} , and Fe^{2+} , via dissolution of clay components as well as release of cations from the interlayer of smectite (Cunningham *et al.*, 2010; Williams and Haydel, 2010; Morrison *et al.*, 2014; Morrison *et al.*, 2016), or co-mingled non-clay minerals. For example, acid-promoted dissolution of the mixed-layered illite-smectite in weathered plagioclase could also promote release of Al^{3+} and Ca^{2+} into solution. In addition, oxidation of intermingled pyrite by O_2 could result in the autocatalytic production of H_2O_2 , which can damage multiple bacterial components, notably membranes, proteins and chromosomal DNA (Morrison *et al.*, 2016; Williams, 2017; Behroozian *et al.*, 2020). Consistent with this, a chemically reduced Fe-rich clay mineral (nontronite rNAu-2) with increased ferrous iron (Fe(II)) content was a potent antibacterial agent against

Gram-negative *Escherichia coli*, by inducing membrane and surface protein damage by hydroxyl radicals produced by the Fenton reaction (Wang *et al.*, 2017; Xia *et al.*, 2020; Guo *et al.*, 2021; Xia *et al.*, 2023). Although Colombian Amazon clays lack significant levels of iron, release of excess Al^{3+} was likely responsible for its antibacterial activity, again damaging bacterial membranes (Londoño *et al.*, 2017). With relevance to their potential in treating wound infections, Oregon Blue clays and their leachates were effective against both Gram-negative and Gram-positive bacteria growing in biofilms, although efficacy was greatest against the latter (Caflich *et al.*, 2018).

In this present study, different clays from Afghanistan, Azerbaijan and Bangladesh were investigated. Anecdotal evidence obtained by the authors links the application of Azerbaijan and Bangladesh local clays to a variety of health-related purposes. In Azerbaijan, Gilabi, mud volcano and green clays have been used for the treatment of skin diseases or for 'cleansing' the stomach since the end of the 19th century. For example, outcrops of green clays from the Surakhany district in Baku are used for both internal and external treatments, Gilabi clays (Seidov and Alizade, 1966) from Amircan, Bulbula, Mashtaga, and Khashoxonu villages of Baku are only employed externally and exploited for washing clothes and hair, and Shirekhey clay from Baku is an ingredient of homemade syrup called pekmez (doşab in Azerbaijani). Similarly, clays and soil are regularly used for personal hygiene and medicine in Bangladesh. In many rural areas eating burned soil while pregnant is not uncommon (the practice of geophagy or pica; Henry and Cring, 2013). Economically disadvantaged or elderly people make small clay balls (at a specific time of the year), preserve them as tablets and consume them for various ailments, while traditional healers give 'enchanted clay' of specific types to treat various illnesses, including psychological conditions. Clay from specific areas is used as a beauty product, for example for deep facial cleansing and for washing hair, kitchen utensils, and dishes.

Despite the local use of the above-described clays for purported therapeutic benefits, the antibacterial or other bioactive properties have yet to be systematically characterized and rationalized based on the clay's specific mineralogy. This knowledge gap is addressed by evaluating the biological activities against bacteria in aqueous clay suspensions and leachates. To help account for differences in observed activities and to attempt to derive easily accessible parameters for prospecting antibacterial clays, the mineralogical and elemental composition of the Azerbaijan, Afghanistan, and Bangladesh clays was characterized.

Materials and methods

Sample collection and preparation

Bulk clay samples were collected from Istalif (34°49'25.80"N 69°07'08.30"E) and Paghman (34°36'24.10"N 68°57'53.91"E) in Afghanistan; Amircan (40°25'26.8"N 49°58'55"E), Bulbula (40°26'7.22"N 49°58'23.61"E), Mashtaga (Yellow: 40°32'32.6"N 49°57'21"E and Green: 40°32'29.8"N 49°57'22.3"E) and Surakhany (40°24'40.5"N 50°00'53.4"E) in Azerbaijan; and Dhaka (23°42'32.8"N 90°23'43.8"E) and Bhola (22°02'58.4"N 90°36'58.3"E) in Bangladesh. Each sample was dried in an oven at 80°C for 48 h and then crushed and ground in a ceramic mortar with a ceramic pestle by hand, homogenized and split into three subsamples for antibacterial assay, geochemical, whole-rock mineralogical and clay mineralogical analyses.

Antibacterial assays using clay suspensions and leachates

Bulk clay samples were autoclaved (VARIO 1528, Dioxon, Wickford, UK) at 121°C for 40 min to sterilize. Clays were mixed with sterile (autoclaved at 121°C for 20 min) deionized water (SDW) or phosphate-buffered saline (PBS; Sigma-Aldrich, Poole, UK) at 0.25 g mL⁻¹ and vortexed for 1 min. PBS was selected to provide suitable physiological conditions without osmotic stress, and also to limit potential effects of clays on suspension pH value that might contribute to bacterial killing. The natural pH of mineral suspensions affects the antibacterial mechanism and thus effects with and without pH buffering were compared. *Escherichia coli* BW25113 (CGSC 7636) and *Bacillus subtilis* strain 168 (ATCC 6051) were used for antibacterial assays as representative Gram-negative and Gram-positive species, respectively. Bacteria were cultivated in Luria-Bertani (LB) broth (Sigma-Aldrich, Poole, UK) to an optical density (OD) at 600 nm of 0.4 equivalent to mid-log phase (approximately 7×10⁷ colony forming units (CFU) mL⁻¹ for *E. coli* and 4×10⁷ CFU mL⁻¹ for *B. subtilis*). Clay suspensions (900 µL) were mixed with 100 µL of bacteria and placed in a shaking incubator (Stuart SI500 Orbital Shaker, Wolfabs, Pocklington, UK) at 150 rpm and 37°C for 24 h. Bacterial viability was determined in colony forming units (CFU) per milliliter by performing 10-fold serial dilutions, with 10 µL aliquots of the 10⁻²–10⁻⁷ dilutions applied to the surface of LB agar plates (Sarstedt, Leicester, UK). Plates were incubated at 30°C for 16–24 h and the number of colonies enumerated. Experiments were performed independently in triplicate.

Aqueous mineral leachates were obtained from bulk clay suspensions prepared as described above using SDW and placed on a test-tube rotator (Tarsons, Kolkata, India) at 40 rpm at room temperature for 24 h. The leachate was further separated, to remove suspended insoluble minerals >0.1 µm, by centrifugation (5000 rpm, 11,600×g) in an MSE MicroCentaur microcentrifuge for 10–30 min. *E. coli* and *B. subtilis* were grown to an optical density at 600 nm of 0.07 and diluted 10-fold to provide an inoculum (approximately 1–2×10⁵ cells per well). Bacteria (50 µL) and leachate (50 µL) were mixed in a 96-well microtiter plate (Sarstedt, Leicester, UK) and incubated in a shaking incubator (150 rpm) at 37°C for 16 h. Optical density readings were obtained at 600 nm on a Spectrostar Nano plate reader (BMG Labtech, Aylesbury, UK). Positive controls without clay leachate and negative controls without bacteria were conducted in parallel. Bacterial viability in CFU mL⁻¹ was determined following exposure to the Dhaka clay leachate by performing serial dilutions and application of 5 µL samples onto LB agar plates.

To establish whether contact with the clay particles was necessary for antibacterial activity, 150 µL of *E. coli* and *B. subtilis* cultures grown to an OD_{600 nm} of 0.4 (mid-log phase) were placed in Mini 6000 Pur-A-Lyzer dialysis tubes (molecular weight cut-off 6–8 kDa; Sigma-Aldrich, Poole, UK) and immersed into either the Dhaka clay suspension (0.25 g mL⁻¹) or SDW in 5 mL tubes. Samples were incubated in a shaking incubator at 37°C for 24 h. Bacterial cultures (100 µL) were recovered and 10-fold serial dilutions generated in SDW as before. Application of 5 µL volumes onto LB agar plates and incubation at 37°C allowed determination of CFU mL⁻¹. All experiments were performed independently in triplicate.

Geochemical analyses

Oven-dried (80°C) bulk clay samples were split into two subsets. One subset of 1 g each was mortar-milled (stainless-steel) and

placed in a pre-heated furnace (1050°C, 1 h) to determine loss on ignition (LOI). These samples were then fused into beads, using a Vulcan Automatic Fusion Machine (Fluxana, Bedburg-Hau, Germany), and used for major oxide composition measurements by X-ray fluorescence (XRF) using a Rigaku Supermini200 WD-XRF Spectrometer (Rigaku Europe SE, Neu-Isenburg, Germany). Major oxide composition was used to generate a discrimination cross plot to identify the provenance area of the clays (Roser and Korsch, 1988) and to calculate the chemical index of weathering (CIW, Eqn 1). Both analyses yielded information about the intensity of chemical weathering in the provenance area (Harnois, 1988):

$$\text{CIW} = [\text{Al}_2\text{O}_3 / (\text{Al}_2\text{O}_3 + \text{CaO} + \text{Na}_2\text{O})] \times 100. \quad (1)$$

Equation 1 includes only the CaO incorporated in the silicate fraction. This was determined by subtracting the amount of CaO present as calcite, dolomite, and gypsum in the bulk sediments, respectively, from the total CaO content of the sediment (Fedó *et al.*, 1995).

The second subset was ground in an agate ball mill (500 rpm, 5 min) and 10±0.2 g each were mixed with a polyvinyl alcohol binder (1% Moviol) and pressed (p = 15 t, 2 min) into 32 mm diameter pellets. Pelleted samples were oven-dried (80°C) for at least 2 h before analysis by XRF (Rigaku NEX-DE ED-XRF spectrometer; Rigaku Europe SE, Neu-Isenburg, Germany) to quantify the minor elements. All XRF measurements were carried out by X-ray Mineral Services Ltd, Colwyn Bay, UK.

X-ray diffraction (XRD)

Randomly oriented samples were prepared from milled (McCrone Micronizing Mill, Hielscher Ultrasonics GmbH, Teltow, Germany; 2 g) bulk clay samples using the front-loading technique and analyzed on a PANalytical X'Pert³ Diffractometer (Malvern Panalytical, Malvern, UK) from 4° to 75°2θ (step size: 0.013°, dwell time: 0.2 s step⁻¹) using CuKα X-ray radiation (λ=1.5406 Å; 40 kV, 40 mA). Mineral quantification was carried out by Rietveld analysis of the XRD patterns using the Autoquan software (Meyer Instruments, Houston, TX, USA).

Clay samples (5 g) were ground using a McCrone micronizing mill and dispersed in deionized water using an ultrasonic bath for approximately 1 h. The clay fraction (<2 µm) was separated from the bulk clay using the Atterberg method (with settling time determined by Stokes' law), and further separated from the solution using centrifugation (4000 rpm, 1792×g) (Abdullayev *et al.*, 2021). Oriented clay fraction mounts were obtained by filter-drying the solids obtained from filtering clay suspension through a Millipore (Merck, Feltham, UK) glass microfiber (0.45 µm). Samples were analyzed as untreated clay, after saturation with ethylene glycol vapor for 24 h to identify smectite and heating at 380°C for 2 h, and after further heating to 550°C for 1 h to identify kaolinite. Clay fraction samples were analyzed on a Philips PW1730 diffractometer (Malvern Panalytical, Malvern, UK) from 3° to 35°2θ at a step size of 0.05° s⁻¹. All XRD measurements were carried out by X-ray Mineral Services Ltd, Colwyn Bay, UK.

Mössbauer spectroscopy

Iron speciation (Fe(II), Fe(III)) in the clay fraction of Mashtaga Green, Mashtaga Yellow, Amircan, and Surakhany clays

(Azerbaijan) and Dhaka and Bhola clays (Bangladesh), and Paghman clay (Afghanistan) was obtained by Mössbauer spectroscopy. Samples were sealed between two pieces of Kapton tape and analyzed in transmission mode with constant acceleration on a MS4 Mössbauer spectrometer (SEE Co., Edina, MN, USA) at both room temperature (293K) and 4K, using a closed cycle cryostat (SHI-850, Janis Research Co., Wilmington, MA, USA). Spectra were calibrated against α -Fe(0) foil and analyzed with the software Recoil, using its Voigt-based fitting routine (Lagarec and Rancourt, 1998).

Scanning electron microscopy

The texture of clay minerals was investigated to help identify authigenic or detrital mineral phases by scanning electron microscopy (SEM) (Abdullayev and Leroy, 2016; Abdullayev *et al.*, 2021) using a JEOL JSM-6610LV microscope (JEOL Limited, Tokyo, Japan) in the Institute of Geology and Geophysics, Azerbaijan National Academy of Science. The SEM was equipped with a low vacuum secondary electron (SE) detector and an Oxford Instruments silicon drift detector for energy-dispersive X-ray spectrometry (EDS) analysis (Oxford Instruments, Abingdon, UK). Clay pieces were placed on SEM stubs and sputter-coated with platinum to reduce charging during analysis. SEM images of minerals present in selected clays are shown in Fig. S1 (see Supplementary material).

Wet-chemical analyses

Aqueous leachates from bulk clays were prepared as described above (0.25 g mL⁻¹ clay in SDW) and centrifuged at 5000 rpm (11,600×g) for 30 min to remove particulates >0.1 μ m. Samples were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) using a PerkinElmer Sciex 6000 (Beaconsfield, UK) to determine the amounts of major and minor elements.

Results and Discussion

Effect of clay suspensions on the viability of Gram-negative and Gram-positive bacteria

To explore the potential of local clays as low-cost antibacterial agents, nine clays from low- and middle-income countries with traditions of clay use in health applications (Williams *et al.*, 2004; Williams and Hillier, 2014) were obtained and investigated. Five clays with purported healing properties were collected from the vicinity of Baku, Azerbaijan. Clays were obtained from Amircan, Bulbula, and Surakhany located in the eastern suburbs of the capital city, while the Green and Yellow Mashtaga clays differ only in their color; their source outcrops are located close to each other, approximately 14 km north of the city. Two Bangladesh clays used as cleansing and medicinal agents were obtained from distinct regions in the Himalayan delta. The first came from the Dhaka district and was obtained from the banks of a pond in Keraniganj, a town situated on the Buriganga river. The second was from Nanglapara, a village in the Bhola district located on an island on the banks of the Meghna river close to the Bay of Bengal. Clay samples from deposits in Istalif and Paghman, two small towns north of Kabul in Afghanistan, are widely used for making clay vessels – pottery is Istalif's main commercial output (Coburn, 2011). Both of the Afghanistan clays are currently being considered for applications in water purification (Hamidi *et al.*, 2023).

Bulk clay minerals were investigated for their antibacterial activities against both Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) species. These two rod-shaped bacteria differ in their cellular structure. Gram-negatives possess both an inner and outer membrane, sandwiching a thin network of peptidoglycan; their outer membrane is enriched with lipopolysaccharides and constitutes a permeability barrier to many hydrophobic antimicrobials. In contrast, Gram-positives have a single membrane surrounded by a dense outer coating of peptidoglycan and teichoic acids. Depending on experimental conditions, either Gram-negatives (Haydel *et al.*, 2008; Cunningham *et al.*, 2010; Behroozian *et al.*, 2016; Zarate-Reyes *et al.*, 2018; Behroozian *et al.*, 2020) or Gram-positives (Otto *et al.*, 2010; Cafilisch *et al.*, 2018; Williams, 2019) show greater susceptibility to antibacterial clays, although broad-spectrum efficacy has also been reported (Morrison *et al.*, 2014; Behroozian *et al.*, 2016; Londoño and Williams, 2016; Cafilisch *et al.*, 2018; Behroozian *et al.*, 2020).

In these experiments, *E. coli* viability over a 24 h incubation period was largely unaffected by exposure to the bulk clays in phosphate-buffered saline (PBS) (Fig. 1A). In fact, both samples from Afghanistan (Istalif, Paghman) and Bangladesh Bhola showed significantly increased bacterial abundance (1.2- to 1.6-fold) when the clays were present (Fig. 1A). This improvement was even more evident with *B. subtilis*, with most of the bulk clays causing increased growth, and hence CFU mL⁻¹, relative to the bacteria exposed to PBS alone (Fig. 1B). In particular, the two Afghanistan clays, Istalif and Paghman, and the Amircan clay from Azerbaijan encouraged substantially improved growth of *B. subtilis* (1.8- to 1.9-fold).

The experiments were repeated with selected bulk clays prepared in sterilized deionized water which would allow the clays to alter the pH value of the aqueous solution (Cunningham *et al.*, 2010; Otto *et al.*, 2010; Williams *et al.*, 2011; Behroozian *et al.*, 2016) and potentially facilitate the release of antibacterial metals such as Fe²⁺ and Al³⁺ (Otto and Haydel, 2013; Morrison *et al.*, 2014; Morrison *et al.*, 2016). The two Afghanistan clays, Istalif and Paghman, improved *E. coli* growth 1.3- to 1.6-fold (Fig. 1C, E), similar to the experiments with PBS (Fig. 1A). Two of the three Azerbaijan clays (Amircan and Surakhany) also significantly increased *E. coli* numbers 1.4-fold (Fig. 1C), which was less apparent with the same clay samples in PBS (Fig. 1A). However, with the Bangladesh Dhaka clay in deionized water there was a greater than 3-log reduction in *E. coli* viability (Fig. 1C, E), from 3.5×10⁸ CFU mL⁻¹ in the control compared with 2.9×10⁵ CFU mL⁻¹ in the treated sample. Such a dramatic effect was not observed either in samples prepared in PBS (Fig. 1A) or for any other clay tested here against *E. coli*.

Similarly, and in agreement with previous reports that Gram-positive bacteria are more susceptible to antibacterial clays (Otto *et al.*, 2010; Cafilisch *et al.*, 2018; Williams, 2019), Dhaka and additionally Paghman, Amircan and Bulbula clays in deionized water significantly decreased the numbers of *B. subtilis* recovered (Fig. 1D, F). While Bulbula and Paghman clays resulted in only 2-fold and 6-fold reductions in bacterial viability, Amircan and Dhaka clays reduced *B. subtilis* viability by 2 and more than 3-log units, respectively (Fig. 1D, F).

Effect of clay leachates on *E. coli* and *B. subtilis* growth

To determine if the antibacterial effects of clays observed in deionized water were due to the release of (antibacterial) substances from the clays into the surrounding fluid, aqueous

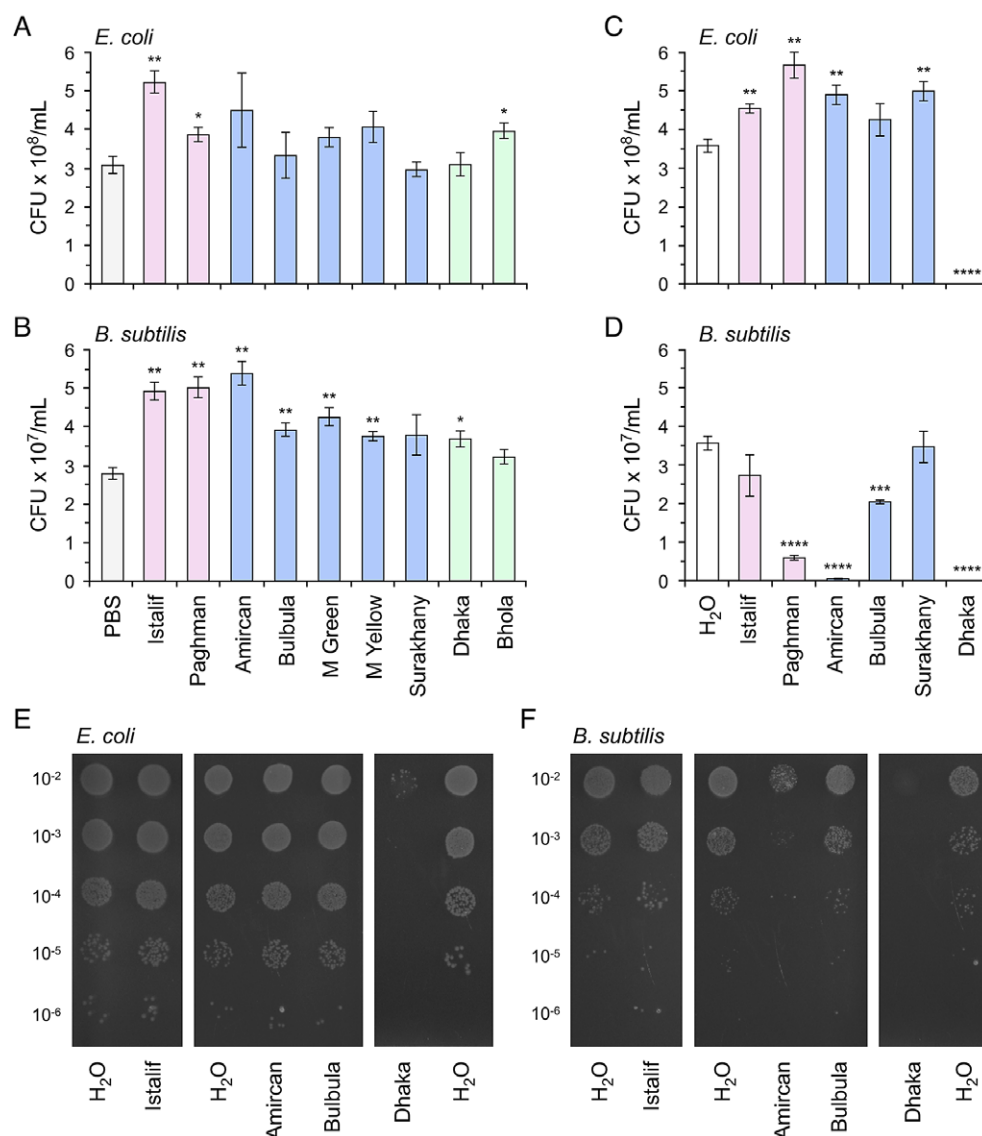


Figure 1. Effect of clay suspensions on the viability of *E. coli* and *B. subtilis*. PBS was added to bulk clays (A,B) to prepare a suspension prior to adding bacteria. Similar suspensions in deionized water were prepared with selected bulk clays (C,D). CFU mL⁻¹ was determined after incubation of plates for 16–24 h at 30°C. Data are the means and standard deviation of three independent biological replicates (*t*-test comparing each clay suspension with the PBS or water control in each case; **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001). Representative images of viability assays conducted in water are shown in panels E and F. Clays were from Afghanistan (pink), Azerbaijan (blue) and Bangladesh (green). M Green = Mashtaga Green; M Yellow = Mashtaga Yellow.

clay leachates were prepared from selected bulk clays and examined for antibacterial activity. Leachates were prepared in deionized water and mixed with either *E. coli* or *B. subtilis*, and bacterial growth was measured by absorbance after overnight incubation at 37°C. None of the leachates, including those from the Dhaka and Paghman clays, inhibited the growth of either the Gram-negative *E. coli* (Fig. 2A) or Gram-positive *B. subtilis* (Fig. 2B). In fact, improved growth was evident in almost all cases, indicating that at least some of the nutrients that promoted bacterial growth in experiments in PBS and deionized water (Fig. 1) were released into solution. It is possible that mobilized particulates and/or colloids in the clay leachates contributed to the measured OD_{600 nm} and, thus, were masking bacteriostatic or bactericidal effects in these experiments. Hence, leachate from the Dhaka clay, i.e. the clay with the greatest antibacterial activity in suspension, was

examined in additional viability assays. *E. coli* and *B. subtilis* were exposed to the Dhaka leachate and CFU mL⁻¹ determined. The leachate caused no significant reduction in bacterial viability with either of the bacterial species in comparison with a water control (Fig. 2C, D). The possibility that bacterial–clay interactions or products of bacterial metabolism could facilitate release of factors responsible for toxicity was also investigated. Leachates were prepared from the Dhaka clay either without bacteria, as before, or with the addition of *E. coli* or *B. subtilis* (OD_{600 nm} 0.4) to the clay suspension (0.25 g mL⁻¹) for 24 h at room temperature. There was no significant difference in the growth of *E. coli* between the clay leachate alone and the *E. coli*-clay-derived leachate (Fig. 2E). However, there was substantially improved *B. subtilis* growth in the *B. subtilis*-Dhaka clay leachate over the leachate prepared in the absence of bacteria (Fig. 2F). This is possibly due to bacterial killing

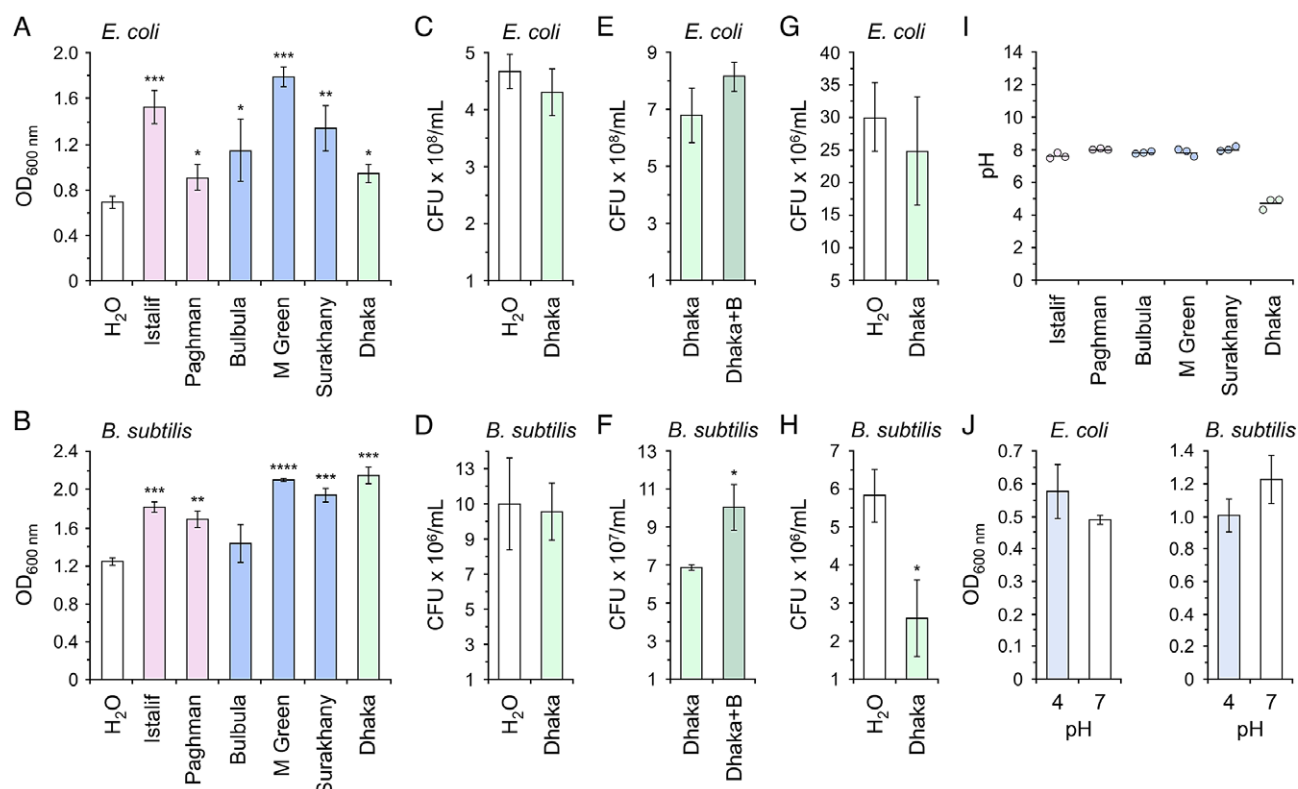


Figure 2. Effect of clay leachates and pH on the growth and viability of *E. coli* and *B. subtilis*. (A,B) Aqueous leachates prepared in sterile deionized water were mixed with *E. coli* (A) or *B. subtilis* (B) and optical density at 600 nm measured after incubation at 37°C for 16 h. M Green = Mashtaga Green. (C,D) Viability of *E. coli* and *B. subtilis* exposed to the Dhaka leachate. (E,F) Effect of mixing bacteria and Dhaka clay on leachate antibacterial efficacy. Leachates were prepared without (Dhaka; green) or with addition of the relevant bacterial species (Dhaka+B; darker green). (G,H) Influence of Dhaka clay contact on antibacterial efficacy. Bacteria were placed in tubes containing a dialysis membrane and immersed in the Dhaka clay suspension or water for 24 h and viability determined. (I) Effects of clays on the pH of aqueous leachates. The pH was measured from leachates prepared in sterile deionized water for 24 h. The horizontal line denotes the mean and circles denote the pH measurements from three independent experiments. (J) Water samples at pH4 and pH7 were mixed with bacteria and absorbance measured after incubation at 37°C for 16 h. Data are the means and standard deviation of three biological replicates (*t*-test comparing each clay leachate with the water control (A–D, G–H), against leachates without or with bacteria added to the suspension (E–F), or water at pH4 compared with that at pH7 (J); **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001).

in the clay suspension releasing metabolites to the leachate that promoted *B. subtilis* growth, although nutrients liberated from the clay itself in contact with bacteria could also be involved.

Further experiments were conducted with the Dhaka clay to determine whether contact with the clay in suspension was necessary for antibacterial activity. Bacteria were placed in tubes containing a dialysis membrane window and immersed in the Dhaka clay suspension. Small molecules, including metal ions, but not clay particles should be capable of traversing the semi-permeable membrane. Viability was measured as before by determining CFU mL⁻¹. No significant change in viability was evident with *E. coli* exposed to the Dhaka suspension relative to the water control (Fig. 2G). Although there was a 2.2-fold reduction in *B. subtilis* viability under the same conditions (Fig. 2H), this was considerably less than the 3-log reduction observed with direct exposure to the clay suspension (Fig. 1D, F).

As the suspension pH value influences metal release from clays (Williams, 2017), the pH of the leachates was determined (Fig. 2I). Deionized water does not provide pH-buffering, and hence the Afghanistan and Azerbaijan clay leachates all had a modestly alkaline pH of between 7.6 and 8.0. In contrast, the Bangladesh Dhaka clay leachate exhibited a slightly acidic pH of 4.9 (Fig. 2I). To confirm whether or not acidic conditions alone were responsible for the antibacterial effect of this clay, *E. coli* and *B. subtilis* were

incubated in water at either pH4 or 7 and optical density measured after 24 h. The results show no significant difference in bacterial growth under these conditions (Fig. 2J), confirming that an acidic pH alone did not adversely affect *E. coli* and *B. subtilis*, in agreement with the known acid tolerance mechanisms present in these two species (Lund *et al.*, 2014).

Metal content of aqueous leachates

In agreement with the observed lack of antibacterial effects of clay leachates (Fig. 2), none contained Al and Fe, or other known toxic elements such as Ag, Cu, Cd, Se, Hg and As (Table S1, Supplementary material) at levels above the maximum tolerable concentrations known (Cu, Zn, Fe: 250 µg mL⁻¹; Mn: 100 µg mL⁻¹; Ni, Pb, Cd: 50 µg mL⁻¹; Nies, 1999), with the exception of the Dhaka clay leachate. This clay exhibited the greatest antibacterial activity in suspensions against *E. coli* and *B. subtilis* (Fig. 1) and its leachate contained much higher levels of Fe, Mn and Zn compared with the other leachates (Table S1, Supplementary material). Hence, it is plausible that the acidic environment found in Dhaka clay suspensions (pH4.9) facilitated the release of these elements. Because antibacterial activity was observed primarily in suspensions of this clay and not in the leachate, it was concluded that the chemical toxicity of the metals was not sufficient to kill the

bacterial species tested (Nies, 1999). It can be deduced that additional physical and/or chemical interactions of the bacteria in contact with the clay is required.

Clay and clay mineral characterization

In an effort to understand the mechanism(s) by which the clay particles might be responsible for the observed antibacterial

activities, the clays and their mineral constituents were examined further. The mineralogical composition of Afghanistan, Azerbaijan, and Bangladesh bulk clays as determined by XRD (Fig. 3A; Table S2 in the Supplementary material) was dominated by quartz, feldspars, calcite and illite. Variations in the relative abundance of the minerals are reflected in the SiO_2 , Al_2O_3 , Na_2O , K_2O , and CaO contents quantified in parallel by XRF (Tables S3 and S4, Supplementary material). Notably, none of the clays assessed by

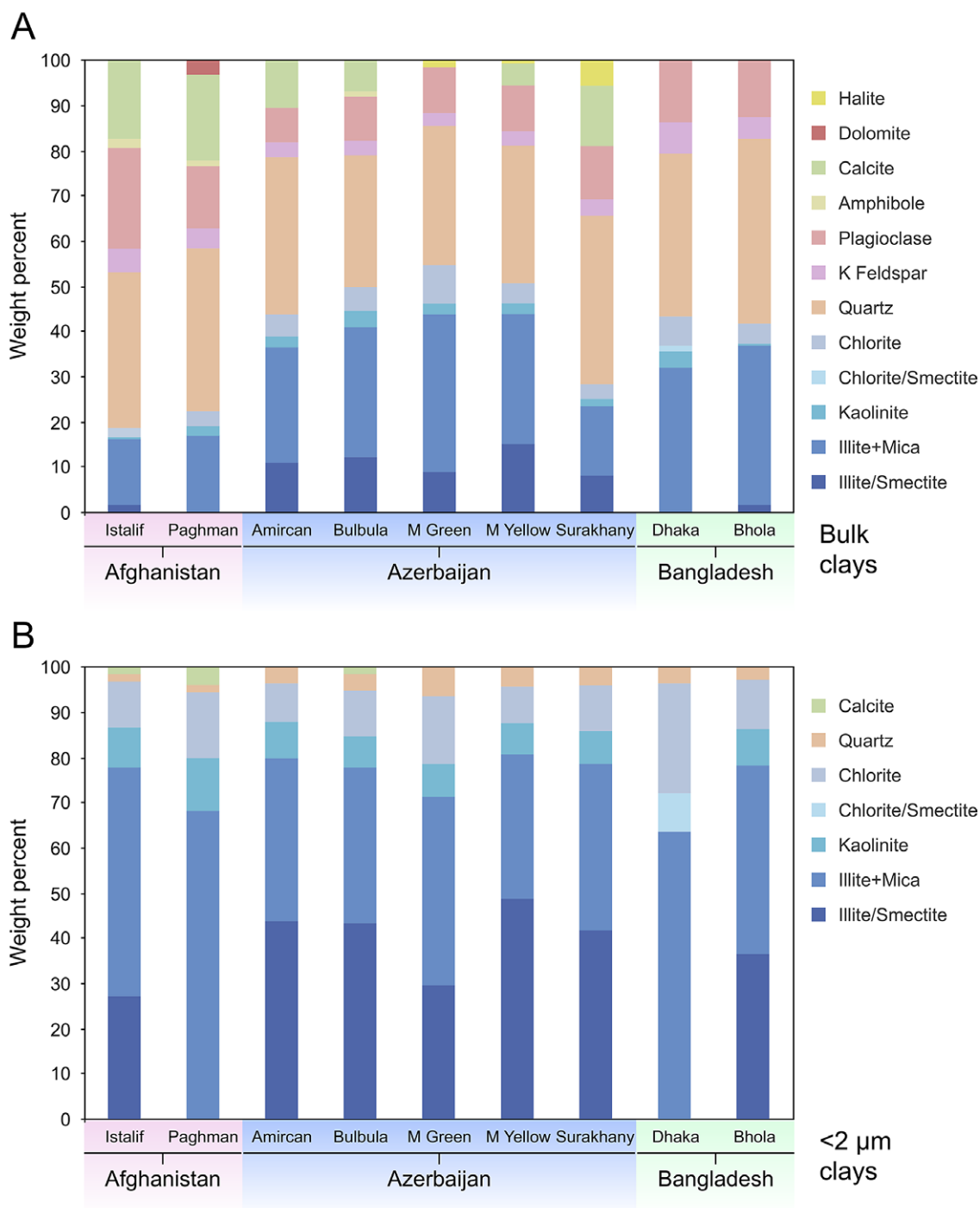


Figure 3. Mineralogical analysis of Afghanistan, Azerbaijan, and Bangladesh clays. (A) Distribution (wt.%) of quartz, K-feldspar, plagioclase, amphibole, calcite, dolomite, halite, and clay minerals in the bulk clay samples. (B) Distribution (wt.%) of clay minerals in the <2 μm clay samples. Small quantities of calcite and quartz can be found in most of these samples. M Green = Mashtaga Green; M Yellow = Mashtaga Yellow.

XRD contained pyrite (Table S2, Supplementary material), a non-clay mineral that has been associated with antibacterial activity of natural clays (Williams, 2017; Gomes *et al.*, 2020).

Further analysis of the <2 μm separated clay fractions was conducted, in which previous studies had identified antibacterial components such as smectite in Oregon Blue clay (Morrison *et al.*, 2016; Williams, 2017). Illite-smectite (rectorite) dominated the Oregon Blue clay and served as a reservoir for interlayer reduced Fe sourced from other mineral components of the clay (Morrison *et al.*, 2014; Morrison *et al.*, 2016). Varying contributions of mixed-layer illite/smectite, illite (+mica), kaolinite, chlorite, mixed-layer chlorite-smectite were found (Fig. 3B; Table S5, Supplementary material) that were linked to differing amounts of MgO, Fe₂O₃, and TiO₂ (Table S3, Supplementary material). Quartz persisted in all <2 μm fractions, and calcite in three, but all other non-clay minerals were absent (Fig. 3; Tables S2 and S5, Supplementary material), most likely being retained in the coarse fraction during the separation process. The relative proportions of mixed-layer illite/smectite, mixed-layer chlorite-smectite, illite, chlorite, and kaolinite were identified in these fractions (Fig. 3), yet with no clear pattern relative to the antibacterial activity, or lack thereof, observed for the different clays. In all air-dried samples, the smectite d_{001} basal-spacing value was approximately 14.0 Å, indicating that the interlayer of smectite is occupied by Ca²⁺, although this could also be due to intercalation of organic matter. In all samples, except those from Paghman and Dhaka, the smectite contained 20–40% illite, which suggests that the clay mineral is a randomly interstratified (R0) mixed-layer illite-smectite. The presence of mixed-layer illite-smectite further implies that the smectite is dioctahedral, as only dioctahedral smectite can transform into illite (Worden and Morad, 2003). In contrast, analysis of the Dhaka sample from Bangladesh revealed the presence of a mixed layer chlorite-smectite instead, consistent with the smectite in these samples being trioctahedral as only trioctahedral smectite transforms into chlorite (Worden and Morad, 2003). Because the clay from Dhaka displayed the highest antibacterial effect toward both *E. coli* and *B. subtilis* (Fig. 1) and it is the only clay containing

chlorite-trioctahedral smectite, it was hypothesized that these mineral components may be linked to the antibacterial activity of this clay.

Because all of the clays contained significant amounts of Fe (4.2–6.7% Fe₂O₃; Table S3, Supplementary material) and the presence of Fe(II) in minerals has been linked to antimicrobial activity (Morrison *et al.*, 2016; Wang *et al.*, 2017; Xia *et al.*, 2020; Guo *et al.*, 2021; Xia *et al.*, 2023), the Fe speciation in selected samples was investigated further using Mössbauer spectroscopy (Figs S2 and S3, Supplementary material). All samples, i.e. those displaying antibacterial activity (Dhaka, Amircan, Paghman) and those that did not (Bhola, Mashtaga Green and Yellow, Surakhany) contained substantial amounts of Fe(II), varying from 21 to 51% (Table S7, Supplementary material), with the Fe(II) content of antibacterial active clays (Dhaka > Amircan >> Paghman) falling within the middle of this range (31–37%). It was concluded that Fe(II) content alone was not predictive for antibacterial activity in the natural clays investigated here.

The values of the hyperfine parameters center shift (CS) and quadrupole split (QS) of the doublets in the Mössbauer spectra collected at 293K were analyzed and found to be typical for octahedral Fe(II) and Fe(III) (Fig. 4A; Dyar *et al.*, 2006). The distribution of the hyperfine parameters' values followed no particular pattern that could explain why some samples displayed antibacterial activity and others not (Fig. S4, Supplementary material). An apparent outlier of the Fe(II) doublet CS values in the sample from Amircan was noted, which could potentially indicate an increased propensity for antibacterial activity, yet this is neither sufficient nor unambiguous, as Fe(II) doublets in clay samples with similar or even higher antibacterial activity (Paghman, Dhaka) do not exhibit these unusual CS values. Interestingly, an additional Fe component in the Mössbauer spectrum of the clay from Surakhany was found with parameters consistent with the presence of FeS (Schröder *et al.*, 2020) (Fig. S4B, Table S6, Supplementary material). This minor component was likely not detected by XRD due to its low abundance (representing 8% of the total Fe in the clay fraction) and/or low XRD crystallinity.

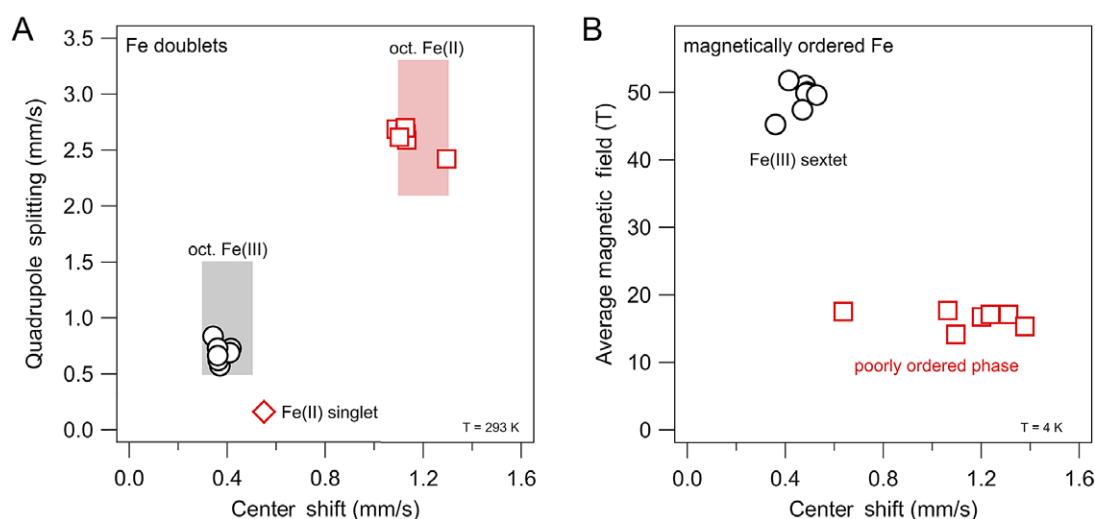


Figure 4. Comparison of hyperfine interaction parameters obtained from Mössbauer spectra of selected clay samples. (A) Center Shift (CS) and Quadrupole Splitting (QS) of the Fe doublets observed in Mössbauer spectra collected at room temperature (293K, spectra in Fig. S2 of the Supplementary material) all fall within the typical range of octahedral Fe(III) (grey shaded area; from Dyar *et al.*, 2006) and octahedral Fe(II) (red shaded area; from Dyar *et al.*, 2006). The Fe(II) singlet values for FeS found in Surakhany clay is also included for comparison. (B) Values of CS and the average magnetic field (H) of the (partially) magnetically ordered Fe phases observed in Mössbauer spectra collected at 4K (spectra in Fig. S3, Supplementary material) cluster together as Fe(III) phases (black circles) and mixed-valent Fe(II)-Fe(III) phases (red squares). More detailed resolution of the data in panels A and B, respectively, is provided in Figs S4 and S5 in the Supplementary material.

Because Surakhany clay was not effective in antibacterial assays (Fig. 1), it is suggested that, in contrast to pure FeS (Cheng *et al.*, 2016; Cheng *et al.*, 2020), its low abundance in natural clays is insufficient for the effective mineral-derived production of OH radicals, which have been linked to antibacterial effects (Wang *et al.*, 2017).

Likewise, Mössbauer spectra collected at 4K were, overall, very similar for all clay samples analyzed. At this low temperature, a proportion of the Fe(III) became magnetically ordered into sextets (Fig. S3, Supplementary material) with hyperfine parameters CS, QS and the average magnetic field (H) typical for Fe(III) phases such as goethite, lepidocrocite or ferrihydrite (black circles in Fig. 4B; Table S6, Supplementary material) (Cornell and Schwertmann, 2003; Murad and Cashion, 2004). Additionally, a significant portion of the spectral area (16–53%; Table S6, Supplementary material) was occupied by a poorly ordered component with CS values between those characteristic of Fe(II) and Fe(III) (cf. Fig. 4A) and H values below those usually obtained for pure Fe(II) minerals ($H < 20T$) (Murad and Cashion, 2004), pointing toward this component being a mixed-valent Fe(II)-Fe(III) phase (red squares in Fig. 4B; Table S6, Supplementary material). This dataset fails to show a particular pattern that could explain why some samples displayed antibacterial activity and others not, suggesting that Fe speciation in the minerals was not a determinant factor in the sample set.

Provenance and origin of clay minerals

In the mineralogical composition of deposits, provenance can be one of the factors that controls mineralogical assemblages, including the chemical composition of clays, the presence of accessory minerals and the abundance of (toxic) metals. Based on the major oxide content of bulk clays (Table S3, Supplementary material), a discrimination diagram (Roser and Korsch, 1988) was constructed which shows that the clays from Afghanistan and Azerbaijan plot into the P2-Intermediate field and clays from Bangladesh lie in the P4-Quartzose field (Fig. 5). In combination with the cross plot diagram of TiO_2 vs Al_2O_3 (Fig. S6, Supplementary material) that identifies eroded deposits in the

provenance (McLennan *et al.*, 1979), the results indicate that the Azerbaijan clays are likely derived from the middle Jurassic diabase, gabbro-diabase, and sandstones of the Greater Caucasus. The Palaeoproterozoic gneiss in the mountains around the Kabul basin was the most important source for the Afghanistan clays, whereas Bangladesh clays were weathering products of the supracrustal rocks and sandstones of the eastern Himalayas. The high CIW values (Eqn 1; Fig. S7, Supplementary material) calculated for all clays indicate that the rocks in the mountains surrounding the Kabul basin, the Greater Caucasus and the eastern Himalayas have experienced similarly intensive chemical weathering conditions in a humid climate. It is therefore suggested that the R0 mixed-layer illite-smectite in the samples (Worden and Morad, 2003) can be an alteration product of dioctahedral smectite under low temperatures (e.g. 47–68°C) in the basins; the amount of illite present in the mixed layer illite-smectite is less than 40%, consistent with the early stage of smectite transformation into illite (Weibel, 1999). The R1 chlorite-smectite mixed layer found in the Bangladesh samples from Dhaka could suggest alteration of trioctahedral smectite (e.g. saponite) under temperatures of 60–70°C (Chang *et al.*, 1986).

Finally, the correlation of minor elements with Al_2O_3 was investigated (Fig. 6) as a proxy for their presence within the interlayer of smectites in illite-smectite mixed layers. Correlations with the Al_2O_3 content were strong for Cs, Co, Cu, and Ni, moderate with two relevant outliers for Zn and Cr, and absent for Pb and As. Those elements likely located within the interlayer of smectites in our samples (Cs, Co, Cu, Ni; most Zn and Cr) could be released into the aqueous phase upon contact with water, providing a mechanism for the presence of these (toxic) elements in the leachates. Only Zn was found in relevant amounts in the leachates (Table S1, Supplementary material), presumably due to its higher solid phase concentration compared with the other elements. In contrast, Pb and As were not located within the interlayers of smectites according to our analysis. Consequently, their presence in the leachates was generally low, with the exception of Pb in leachates of Mashtaga Green (60 ppm; Table S1, Supplementary material), consistent with the elements' low solid phase concentrations and a mineral dissolution mechanism, which would be less facile and slower than release from smectite interlayers.

Conclusions

The results revealed that most of the clays from the three different study areas do not possess antibacterial properties, despite the traditional and current use of Azerbaijan and Bangladesh clays for cleansing and therapeutic purposes. This is perhaps not too surprising as only 5–10% of natural clays are estimated to exhibit antibacterial activity (Williams, 2017; Williams, 2019). Most of the clay suspensions and leachates actually promoted bacterial growth, with the latter findings indicating that some of the nutrients responsible for promoting bacterial growth must be readily released into solution. Similar results were observed with the Wyoming SWy-1 bentonite clay (Williams *et al.*, 2004) and some French Green clays (Haydel *et al.*, 2008; Williams *et al.*, 2008) and probably result from the provision of additional organic and inorganic nutrients, likely including carbohydrates (Sezonov *et al.*, 2007; Dong *et al.*, 2022), organic material from diverse bacterial species found in clays (Svensson *et al.*, 2017) or, as confirmed by these measurements, high levels of Na, Mg, K, and

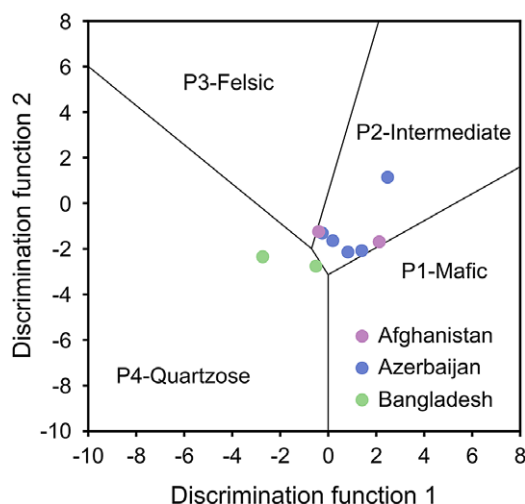


Figure 5. Discrimination plot of discriminant function 1 and 2 showing a mixed provenance for the argillaceous rocks from the Afghanistan, Azerbaijani and Bangladesh clays. The four main provenance groups are P1-mafic, P2-intermediate, P3-felsic and P4-quartzose.

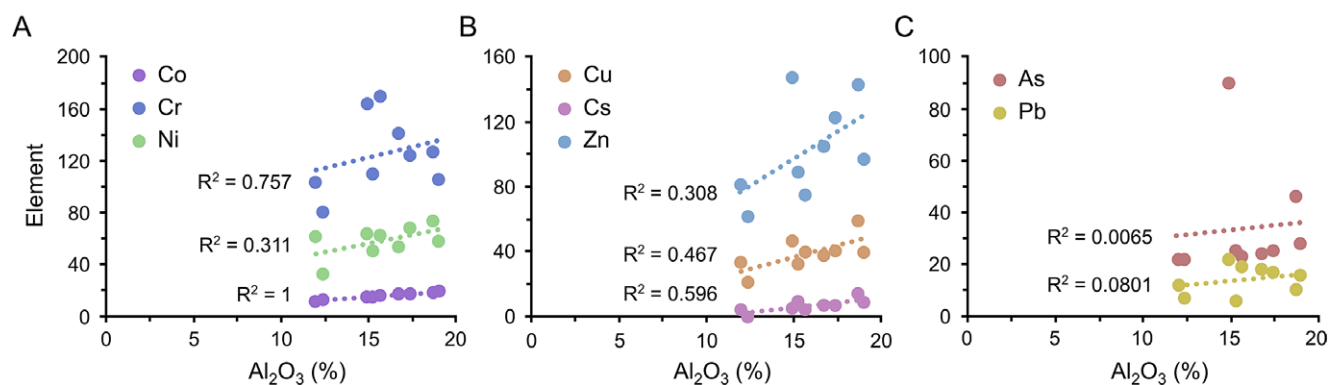


Figure 6. Content of selected trace metals in Afghanistan, Azerbaijan and Bangladesh clays as a function of clay Al_2O_3 content, which is used as a proxy for the abundance of smectites. (A) Co, Cr, and Ni; (B) Cu, Cs, and Zn; and (C) As and Pb.

Ca in leachates (Table S1 Supplementary material) that help stabilize osmotic stresses on the bacteria when incubated in water. The findings that the natural clay deposits stimulated bacterial growth either as bulk clay suspensions, purified clay mineral fractions or as aqueous leachates suggest caution in administering uncharacterized clays for beauty, cleaning, and medical applications.

Antibacterial testing further identified likely bacteriostatic clay suspensions (Afghanistan: Paghman; Azerbaijan: Amircan, Bulbula) with activity against the Gram-positive *B. subtilis* but not the Gram-negative *E. coli*. The Bangladesh Dhaka clay, however, possessed bactericidal activity against both bacterial species, and activity correlated with measured acidic conditions (pH4.9), which are known to assist the solubilization and release of toxic metals (Cunningham *et al.*, 2010; Williams and Haydel, 2010; Morrison *et al.*, 2014; Morrison *et al.*, 2016; Behroozian *et al.*, 2020). Higher levels of Mn and Zn, and particularly Fe, present in the leachate from this clay (Table S6, Supplementary material) are good candidates in terms of recognized antibacterial efficacy (Londoño *et al.*, 2017; Williams, 2019; Behroozian *et al.*, 2020), even though the Dhaka leachate itself did not show antibacterial activity. It is feasible that toxic metal ions are delivered on contact with particles in suspension, as noted previously (Xia *et al.*, 2023), and may function synergistically to kill bacteria especially at low pH (Williams, 2019). Moreover, the Dhaka clay contained very little of the smectite that is typical of many antibacterial clays and no significant amounts of accessory minerals such as the Fe-sulfide pyrite that have been linked to acidic, high metal and consequently antibacterial conditions (Morrison *et al.*, 2014; Morrison *et al.*, 2016; Williams, 2017). Hence, alternative mechanisms that require the presence of the clay itself must be considered, with the presence of chlorite-trioctahedral smectite as the only identifiable unique characteristic that is absent in all of the other clays not displaying antibacterial activity. It is speculated that Dhaka clay's chlorite-smectite might contain divalent metals such as Fe, Mn, and Zn as impurities in the brucite sheet, which could explain their release into deionized water at mildly acidic conditions (pH4.9). This hypothesis would also allow for increased localized metal release upon direct contact of the chlorite-smectite with bacteria as a plausible mechanism for the increased antibacterial activity observed here.

Lastly, dramatically different results of antibacterial activity when clays were tested in PBS compared with in sterile deionized water demonstrate the importance of using relevant experimental conditions for the desired application. In particular, pH buffering may be undesirable in clay suspensions for medical applications as

others have demonstrated (Morrison *et al.*, 2016), and confirmed here, because the desired antibacterial outcomes are often linked to induced lowering of the pH value that subsequently promotes the release of toxic metals. Moreover, this logic also suggests that if pH buffering is required in the desired application, results from experiments without pH buffering may be indicating unrealistically high antibacterial effects and care needs to be taken when translating and transferring results from highly controlled laboratory experiments to application under real-world conditions.

Supplementary material. The supplementary material for this article can be found at <http://doi.org/10.1017/cmn.2024.7>.

Data availability statement. The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Competing interest. The authors declare none.

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