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Rapid and reliable detection of bacterial endospores in environmental samples by diagnostic electron microscopy combined with X-ray microanalysis

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Abstract

Diagnostic negative staining electron microscopy is a front-line method for the rapid investigation of environmental and clinical samples in emergency situations caused by bioterrorism or outbreaks of an infectious disease. Spores of anthrax are one of the diagnostic targets in case of bioterrorism, because they have been used as a bio-weapon in the past and their production and transmission are rather simple. With negative staining electron microscopy bacterial spores can be identified based on their morphology at the single cell level. However, because of their particular density, no internal structures are visible which sometimes makes it difficult to distinguish spores from particles with a similar size and shape that are frequently present in environmental samples. Spores contain a high concentration of calcium ions besides other elements, which may allow a proper discrimination of spores from other suspicious particles. To investigate this hypothesis, negative staining electron microscopy, using either transmission or scanning electron microscopes, was combined with energy dispersive X-ray microanalysis, which reveals the element content of individual nanoparticles. A peak pattern consisting of calcium, sulphur and phosphorus was found as a typical signature within the X-ray spectrum of spores in various Clostridium and Bacillus species, including all strains of anthrax (Bacillus anthracis) tested. Moreover, spores could be reliably identified by this combined approach in environmental samples, like household products, soil or various presumed bioterrorist samples. In summary, the use of X-ray spectroscopy, either directly in the transmission electron microscope, or in a correlative approach by using scanning electron microscopy, improves the emergency diagnostics of suspicious environmental samples.

Introduction

Spores of *Bacillus anthracis* (anthrax) have been used as a biological weapon and are considered as putative agents for bioterrorism (Moran et al., 2008). In cases of their presumed distribution into the environment, accidentally or intentionally, a diagnosis must quickly prove or rule out the presence of infectious spores to allow a better risk assessment and case management (Bossi et al., 2006). Diagnostic electron microscopy is one method of the diagnostic panel which is available for the analysis of putative bioterrorist samples regarding pathogenic agents. The role of electron microscopy is to provide an overview of the micro and nanoparticles within a sample and, in case of a suspicious finding, to give a hint towards a pathogen and thereby speeding up the whole diagnostic process. The major advantage of diagnostic electron microscopy is that it does not exclude any pathogen from detection, even if it has been altered by mutation or genetic engineering. Moreover, it is comparatively fast, gives an orientation about all particles included in a sample already at the beginning of an investigation and provides a quick and independent control at later stages (Gentile and Gelderblom, 2005).

Negative staining electron microscopy is the first choice for diagnostic electron microscopy of infectious microorganisms, including viruses, in clinical or environmental samples, basically because preparation of samples is quick and simple (Hazelton and Gelderblom, 2003). A huge collection of reference images recorded from negatively stained microorganisms is available in literature and

provides a solid basis for the diagnostics. Ultrathin section electron microscopy can reveal additional information for a diagnoses (Miller, 1986 and Curry et al., 2006), but usually needs significantly more preparation time than negative staining electron microscopy, even if rapid protocols are employed (Laue, 2010).

The detection of spores by negative staining electron microscopy is generally possible (Laue and Bannert, 2010). Spores that possess a typical exosporium, like spores of anthrax, can be distinguished from spores which are lacking this particular outer coat. However, because of the high density of the spores, no internal structures can be used for identification or a further classification (Laue et al., 2007). In environmental samples the discrimination between spores and other particles of similar size, shape and density, is not always trivial (Fig. 1). Spores contain a considerable amount of calcium within the spore core and sometimes other particular elements (e.g. manganese) (Setlow, 2006), which could help to distinguish spores from other, for instance inorganic particles. Investigation of spores by scanning-transmission electron microscopy and X-ray spectroscopy, using thin cryosections, could reveal the main element composition of the spore and their distribution among the different structures (Scherrer and Gerhardt, 1972, Stewart et al., 1980 and Stewart et al., 1981). In addition to calcium, phosphorus, sulphur, manganese and silicon were found. However, not all of the elements could be detected in every species and strain. At least calcium and phosphorus could be detected in every spore by X-ray microanalysis. Entire spores, so far, were only analysed by X-ray microanalysis in conjunction with scanning electron microscopy to compare the calcium content among different species (Hintze and Nicholson, 2010) or to follow the release of calcium during germination (Bassi et al., 2009).

To check if the element composition of spores can be used as an additional criterion to discriminate spores from other particles in environmental samples, we have analysed spores of different species and strains as well as mixtures of spores and environmental samples by using X-ray spectroscopy in conjunction with negative staining electron microscopy.

Materials and methods

Bacterial strains and generation of spores

Spores of *Bacillus subtilis* (ATCC 6633) were used as a model for systematic testing of the imaging and the X-ray microanalysis. Bacteria were cultivated on agar plates followed by multiplication in LB medium (10 g tryptone, 10 g NaCl, 5 g yeast extract) at 37 °C on a shaker for 16 h prior to sporulation. Sporulation was induced by exchange of the LB medium for a sporulation medium according to Sterlini and Mandelstam (1969). After a few days, almost all cells produced spores which could be monitored by phase-contrast microscopy. After washing of spores twice with distilled water, a small probe was embedded for ultrathin section electron microscopy for assessment of purity and unaffected ultrastructure (Laue et al., 2007). Spores were stored in Hepes buffer (0.05 M, pH 7.4) at 5–10 °C until use.

To check for the universality of the results that were obtained by using spores from *Bacillus subtilis*, spores of the following bacterial strains were analysed: *Bacillus anthracis* 527, *Bacillus anthracis* Stamatin Sokol, *Bacillus anthracis* Sterne strain 34F2, *Bacillus anthracis* UDIII-7 (see Klee et al., 2006 for a molecular typology of the different anthrax strains), *Bacillus atrophaeus* ATCC 9372, *Bacillus cereus* ATCC 10987, *Bacillus stearothermophilus* (gke Steri-Record; gke GmbH, Germany), *Bacillus thuringiensis* (DSM 350), *Clostridium botulinum* NCTC 7272, and *Clostridium difficile* NCTC 13366. Spores were produced according to a European standard procedure (prEN 14347:2001 [D]) and stored at 4 °C in double distilled water until use.

Mock and test substances

Different mock and test substances were used to test X-ray spectroscopy in conjunction with negative staining electron microscopy and to screen environmental samples for particles (size, shape, and density) that are similar to spores. Nanoparticles of colloidal gold (100 nm; British Biocell) and polystyrene (200 nm; Nanosphere, Duke Scientific) served as a control. Bentonite, a potent absorber material, was used as a source for silicon crystals. It was also used to establish the detection limit of

the new approach, by spiking an aqueous suspension of bentonite (1 mg/ml) with a defined number of *Bacillus subtilis* spores (see Section 3.4).

Various household products with unknown composition were tested, including a cleaner (Sidol), a polish (Pulex), pepper and a particulate adsorber for cat faeces. Soil samples of different origin were screened. Numerous inactivated (10% formaldehyde and 0.05% glutaraldehyde in 0.05 M Hepes) samples of unknown composition and origin, which were delivered as presumed bioterrorist samples, were also included in the testing. Vegetative bacteria (*Escherichia coli*; *Bacillus subtilis* ATCC 6633) served as an additional control to check if they could be distinguished from spores. All powders or solid materials were suspended in distilled water to produce a suspension which was then used for the experiments.

Inactivation by chemical fixation

Samples which may contain contagious microorganisms should be inactivated to minimise the risk for an infection of laboratory personnel. Therefore, all infectious samples were inactivated by chemical fixation in 10% formaldehyde or paraformaldehyde and 0.05% glutaraldehyde in 0.05 M Hepes buffer (pH 7.4) for at least 2 h at room temperature (Kuehn et al., 2009). To evaluate the difference between inactivated and native samples, spores of *Bacillus subtilis* were either analysed with or without inactivation by chemical fixation. In some experiments spores of *Bacillus subtilis* were fixed with 2% paraformaldehyde in 0.05 M Hepes buffer.

Negative staining of samples

Spores were adsorbed at the sample support, washed and stained by heavy metals according to evaluated protocols (Laue and Bannert, 2010). As a standard sample support, copper grids (400 or 300 mesh; AGAR Scientific) that have been coated with plastic (pioloform or formvar) and carbon (to provide a better heat distribution and mechanical stability) were used. To make the surface more hydrophilic and sticky, the carbon reinforced plastic film was covered by the cationic stain alcian blue (1% in 1% acetic acid, 10 min; Laue and Bannert, 2010). After washing of the grids on four droplets of double distilled water, 10 µl of the spore suspension was dispensed directly onto the coated grid surface. During the incubation time of 10 min, spores sedimented by gravity onto the grid surface. Then, grids were washed on three droplets of double distilled water and were briefly (a few seconds) brought into contact with a droplet of the heavy metal stain. Finally, the excess of the staining solution was removed from the grid surface by means of a filter paper.

The following staining solutions were used at a concentration of 0.5% in double distilled water: uranyl acetate (UA), phosphotungstic acid (PTA), and methyl amine tungstic acid (MAT). In some experiments staining was omitted for comparison with stained samples. In those cases, grids were dried by the help of a filter paper after the final washing steps.

Airfuge (Beckman) sedimentation of samples directly onto the surface of the grid was performed as described by Laue and Bannert (2010). Briefly, alcian blue coated grids were fitted into particular grid adaptors (Type S/N 833101329, Beckman; No. 11093, Laborgeraete Beranek, Weinheim, Germany) and immersed into 80 µl of sample suspension within a centrifugation vial (Polyallomer, 5 × 20 mm, 240 µl). Centrifugation was done for 10 min at 20 psi (around 120000 *g*) using a fixed-angle rotor (A100-18, 18°). Grids were released from the adaptors using 80 µl of distilled water and stained with 0.5% uranyl acetate.

Imaging and X-ray microanalysis

Imaging and analysis by energy dispersive X-ray spectroscopy were done with a transmission electron microscope (Libra 120, Carl ZEISS, Germany) that was equipped with a digital camera (Proscan, 2 × 2k) and an X-ray detector (Sapphire, EDAX; Si/Li detector with Beryllium window, 132 eV resolution at Mn Kα). For X-ray analysis the samples were tilted 15° towards the detector and the microscope was operated at 120 kV acceleration voltage and a beam current of 7–10 µA. Further parameters for spectrum collection, like for instance time, were determined experimentally (see Results).

Scanning electron microscopy (SEM) was done by using two desktop microscopes from Hitachi (TM-1000 and TM-3000), both equipped with X-ray detectors (Bruker Quantax 50 or 70, respectively). Grids were fitted into a special grid holder, that was self-constructed by using a sample holder tip (retainer) of a Jeol transmission electron microscope glued on a 22° sample holder (Supplemental Fig. 1) or a standard multigrid-holder for SEM (No. G3662A; Plano, Germany). While spectra were collected in spot mode at the TM-1000, the analytical mode was used at the TM-3000. Acceleration voltage was 15 kV with both systems. Energy resolution was equivalent to that of the Sapphire detector.

All of the spectra were displayed using the Origin (Microcal Software Inc.) data plotting software. Spectra recorded with the Quantax detectors were filtered by adjacent averaging (10 points) to improve visibility of the major peaks and to make them comparable to the spectra recorded with the Sapphire detector, which reveals less information

Results

Spores reveal a typical spectral signature by X-ray microanalysis

In order to find the typical X-ray spectrum of untreated and unstained spores, experiments were conducted, in which spores of *Bacillus subtilis* were adsorbed at the surface of a sample support (carbon-reinforced plastic film on a copper grid) followed by three quick washes with distilled water. After drying, spores were analysed in the transmission electron microscope by X-ray spectroscopy. The resulting spectra revealed peaks of phosphorus, sulphur and calcium as typical signature (Fig. 2). While the relative peak height of the three elements was almost constant within a given population of spores, it could differ between different sporulation batches or species. Other elements, like magnesium, aluminium or silicon, could be detected as well, but were not present in all spores or were generally found in measurements of the background. Copper was found regularly in the spectra and derived from the copper grids used as a support. However, the copper signal did not cover any of the typical peaks. Experiments with chemically fixed and therefore inactivated spores qualitatively gave the same spectral pattern (not shown, but see Supplemental Fig. 2 and spectra of *Bacillus anthracis* spores, which were always used inactivated). Only few spores of a population did not show the entire typical peak pattern (3 of 52 spores in systematic measurements of fixed and unfixed spores).

Optimal parameters for preparation and diagnostic X-ray microanalysis of spores in the TEM

Negative staining electron microscopy usually involves staining and stabilisation of particles by heavy metal staining, which finally allows a diagnosis based on structural features. However, heavy metal staining might compromise the analysis by X-ray spectroscopy. To check the influence of heavy metal staining on the typical X-ray peak pattern, that has been recorded for unstained spores, *Bacillus subtilis* spores were stained with either of the following staining solutions at a concentration of 0.5% in distilled water: phosphotungstic acid (PTA), uranyl acetate (UA), and methyl amine tungstic acid (MAT). The typical peak pattern was not compromised by the negative staining (Fig. 3). However, the PTA contaminated the sample with phosphorus, which is one of the elements needed for the identification of a typical spectrum (Fig. 3B). Moreover, tungsten (i.e. the Mα peaks) may cover the rather small phosphorus peak making it invisible for an identification (Fig. 3B, D). UA may mask the important calcium peak, but even with highest peaks of uranium the calcium (Kα) peak was always clearly detectable (Fig. 3C).

To find out the optimal spectrum collection time, spores were analysed for different time periods (30, 60, 120, 200, 500, and 1000 s). Although the calcium peak appeared already during the first seconds, a collection time of 200 s was usually necessary to discriminate all relevant peaks from the continuum (not shown).

X-rays were collected from an area illuminated by the electron beam which was limited by a condenser aperture (30 µm) to an area of approximately 0.8 µm in diameter (see Fig. 2A). Changing the illumination system of the microscope to spot illumination, which reduces the illumination area to about 40 nm and increases beam intensity, did not change the signal significantly (not shown) but increased

the probability to miss one of the specific elements because they are not evenly distributed within the spore.

The typical X-ray spectrum can be obtained from different *Bacillus* species and strains and from different *Clostridium* species when using a sensitive spectrometer

Spores of different *Bacillus* species and strains were prepared by negative staining for analysis by X-ray spectroscopy in the TEM. Most of the preparations allowed the discrimination of the typical peaks (P, S, Ca) within the X-ray spectrum recorded from the spores (Fig. 4A). However, in some species, especially in some strains of *Bacillus anthracis*, spectra missed the phosphorus or sulphur peaks (Fig. 4B). Since the spectrometer used at the TEM in this study was only able to detect elements down to sodium and the overall sensitivity was low in comparison to spectrometers available today, samples were analysed in a table top scanning electron microscope equipped with a silicon drift detector at 15 kV. Grids were placed in a grid holder where the beam could pass through (see Supplemental Fig. 1) and X-ray analysis was performed by using the spot mode. The typical pattern of peaks could be recorded for all *Bacillus* species and strains (see Materials and methods) tested so far (Fig. 4 and Fig. 5). Although peaks were small, discrimination was reliable and reproducible (Fig. 5; see also Supplemental Fig. 2).

Spores of *Clostridium botulinum* and *Clostridium difficile* were also analysed for their X-ray spectrum using the TM-1000 table top scanning electron microscope. Both species revealed the typical spectral signature in their X-ray spectrum (Supplemental Fig. 3).

Spores can be clearly identified in environmental samples by using morphological criteria and the typical composition of elements

In environmental samples discrimination of putative spores from other particles, e.g. inorganic crystals, is frequently not safe by using morphological criteria only. By employing X-ray microanalysis and the measurement strategies established in the preceding experiments, it was possible to analyse the element composition of individual particles in negatively stained environmental samples. In the different samples tested, particles were found which resembled spores regarding their morphology. However, by X-ray microanalysis they never showed the typical spectral signature of phosphorus, sulphur and calcium that can be recorded for spores. Even if the spectrum of a non-spore particle showed an overlap with the typical spectrum of spores, complete identity was never found (Fig. 6A–D).

In mixtures of *Bacillus subtilis* spores with different mock substances (e.g. household products) or complex environmental samples, it was always possible to discriminate the spores clearly from other particles (Fig. 6E, F). To check the reliability of the approach systematically, a test panel of twenty environmental samples from the institute's stock of investigated putative bioterrorist samples (all of them negative for pathogens) was partially mixed with spores of *Bacillus subtilis* (final concentration was 10^8 spores/ml), blinded to the investigator and analysed by negative staining electron microscopy followed by scanning electron microscopy and X-ray microanalysis. In all cases an unequivocal identification of spores was possible and no false positive diagnosis was recorded. In three of the twenty samples X-ray microanalysis was essential to exclude the suspicion that spores are present in a sample which was raised by the preceding morphological analysis. A representative image from such an analysis is shown in Fig. 7 (see also Supplemental Fig. 4).

To get an idea about the detection limit of the combined approach between morphological investigation and X-ray microanalysis, a suspension of bentonite (1 mg/ml) was spiked with spores of *Bacillus subtilis* at different defined final concentrations. Ten samples per defined spore concentration were systematically inspected with a scanning electron microscope (TM-1000) by screening 22 meshes using a regular pattern which involves all regions of the sample (Laue and Bannert, 2010). Particles revealing the morphology of spores (size, shape, and density) were analysed by X-ray microanalysis to check the X-ray spectrum for the typical signature of spores. By using this strategy, the detection likelihood was 100% (10/10 positive samples) for a spore concentration of 10^6 spores/ml and 80% (8/10 samples were positive) for a concentration of 10^5 spores/ml. Since only few spores could be found in samples prepared from suspension that have been spiked with 10^6 spores/ml, the preparation of lower concentrations was done by using airfuge sedimentation.

Vegetative bacteria are usually morphologically distinguishable from spores. However, we analysed vegetative bacteria of *Escherichia coli* and *Bacillus subtilis* by X-ray microanalysis. Their X-ray spectra were rather variable (even within members of the same population) and showed minor peaks of sulphur, phosphorus, magnesium, chloride and/or nitrogen in different combinations, besides major peaks of carbon, oxygen and systemic peaks. Significant peaks of calcium were never recorded (not shown).

Discussion

Calcium, phosphorus and sulphur could be regularly detected in spores of several *Bacillus* and *Clostridium* species by using transmission electron microscopy or scanning electron microscopy in conjunction with energy dispersive X-ray spectroscopy (EDX). With all strains and preparations the calcium peak was about the same or larger than the smallest of the phosphorus and sulphur peaks. As a conclusion of our observations, we suggest to consider a spectrum as spore-specific, if the following criteria are met: (1) presence of specific peaks of phosphorus, sulphur and calcium; and (2) height of the calcium peak should be equal or higher than the smallest of the peaks of phosphorus and sulphur.

In former studies on the element composition of spores these characteristic elements have been detected in sections of different *Bacillus* species (Scherrer and Gerhardt, 1972, Stewart et al., 1980 and Stewart et al., 1981) and also in entire spores (Bassi et al., 2009 and Hintze and Nicholson, 2010). However, the present study shows that the particular element signature can be used to discriminate spores from other particles present in environmental samples. The prominent calcium content alone is not sufficient for an unequivocal discrimination from non-spore particles, because calcium is often present in inorganic crystals while no other elements are showing up. Thus the detection of the phosphorus and sulphur at similar or lower count rates than the calcium is necessary for a reliable discrimination of spores from other particles with a similar morphology. It remains however unclear if particles with similar characteristics (morphology and EDX spectrum) are present in environmental samples at all or not. Although our test samples cover a wide range of substances, including presumed but negative bioterrorist samples, we cannot rule out that such particles exist. So far, we have not recognised any false positive diagnosis.

Detection of spores by a combination of morphology and EDX seems to be robust and reproducible, if a standard negative staining preparation using uranyl acetate, which does not cover any of the specific signals, is used. The spectroscopy was successful with different microscopes and spectrometers for most spores and a spectrum collection time of 200 s. However, we recognised that at least one combination of microscope and spectrometer (Zeiss Libra 120 and EDAX Sapphire) was not able to detect the characteristic elements in all bacterial strains tested, although they were present in spectra collected with other machines. This discrepancy could be due to a difference in the sensitivity of the EDX detector (Si/Li versus Silicon drift detector). As a consequence, a careful pre-analysis of the detection capabilities of the microscope and the X-ray detector has to be performed before using measurements of the element composition as an additional criterion for a diagnosis. Spores of *Bacillus atrophaeus* seem to be a good test object, because of their comparatively low calcium content in our measurements and their commercial availability as a bioindicator to test the success of sterilisation procedures. Further validation of the detection procedures should involve spores of possible target species, e.g. spores of different *Bacillus anthracis* strains, and the inactivation procedure used.

Spores of different species and strains reveal differences in their X-ray spectra, mainly concerning the absolute height of the signal and sometimes also the relative height of the typical peaks. However, those differences are not robust enough to use them for specific species detection. The calcium content may also depend on the conditions during sporulation (Bassi et al., 2012). Discrimination of spores from different *Bacillus* species at the single particle level so far is only possible by using a combination between light microscopy and Raman spectroscopy (Stöckel et al., 2012). However, the method still needs a purification process to pre-process the sample. A sequential correlative approach by scanning electron microscopy, providing a higher spatial resolution than light microscopy as well as a large field of view, followed by Raman spectroscopy could be a promising alternative to the methods presented so far.

The combination of morphological with chemical criteria to detect spores is useful for the rapid detection of spores in environmental samples. It is also likely that the method is applicable for the detection of spores in other complex samples (e.g. food, clinical samples), because spore detection was successful in a variety of environmental samples with different chemical features. Detection of spores was feasible by using even simple scanning electron microscopes that are equipped with silicon drift detectors, like the desktop scanning electron microscopes used in this study. For a more generic diagnosis of environmental samples regarding pathogens including viruses, transmission electron microscopy in conjunction with EDX must be used, because of the resolution limit and time needed for the formation of suitable images in most scanning electron microscopes. In this paper we have shown that this method is compatible with the standard negative staining preparation and inactivation procedures using aldehydes. It not only reveals the morphology of particles at the highest spatial resolution but also allows the measurement of the chemical composition of a suspicious particle within 200 s. Thus, the method is valuable for the screening of many particles in a sample within a reasonable time. However, a further reduction of spectrum collection time would be beneficial to speed up the EDX analysis of individual spores. The measurement of elements not necessarily needs to be conducted in the same microscope in which the morphological inspection is performed. Correlative procedures, like those shown in this paper, which involve a screening of a sample by transmission electron microscopy followed by scanning electron microscopy in conjunction with EDX, can also be applied.

Our experiments on the detection limit indicate that at least high concentration of spores (i.e. $\geq 10^6$ spores/ml) can be reliably (i.e. 100% detection likelihood) detected in a short time. This finding is in the range of the detection limit that has been established for pure spore suspensions (Laue and Bannert, 2010). It is notable that the method is extremely useful to exclude the presence of a high concentration of spores within a complex sample, which may help to facilitate risk assessment or to sort out relevant samples for a further analysis. Detection of putative bioterrorist agents in complex samples, like environmental samples, usually is difficult and may be impaired, regardless of the method employed (Kurth et al., 2008). As a consequence, different methods should be used in parallel to analyse complex samples for pathogens. In summary, the combination of morphological and chemical measures leads to a reliable detection of spores in environmental samples by electron microscopy and improves our panel of methods for the detection of putative bioterrorist samples.

The following are the supplementary data related to this article.

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Tables and Figures

Figure 1. Negative staining electron microscopy of a putative bioterrorist sample of unknown composition (“white powder”). A. Overview of a typical area showing crystal-like particles of different sizes and shapes, some of them resembling spores. B, C. Comparison between a crystal-like particle (B) from a putative bioterrorist sample and a spore of *Bacillus subtilis* from a reference sample (C). Both are almost impossible to distinguish without any doubt based on morphology.

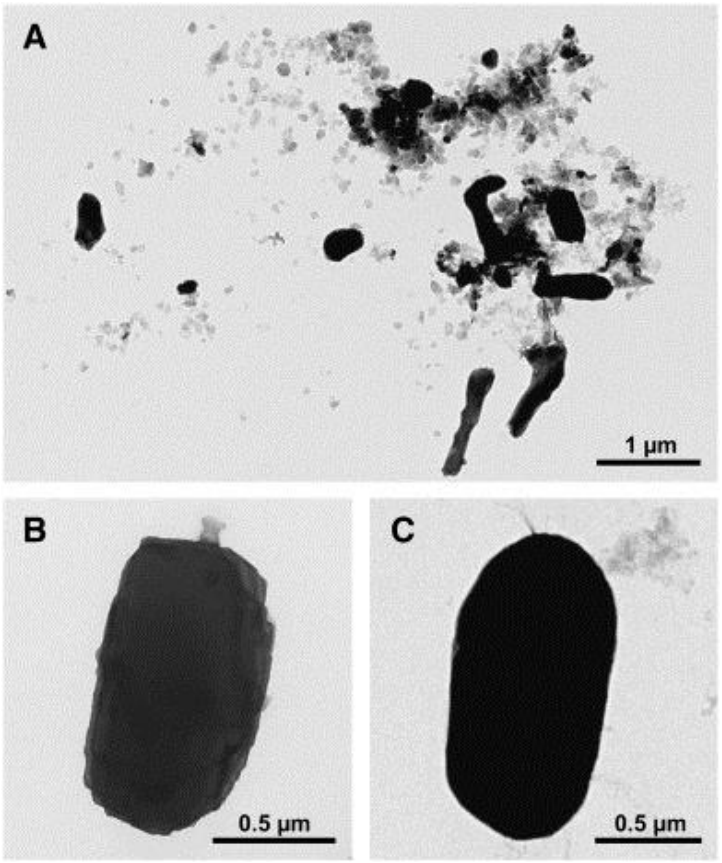


Figure 2. Typical X-ray spectrum of an untreated *Bacillus subtilis* spore. A. Transmission electron microscopy of a spore. The ring indicates the area which was illuminated by the electron beam for collection of the X-rays. B. X-ray spectrum of the spore in (A), which shows a prominent peak of calcium (Ca), besides peaks of phosphorus (P) and sulphur (S). The copper (Cu) signal derived from the sample support (i.e. the grid).

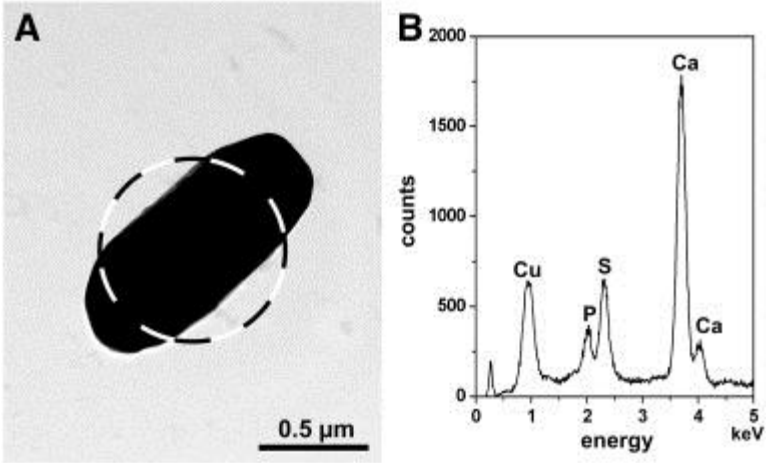


Figure 3. Comparison of X-ray spectra of spores after negative staining with different stains. A. Control without any heavy metal staining. B. Phosphotungstic acid (PTA). C. Uranyl acetate (UA). D. Methyl amine tungstic acid (MAT). (Ca) calcium, (Cu) copper, (P) phosphorus, (S) sulphur, (U) uranium, and (W) tungsten.

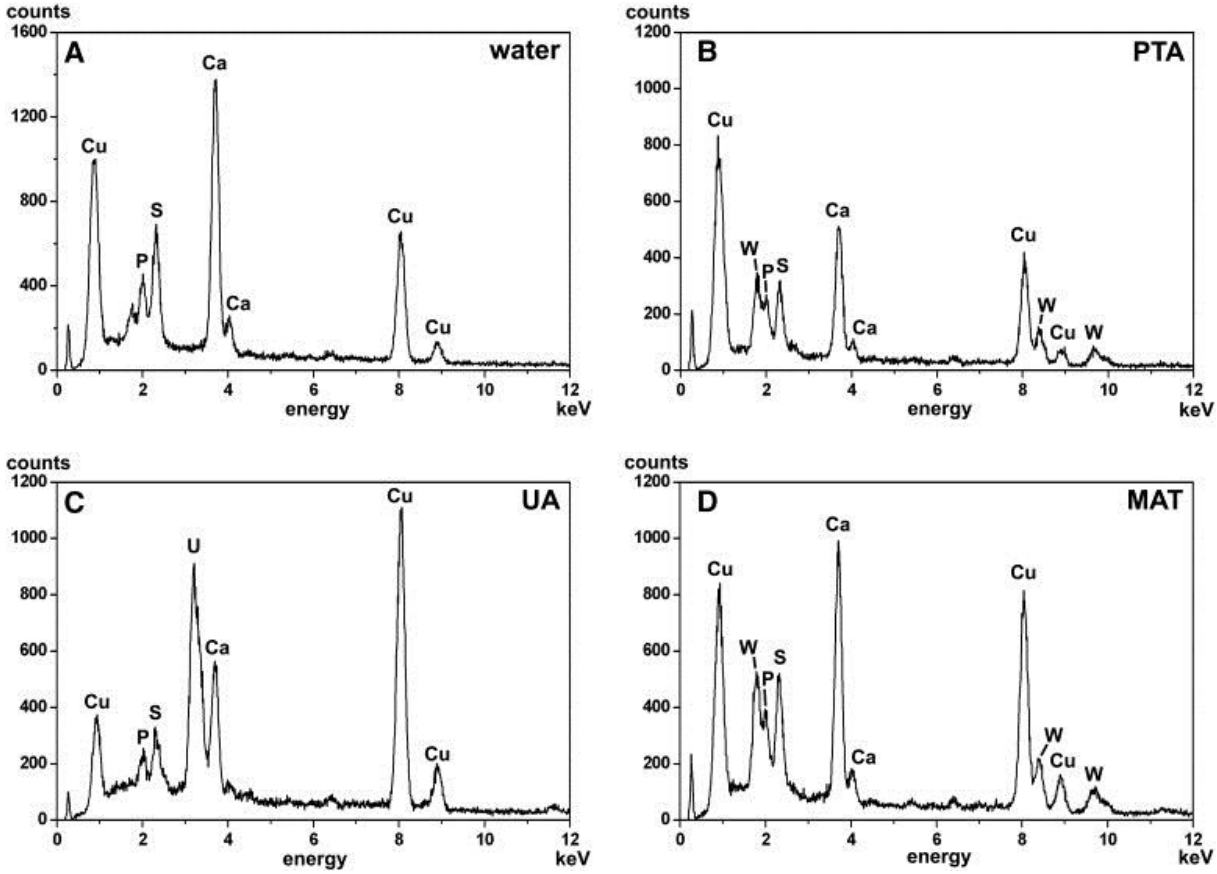


Figure 4. X-ray spectra of negatively stained *Bacillus anthracis* spores. A, B. Spectra of two spores from different strains recorded using a transmission electron microscope in conjunction with a Si/Li X-ray spectrometer. While spores of strain 527 (A) show the typical spectral signature, spores of the Sterne strain 34F2 (B) reveal only the calcium and the phosphorus peak. C. X-ray spectrum of a spore of the Sterne strain 34F2 acquired by using a silicon drift detector (SDD) and a table top scanning electron microscope. The spectrum shows the typical signature of calcium (Ca), phosphorus (P) and sulphur (S) peaks. In addition, peaks of magnesium (Mg) and silicon (Si) are visible. Like the copper peak, silicon is a systemic peak, which could be recorded from bare plastic support film and background areas.

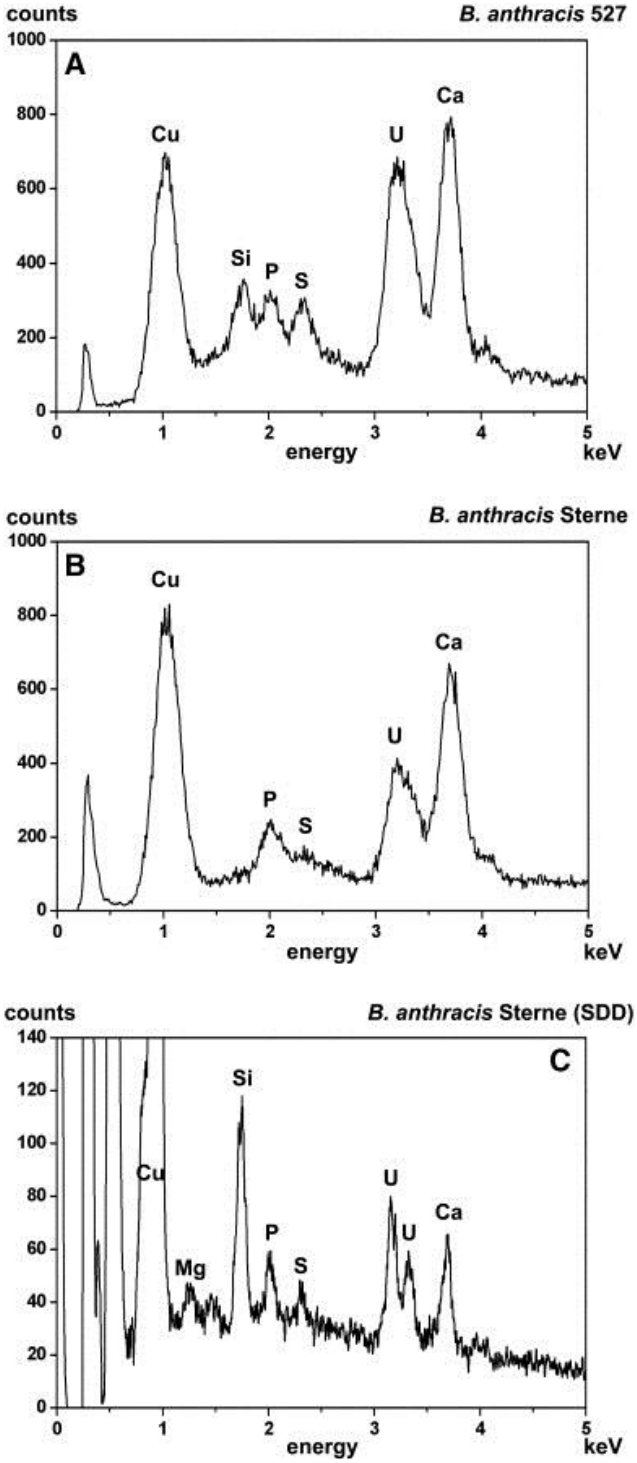


Figure 5. X-ray spectra of spores of different negatively stained *Bacillus* species. Spectra were collected with a scanning electron microscope that was equipped with a silicon-drift X-ray detector. A. *Bacillus anthracis* 527. B. *Bacillus anthracis* Stamatin Sokol. C. *Bacillus atrophaeus* ATCC 9372. D. *Bacillus cereus* ATCC 10987. E. *Bacillus subtilis* ATCC 6633. F. *Bacillus thuringiensis* DSM 350. (Al) aluminium, (Ca) calcium, (Cu) copper, (Mg) magnesium, (P) phosphorus, (S) sulphur, (Si) silicon, and (U) uranium.

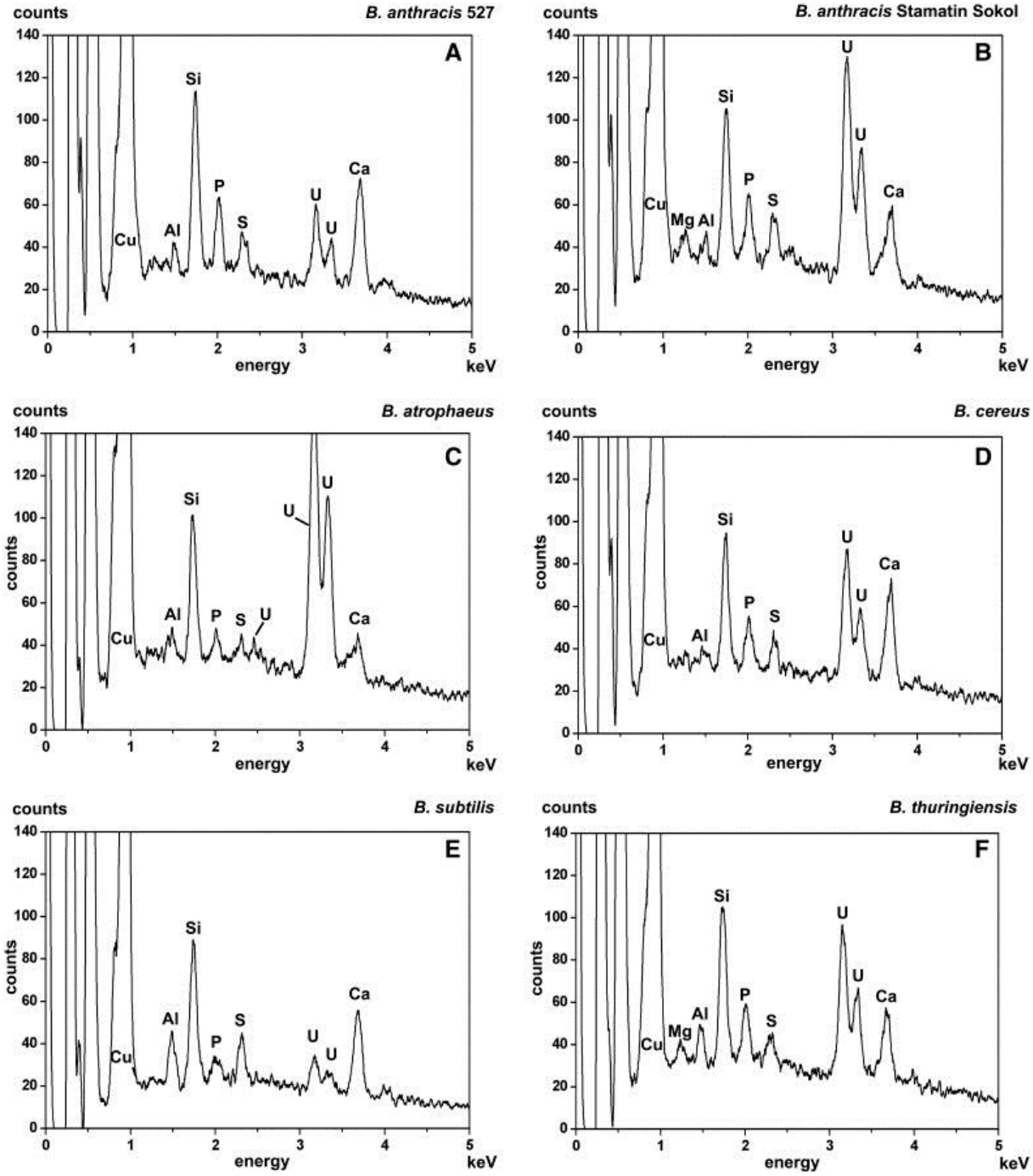


Figure 6. Comparison of X-ray spectra of spores with spectra of particles in environmental samples that possess a similar morphology as spores. Samples were negatively stained and spectra were recorded with a transmission electron microscope using a Si/Li X-ray detector. A, C. Bright-field image of a spore of *Bacillus subtilis* (A) and the corresponding X-ray spectrum, which shows the typical peaks of phosphorus (P), sulphur (S) and calcium (Ca). B, D. Bright-field image (B) and X-ray spectrum (D) of a crystal-like particle of an environmental sample which possesses about the same size and shape as a spore. The spectrum shows aluminium (Al), silicon (Si) and calcium (Ca) as major relevant peaks which are different from the typical spectrum of spores. E, F. Spectra from two particles in a mixture of *Bacillus subtilis* spores and a household polish. While both particles look similar in bright-field transmission electron microscopy (insets), spectra indicate clearly that particle in (E) is a spore and particle in (F) is a component of the polish, because spectrum in (E) shows the typical spectral signature of spores (P, S, and Ca) and the spectrum in F reveals calcium (Ca), manganese (Mn) and iron (Fe) as relevant peaks.

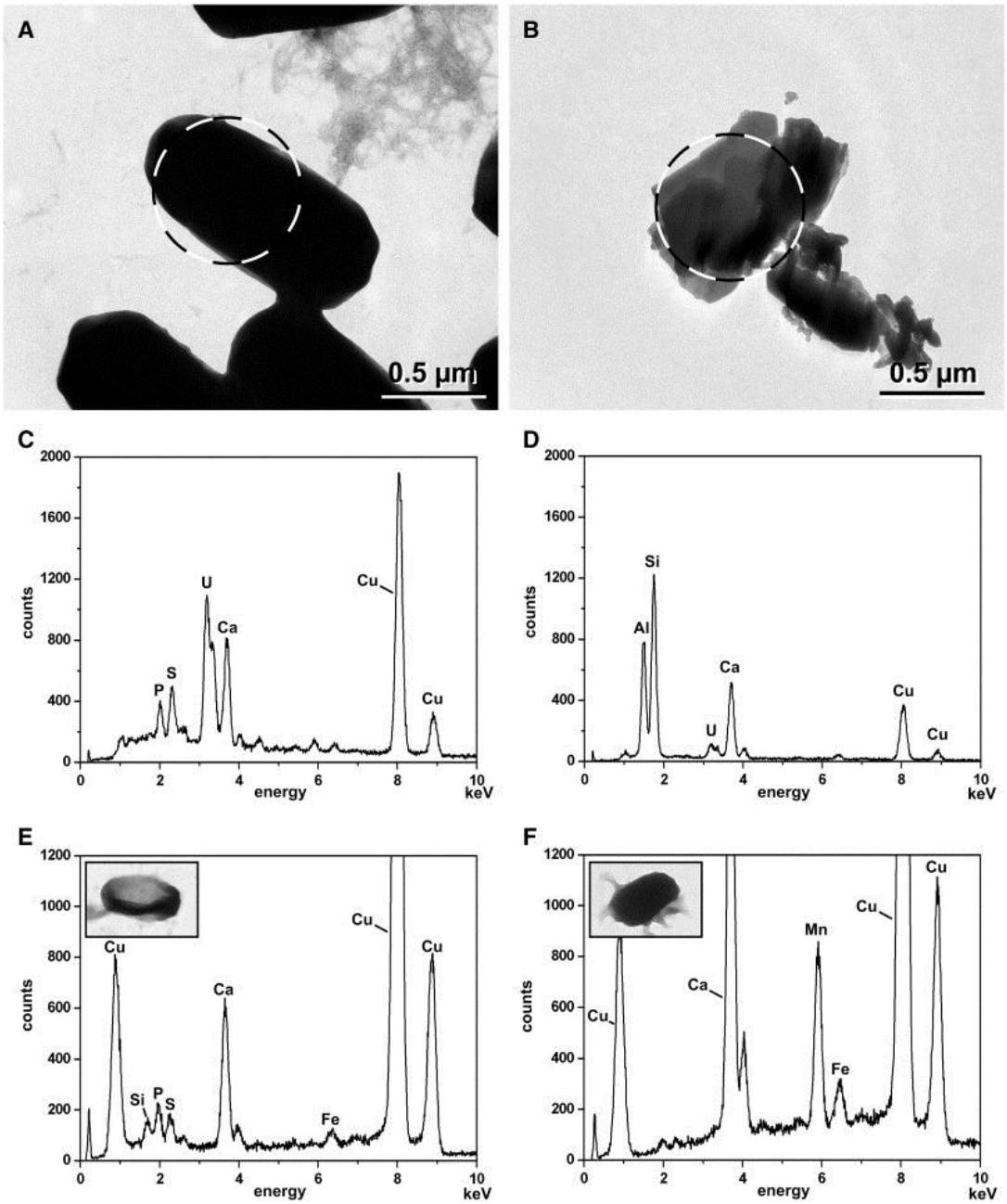
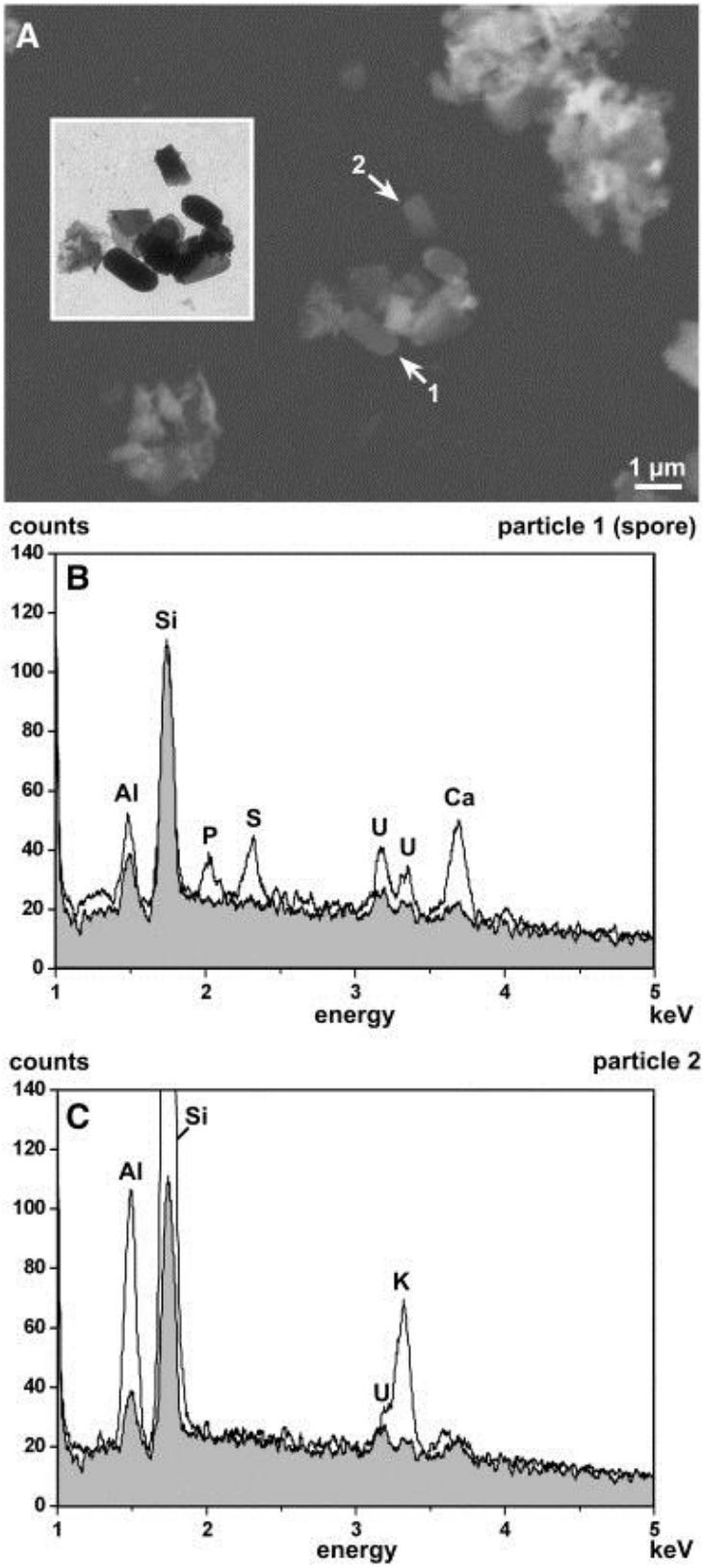
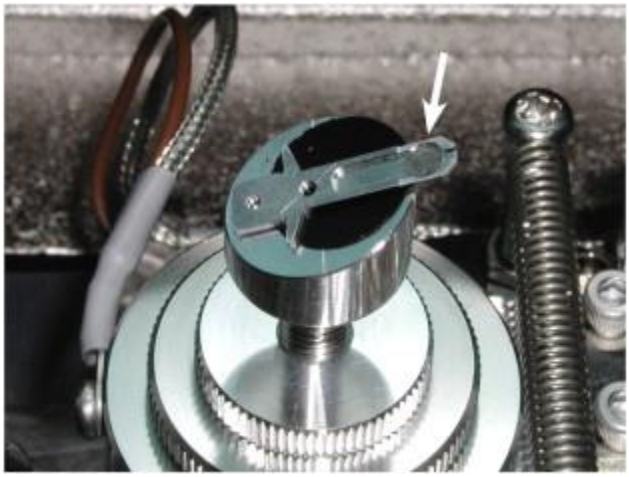


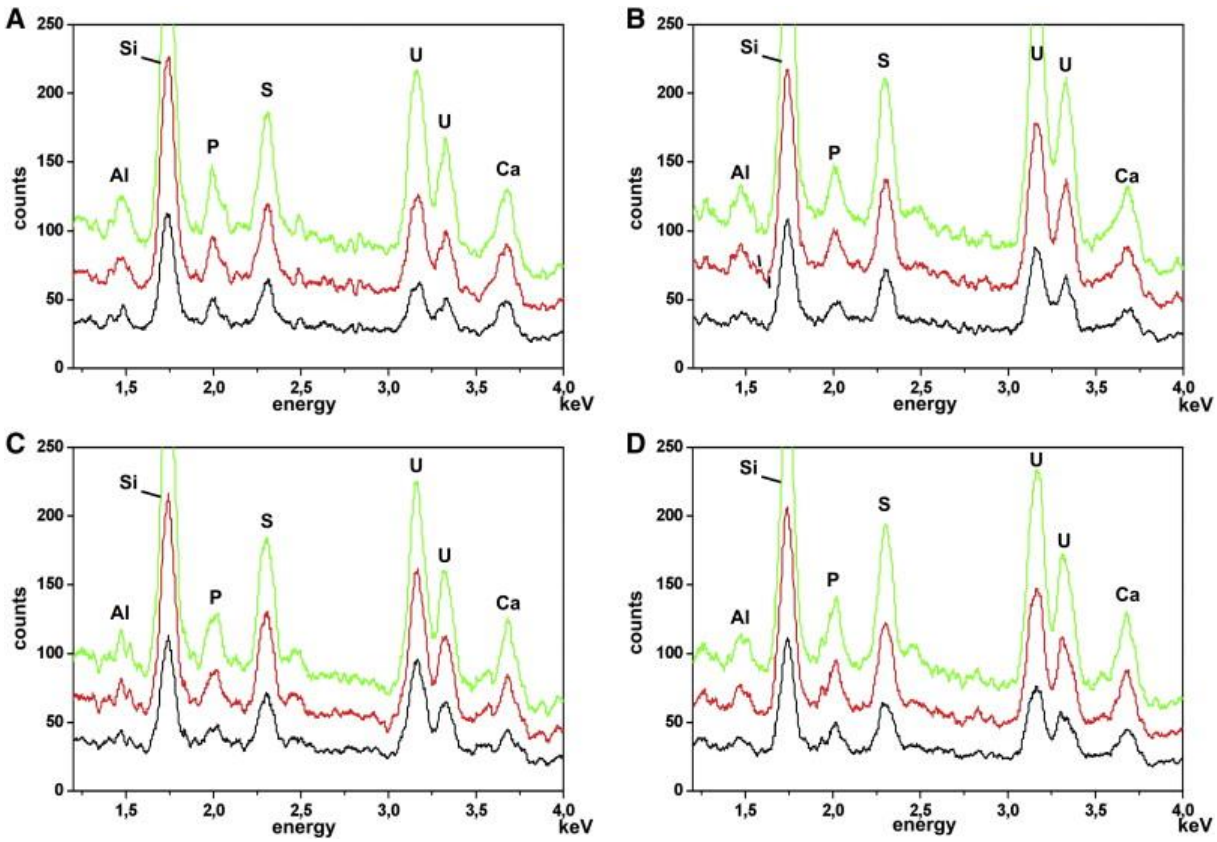
Figure 7. Diagnosis of a suspected bioterrorist sample that has been spiked with spores of *Bacillus subtilis*. Analysis was done by using transmission electron microscopy for screening and morphological typing of negatively stained particles followed by scanning electron microscopy in conjunction with X-ray microanalysis for revealing the element composition of particles. A. Scanning electron micrograph and corresponding transmission micrograph (inset) showing a cluster of suspicious particles. B. X-ray spectrum of particle 1 shows the characteristic peaks of phosphorus (P), sulphur (S) and calcium (Ca), indicating that particle 1 is indeed a spore. Grey curves represent background measured at locations on the support which are virtually free of particles. C. X-ray spectrum of particle 2 shows peaks of aluminium (Al), silicon (Si) and potassium (K) that are clearly above background levels (grey curves) and different from the spectral signature of a spore.



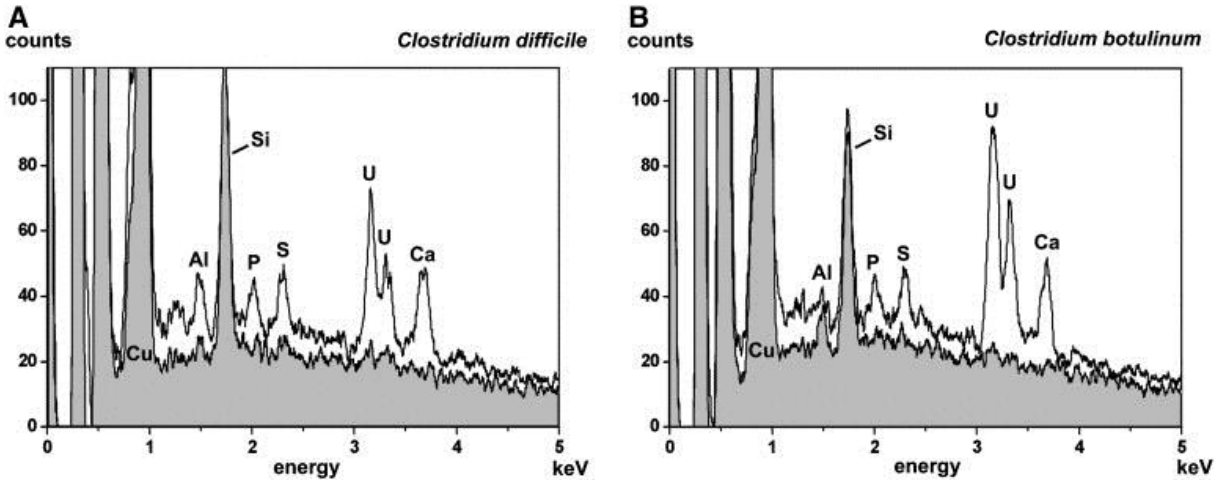
Supplemental Figure 1. Sample holder to fix grids at an angle of 22° in the Hitachi TM-1000 desktop scanning electron microscope for an efficient collection of X-rays. The removable tip (retainer) of a sample holder of a Jeol transmission electron microscope was fixed on an aluminium stub, which has an inclination of 22°, by an adhesive carbon tape. The hole of the tip was placed beside the stub to avoid beam interaction with the support.



Supplemental Figure 2. A–D. Reproducibility of the typical X-ray spectrum in spores of the same population after negative staining with uranyl acetate. Twelve spores of *Bacillus subtilis* (fixed in 10% paraformaldehyde and 0.05% glutaraldehyde in 0.05 M Hepes) were randomly selected from one grid in a desktop scanning electron microscope (TM-1000) and analysed with an X-ray detector (Quantax 50; spectrum collection time: 200 s). The recorded spectra (A–D) show the typical element composition of spores (S, P, and Ca) and reveal a high degree of similarity.



Supplemental Figure 3. X-ray spectra of negatively stained *Clostridium* spores. A. *Clostridium difficile* NCTC 13366. B. *Clostridium botulinum* NCTC 7272. In both species the typical spectral signature of spores, phosphorus (P), sulphur (S) and calcium (Ca) can be clearly distinguished from background measurements (grey area). Silicon (Si) and aluminium (Al) are, to a substantial extent, systemic peaks and uranyl (U) derived from the staining of the spores.



Supplemental Figure 4. Element maps from X-ray microanalysis of a spore in a negatively stained environmental sample. The backscattered electron detector image (BSE) shows a spore-like particle (arrow) which is surrounded by particles of different shape and electron scattering. X-ray intensities for selected elements are shown as colour-coded intensities for every pixel of the image. The spore-like particle contains calcium (Ca), phosphorus (P) and sulphur (S). The other particles reveal silicon (Si), some sulphur (S), magnesium (Mg) and/or phosphorus (P).