

# Disinfection chemicals mode of action on the bacterial spore structure and their Raman spectra

## Supporting information

Dmitry Malyshev<sup>†</sup>, Tobias Dahlberg<sup>†</sup>, Krister Wiklund<sup>†</sup>, Per Ola Andersson<sup>‡,¶</sup>, Sara Henriksson<sup>§</sup>, Magnus Andersson<sup>†,\*</sup>

<sup>†</sup>Department of Physics, Umeå University, Umeå, Sweden

<sup>‡</sup>Swedish Defence Research Agency (FOI), Umeå, Sweden

<sup>¶</sup>Department of Engineering Sciences, Uppsala University, Uppsala, Sweden

<sup>§</sup>Umeå Core Facility for Electron Microscopy, Umeå University, Umeå, Sweden

\*Corresponding author Magnus Andersson

Email: [magnus.andersson@umu.se](mailto:magnus.andersson@umu.se)

Phone:+46 90 786 6336

## Table of content

Experimental methods	S2
Laser Tweezers Raman Spectroscopy (LTRS) setup	
Sample preparation and LTRS measurements	
Supporting figures	S3
Figure S1. Changes of the 1400 cm <sup>-1</sup> Amide peak.	
Figure S2. TEM of untreated spores.	
Figure S3. TEM of sodium hypochlorite treated spores.	
Figure S4. TEM of peracetic acid treated spores.	
Figure S5. Change in the Raman spectrum of a single spore over time.	
Figure S6. Serial dilutions drop counts of samples treated with sporicidal chemicals after 10 and 30 minutes incubation.	
Figure S7. Serial dilutions drop counts of samples treated sporicidal chemicals for 1 minute	
Figure S8: Additional fields of view of <i>B. thuringiensis</i> treated with 750 ppm chlorine dioxide for 30 min (24 SEM and 24 TEM fields of view).	
Figure S9: Additional fields of view of <i>B. thuringiensis</i> treated with 0.5 % sodium hypochlorite for 30 min (24 SEM and 24 TEM fields of view).	
Figure S10: Additional fields of view of <i>B. thuringiensis</i> treated with 1 % peracetic acid for 30 min (24 SEM and 24 TEM fields of view).	

## Experimental methods

### Laser Tweezers Raman Spectroscopy (LTRS) setup

To prevent laser beam back reflections that might cause laser beam intensity flickering and mode jumping, we use an optical isolator. We pass the laser through a narrow line filter (center wavelength 808 nm, 3.1 nm Bandwidth, Edmund Optic) to improve the Raman signal. To ensure a diffraction-limited spot-size, we spatially filter and expand the beam to fill the back aperture of the microscope objective before coupling the laser into the microscope. We couple the laser into the microscope using a dichroic shortpass mirror with a cut off wavelength of 650 nm. This dichroic mirror serves two purposes, separating the spectrograph side from the conventional imaging side and directing the laser into the microscope objective. We use a 60x water immersion objective (UPlanSApo60xWIR, Olympus) with a numerical aperture of 1.2 and a working distance of 0.28 mm that focuses the beam and forms the trap. With this water immersion objective, we can use the full working distance to position the trap without impacting the trap performance due to spherical aberrations. This freedom in positioning improves signal quality as it allows us to trap objects far away from surfaces that can introduce noise in the form of unwanted Raman scattering and fluorescence.

To perform accurate positioning of the laser focal spot, we aligned the beam so the focal spot coincides with the focal plane of the microscope objective. A diffraction-limited spot of ~800 nm and a depth of field of ~700 nm is created that we can position with nm-resolution in the lateral and axial plane using both a motorized microscope stage (SCAN IM120x100, Märzhauzer Wetzlar) and a piezo stage (PI-P5613CD, Physik Instruments). Since the objective strongly focuses the laser beam ~65°, the intensity drops fast from the focal spot, both in the lateral and axial directions. We estimate the intensity in the focal spot to 7.81 kW/mm<sup>2</sup> by measuring the effective power (7 mW) before the objective and using the properties (transmission, focal length, etc.) of the objective from the manufacturer.

To visualize the sample during measurement, we use an LED lamp with a center wavelength of 470 nm (M470L4, Thorlabs). As the light emitted by the LED has a bandwidth of 26 nm and is located far from the spectral working region of the spectrometer, we can do simultaneous visual imaging and Raman spectroscopy without added spectral noise. To acquire the images, we use a 1920 x 1440 pixel CMOS camera (C11440-10C, Hamamatsu).

To control the sample temperature, we used a home-made software-driven PID regulator, design available upon request. We built the regulator using a micro-controller (STM32F103C8T6, STMicroelectronics), heating foil (Calesco), which we drive using a dual H-bridge (L293D, Texas Instruments), and a thermistor (B57550G1103F005, TDK Electronics) connected through a Wheatstone bridge to a 16-bit ADC (ADS1115, Texas Instruments). To accurately measure the temperature of the sample, we place the thermistor and the heating foil on the nose-cone of the objective, close to the sample. This setup lets us control the sample temperature with sub-mK resolution and an accuracy of 0.1 K. To measure the influence of temperature drifts and noise in general, we use Allan variance to analyze our setup.

### Sample preparation and LTRS measurements

We prepare *B. thuringiensis* (ATCC 35646) spores using BBLTM AK agar 2 Sporulating plates. The spores are harvested from the plates and the suspension homogenised by vortexing the suspension with glass beads. The spores are then washed by centrifuging them in deionised water and discarding the supernatant. This is repeated twice. Spore batches are kept in MQ water at 4 °C until use. A working stock spore suspension is made with a concentration of 10<sup>6</sup> spores per ml. To re-suspend the spore stock we vortex them at 2,800 rpm (VM3 Vortex, M. Zipperer GmbH) for 10 seconds. A sample is made by adding a 1 cm diameter ring of 1 mm thick vacuum grease on a 24 x 60 mm glass coverslip. Then we add 5 µl of the diluted spore suspension inside the ring.

We then proceed to add 5 µl of sporicidal chemical on top of the spore suspension, for a final concentration of 750 ppm chlorine dioxide (DKDOX 1500, used at 50 %), 1 % peracetic acid (Acros Organics 35 % stock, diluted 2 % stock prepared on the day of experiment) or 0.5 % sodium hypochlorite

(commercial bleach, Klorin brand), respectively, and seal the sample by placing a 23 x 23 mm cover slip on top the grease ring. We immediately start a timer to keep track of how long the spores have been exposed to the chemical. We then place the sample in the LTRS-system and locate a single free floating spore for Raman measurements. The Raman spectrum is recorded using 30 s acquisition time and 2 accumulations per spectra to achieve a temporal resolution of 60 s measured in the range of 600-1400. The spectral resolution is  $<2\text{ cm}^{-1}$ .

We use a modified version of the above procedure to measure the Raman spectra of spore components (DNA and DPA) and of sporicidal chemicals. Anhydrous DNA and DPA were sourced from Sigma-Aldrich. Instead of using glass cover slips, a pair 0.25 mm thick quartz cover slips (Alfa Aesar) is used to eliminate the broad Raman peak from the glass. To allow the quartz cover slips to be washed and reused, the grease ring is replaced with a PDMS ring (which can be easily removed). Since there is no particle to trap in these samples, the laser focal point is set  $150\text{ }\mu\text{m}$  above the quartz surface.

## Supporting figures

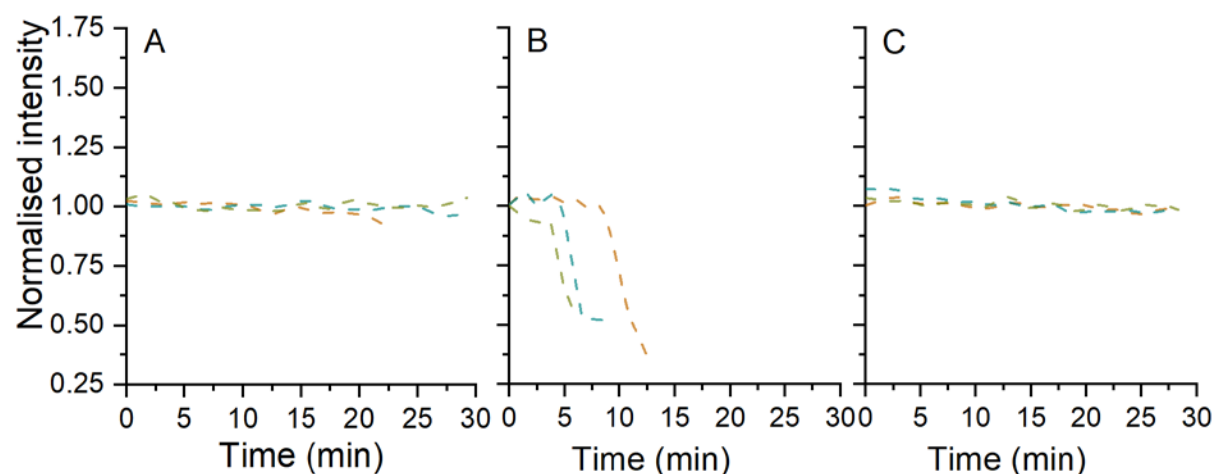


Figure S1. Changes in the normalised intensity of the  $1400\text{ cm}^{-1}$  (Amide) peak over time with chlorine dioxide (A), sodium hypochlorite (B) and peracetic acid (C) ( $n=3$  for each chemical). Aside from the Raman peak studied, the experimental conditions are the same as those used in Figure 2. There was no reduction in the peak for chlorine dioxide or peracetic acid.

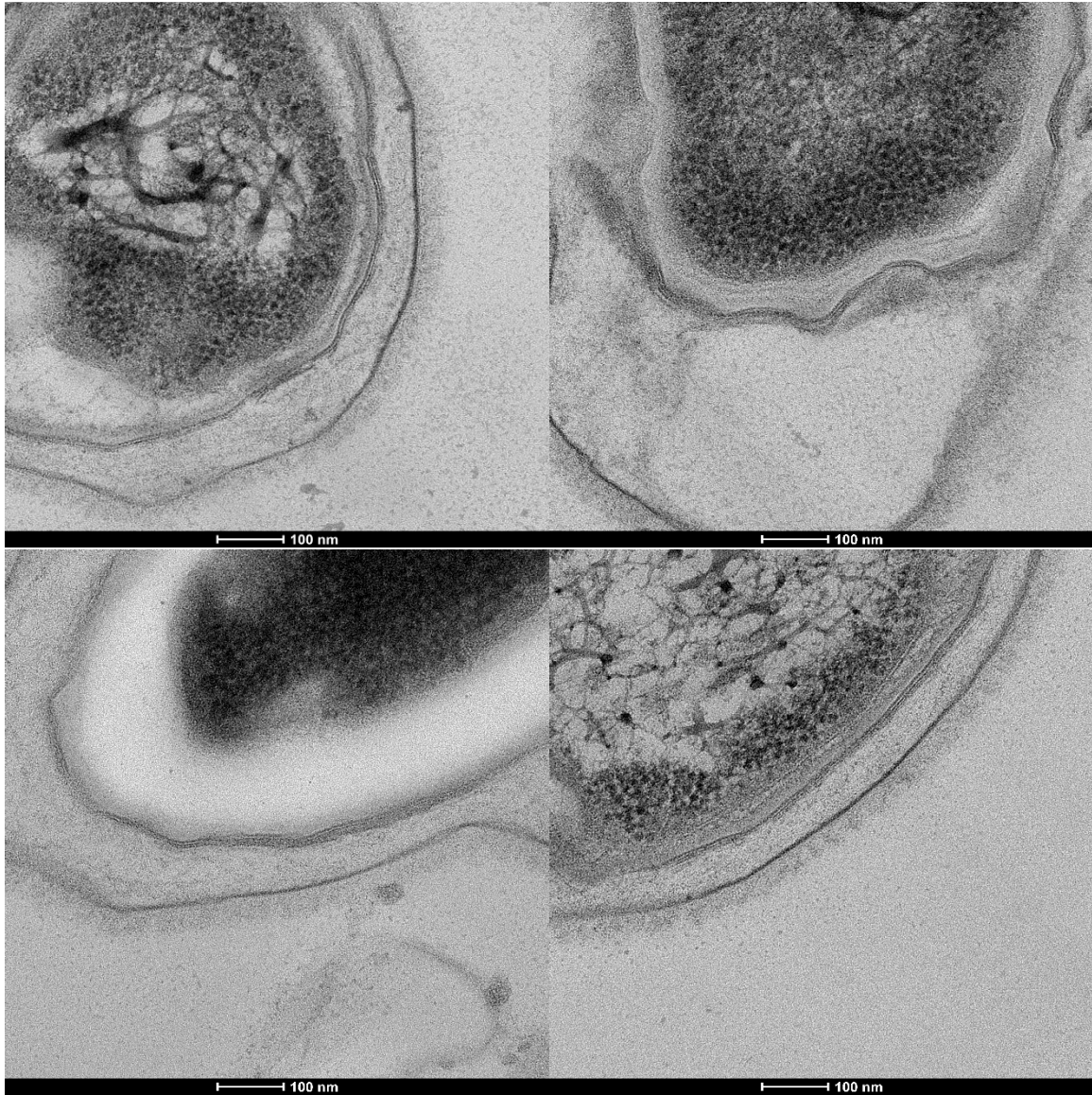


Figure S2. Additional fields of view of untreated *B. thuringiensis* spores. Note the continuous unbroken lines of the spore coat layers.

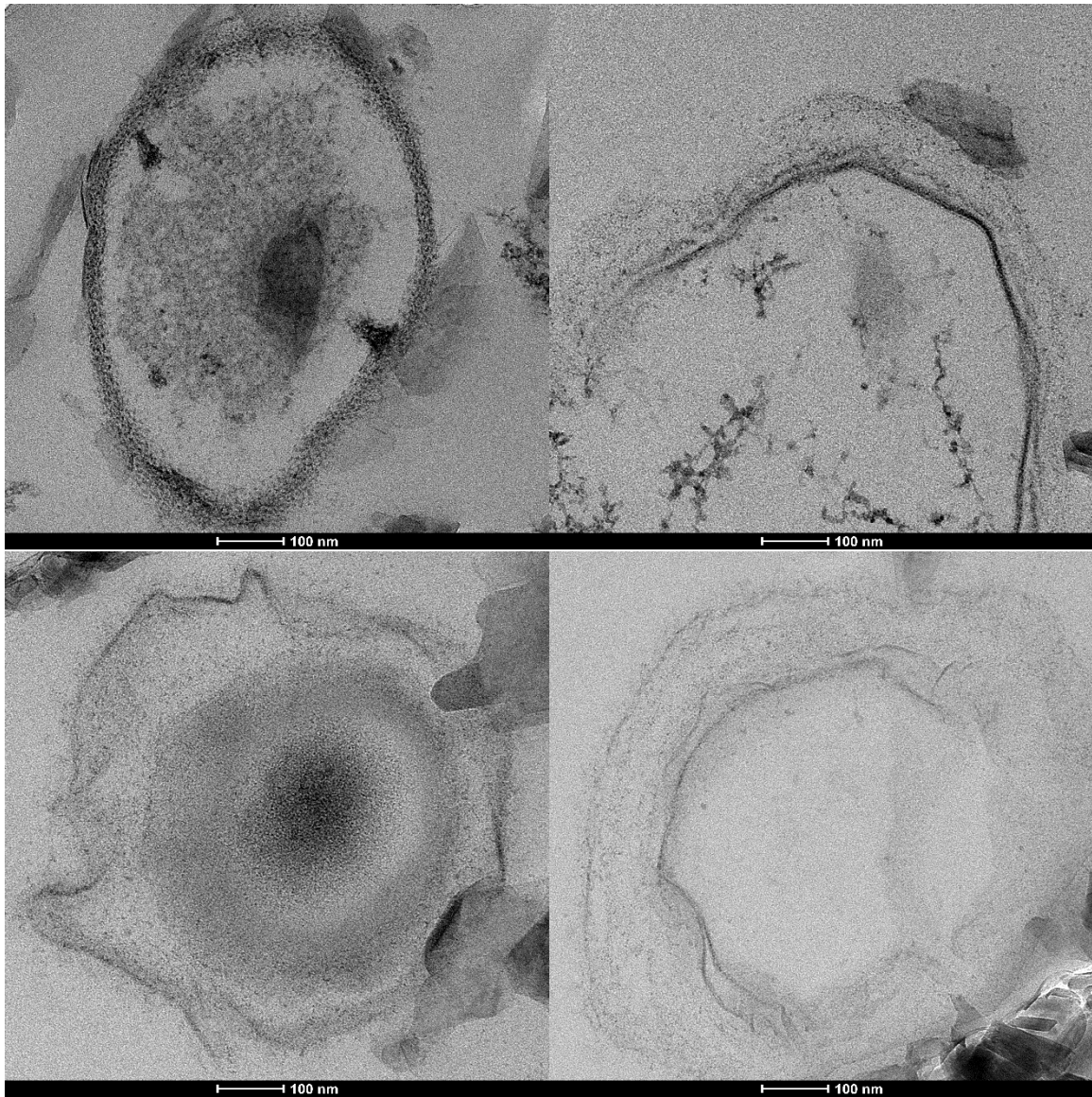


Figure S3. Additional fields of view showing spores degraded by 30 min incubation in 0.5% sodium hypochlorite (some of these spores are also included in S9).

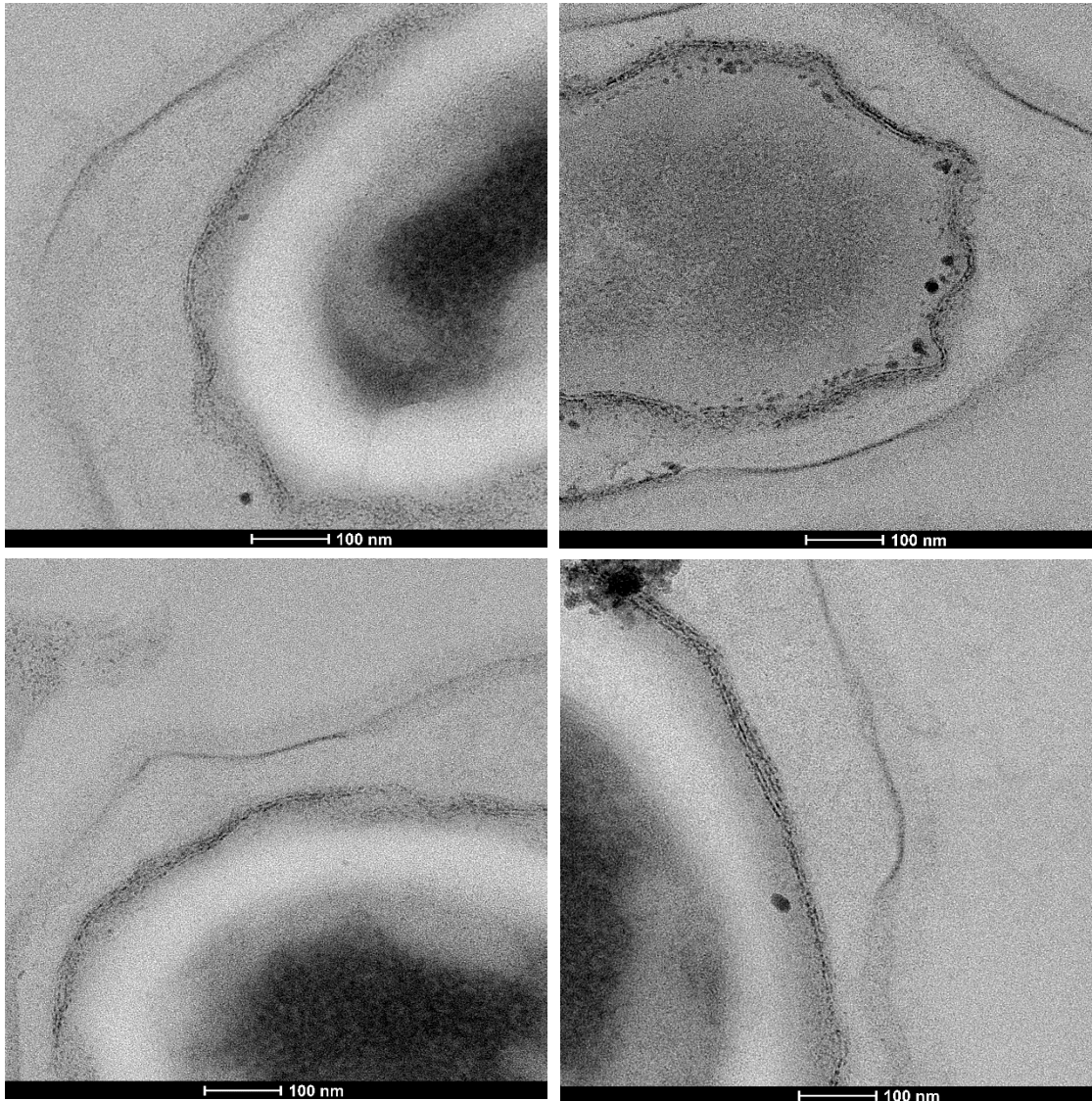


Figure S4. Additional fields of view showing damage to the spore coat from 30 min incubation with 1% peracetic acid. Note the fragmentation of the spore coat. (some of these spores are also included in S10).

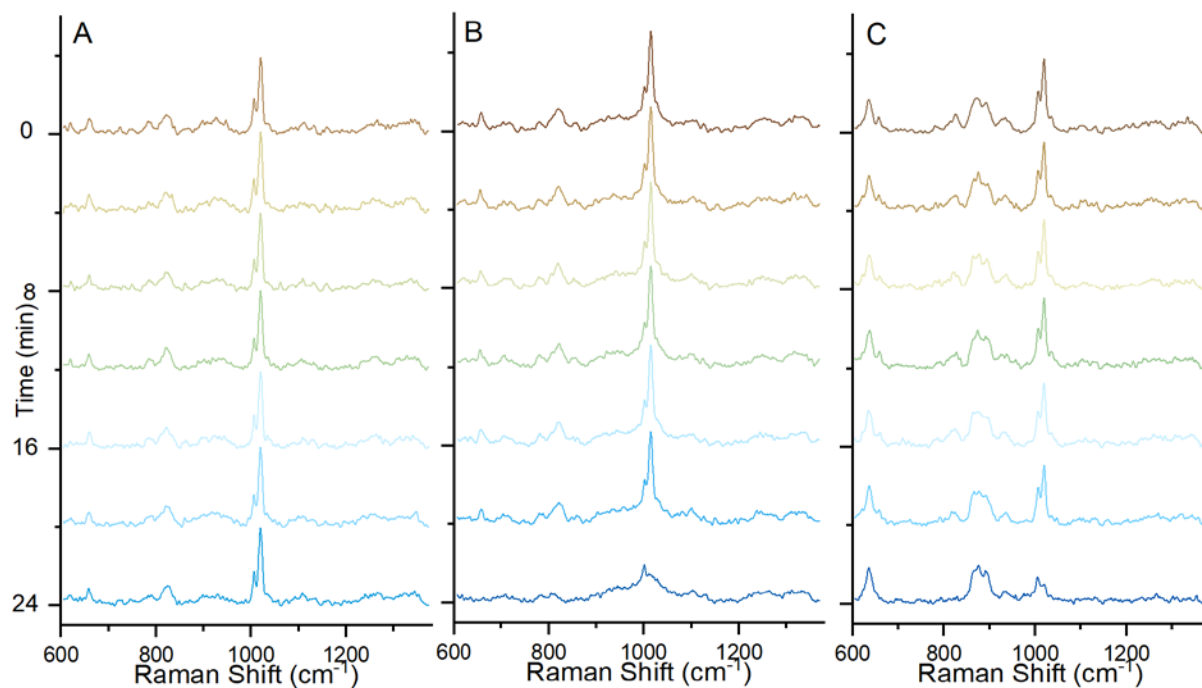


Figure S5. Change in the Raman spectrum of a single spore over time with A) 0.075 % chlorine dioxide B) 0.5 % sodium hypochlorite and C) 1 % peracetic acid. Measurements were taken every 4 minutes.

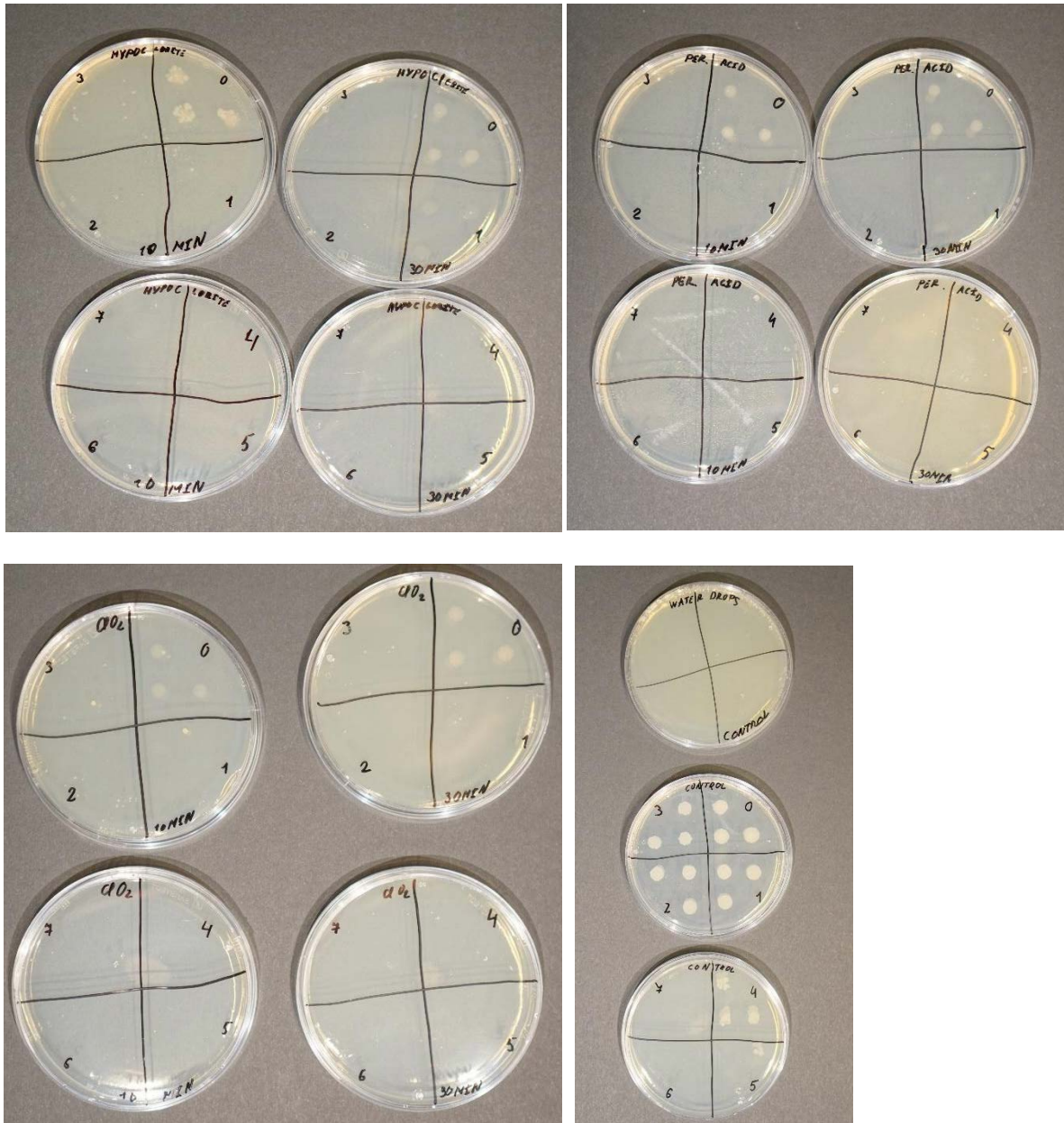


Figure S6. Serial dilutions drop counts of samples treated with sporicidal chemicals after 10 and 30 minutes incubation (numbers indicate dilution  $10^x$ , with 0 being  $10^0$ , undiluted suspension). Compared to the control, peracetic acid showed a complete inactivation of the spores after 10 minutes (minimum 5 log reduction). Sodium hypochlorite showed a 4 log reduction after 10 min and 5 log reduction after 30 min. Chlorine dioxide showed a 5 log reduction after both 10 minutes and 30 minutes. Photos were taken by Dmitry Malyshev.



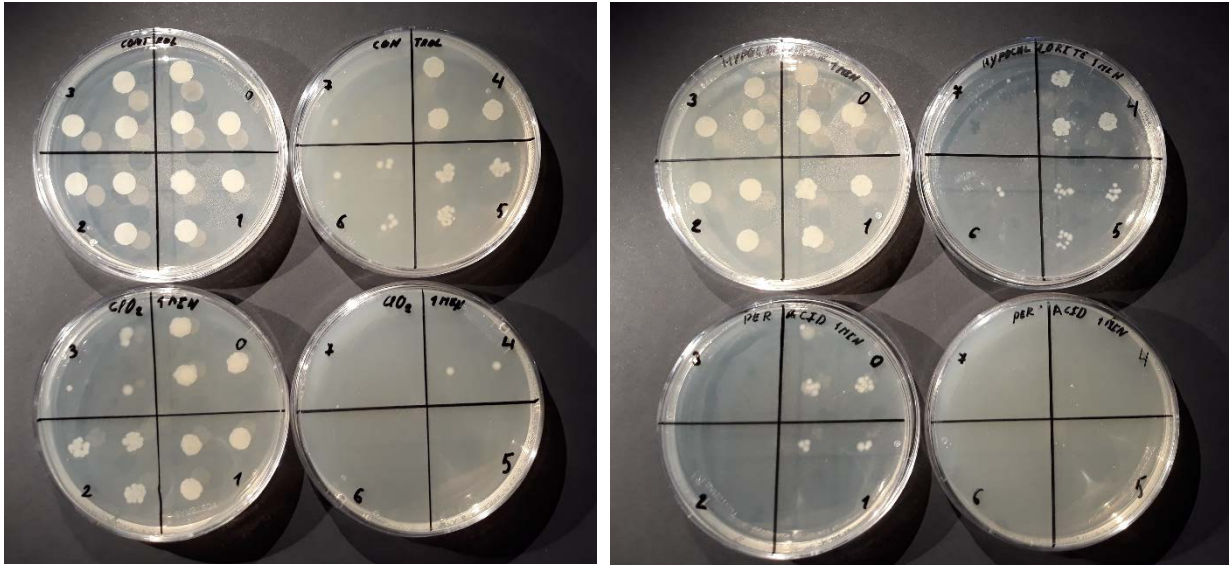
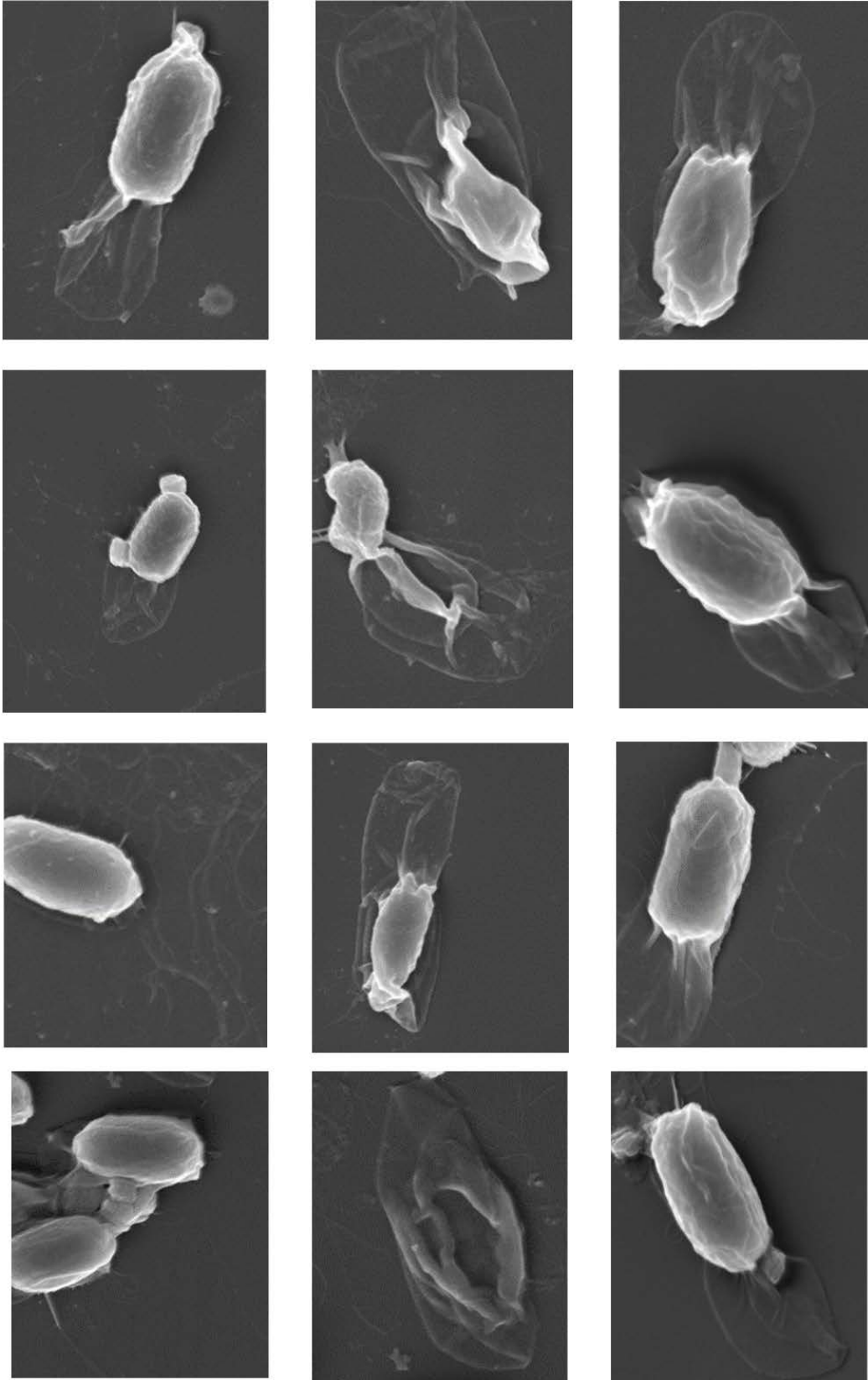
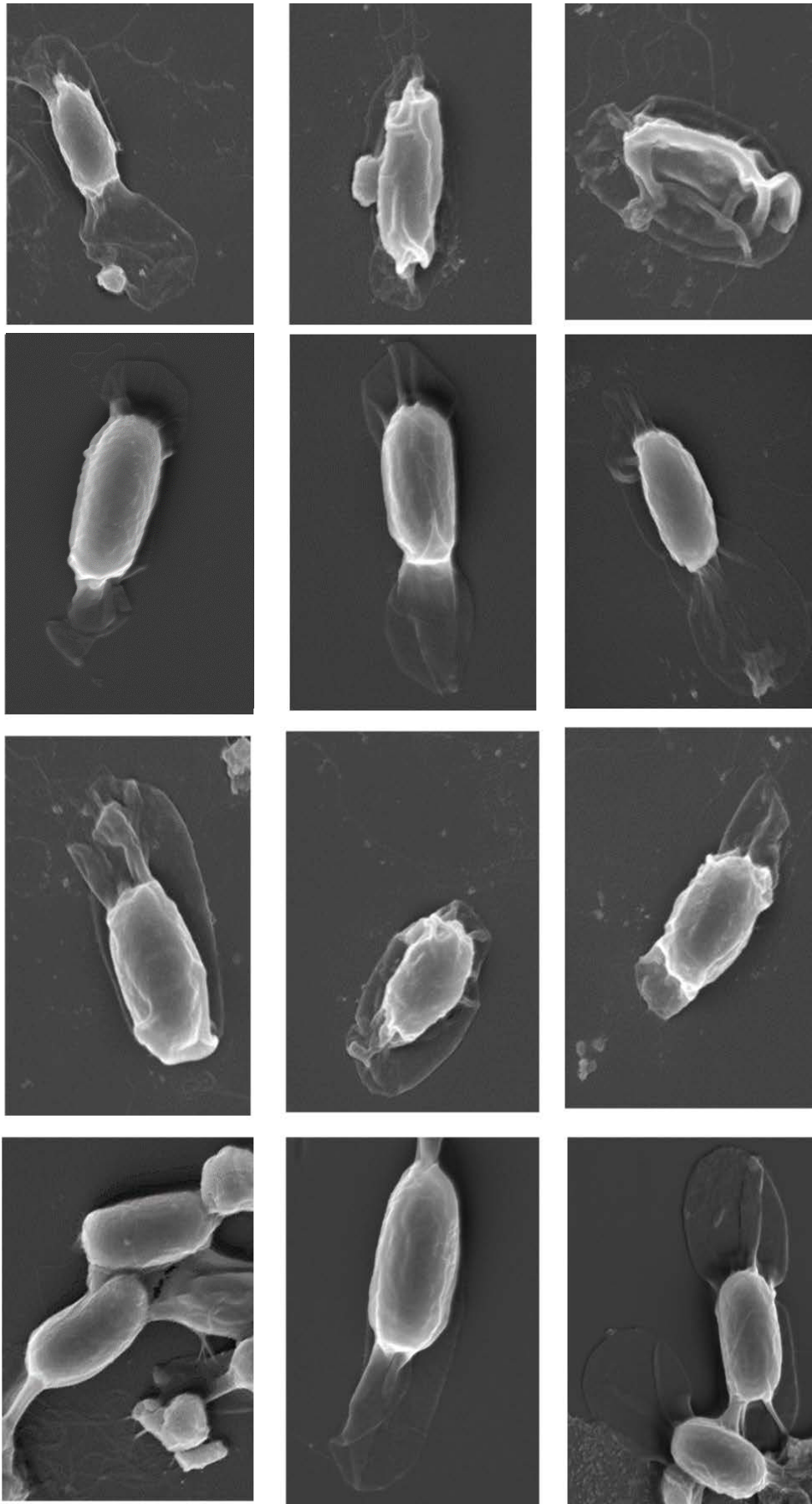


Figure S7. Serial dilutions drop counts of samples treated with sporicidal chemicals after 1 minutes incubation. Compared to the control, chlorine dioxide showed a 3 log reduction after 1 min. Sodium hypochlorite showed a 1 log reduction after min. peracetic acid showed a 5 log reduction after 1 min; note the pale circles at  $10^0$  dilution for peracetic acid are dried drops, they are not colonies). Photos were taken by Dmitry Malyshev.

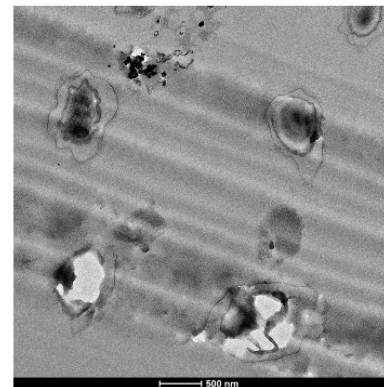
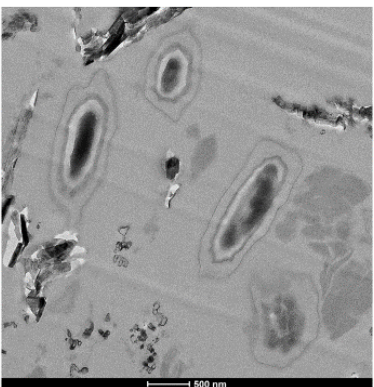
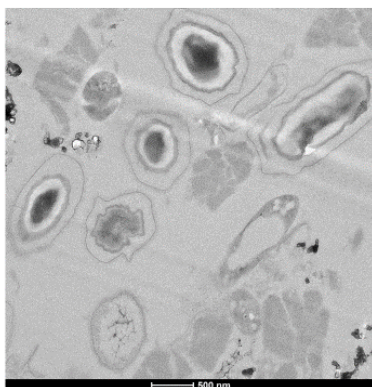
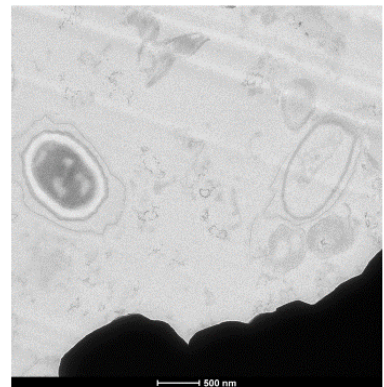
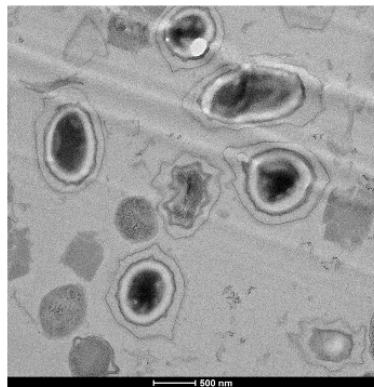
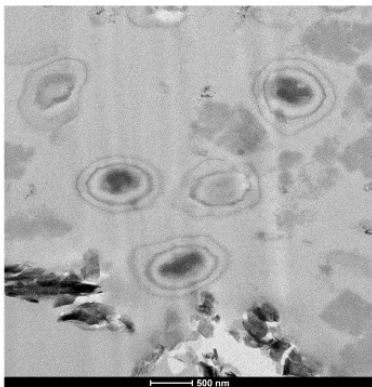
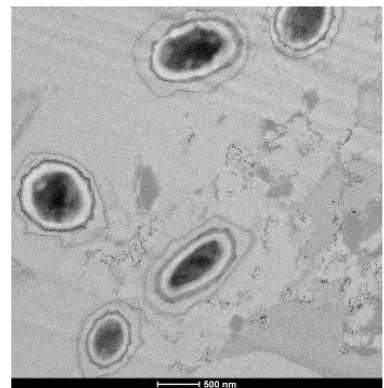
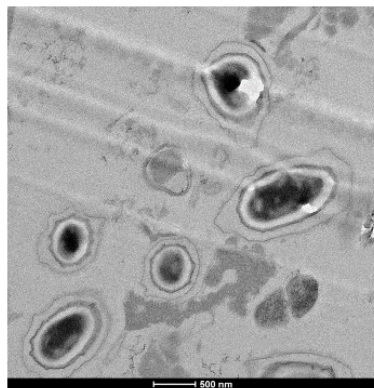
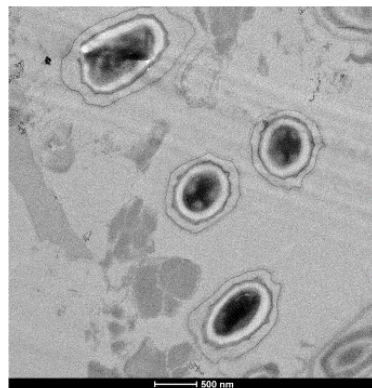
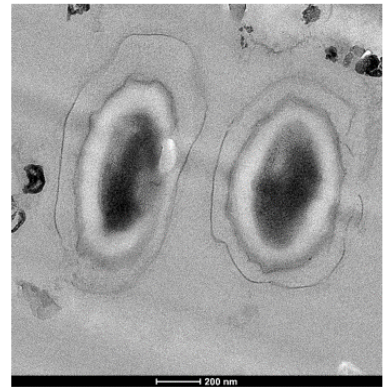
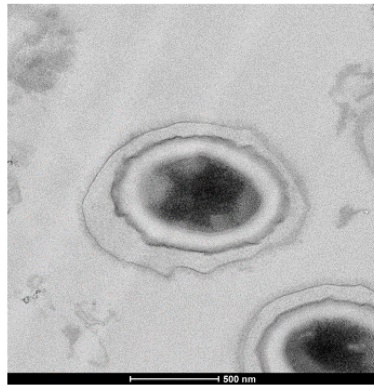
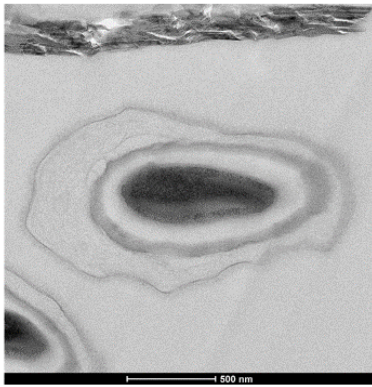
Supporting information



Supporting information



Supporting information



Supporting information

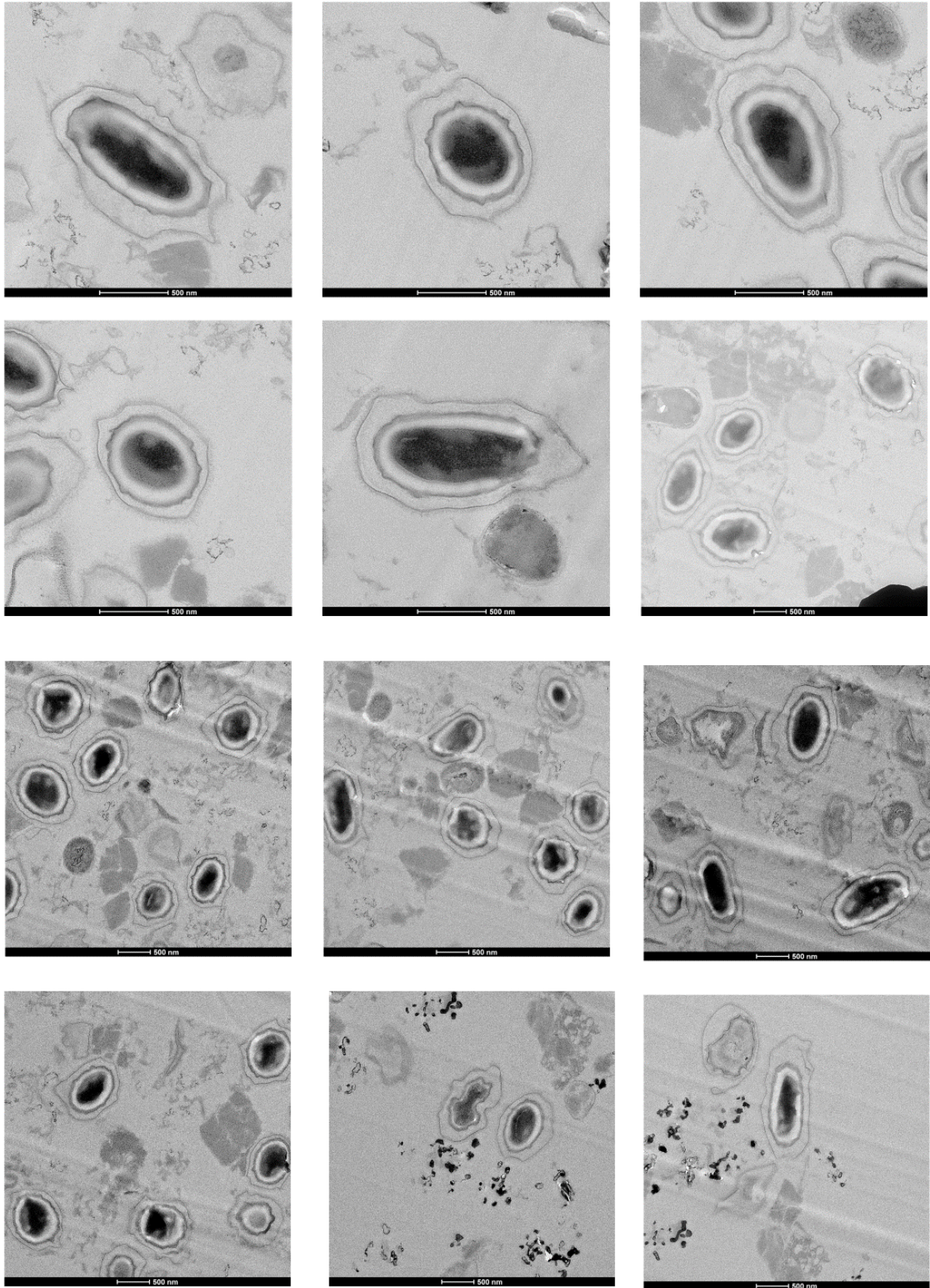
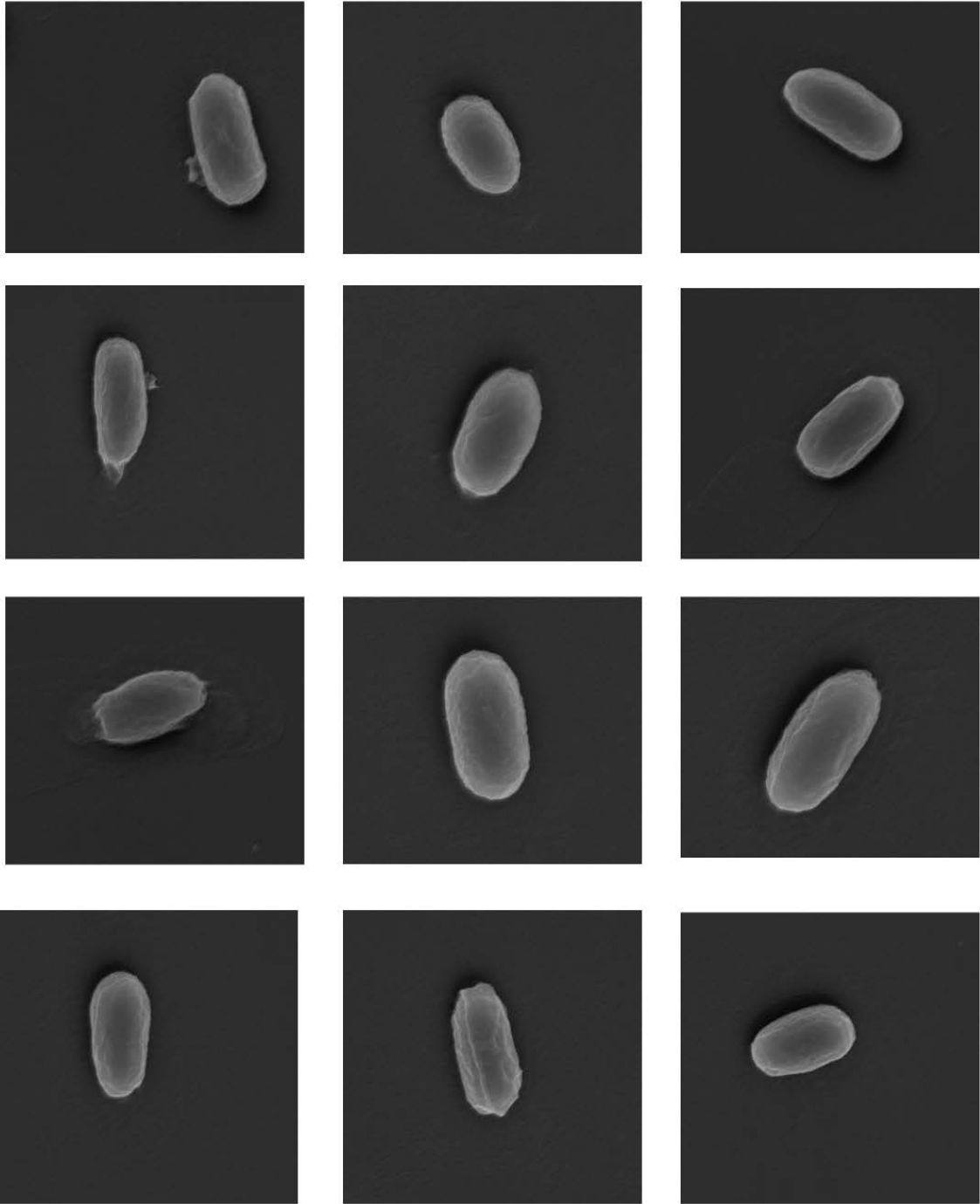
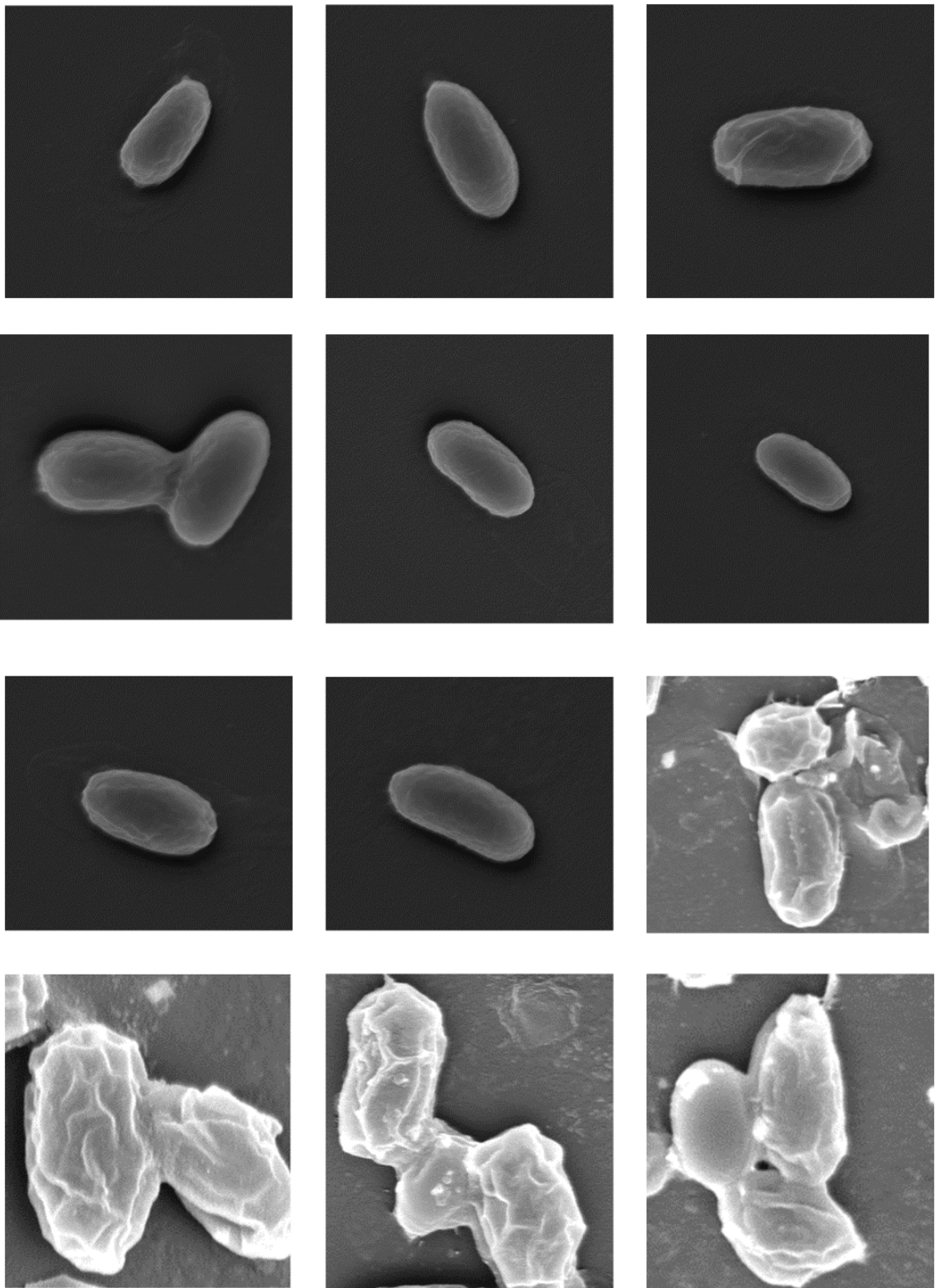


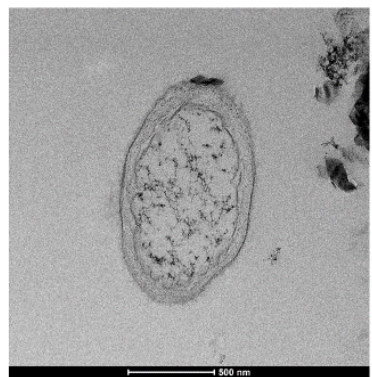
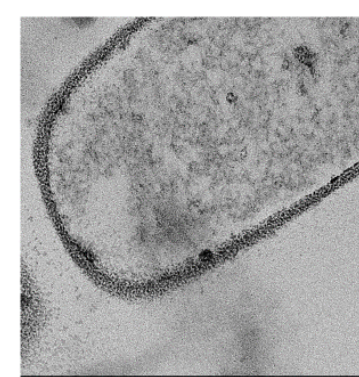
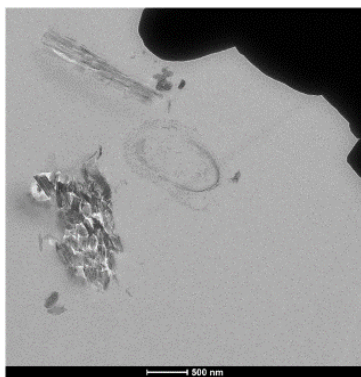
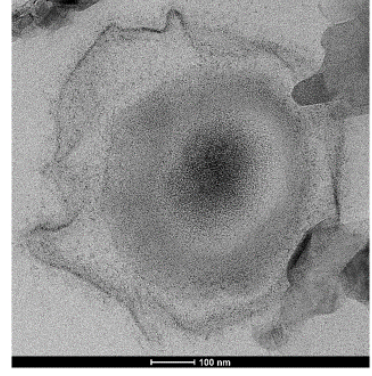
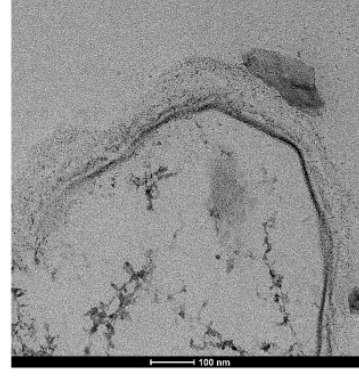
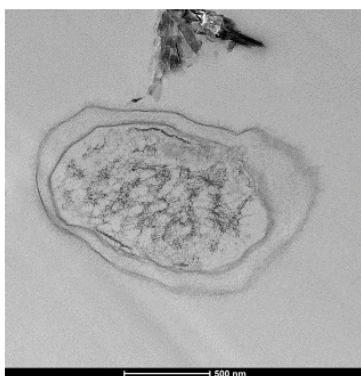
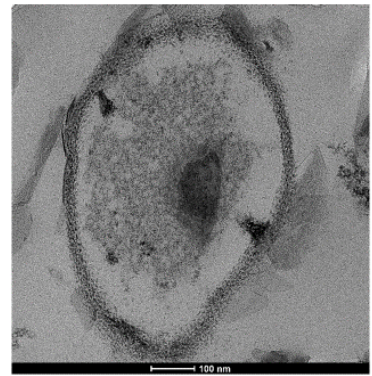
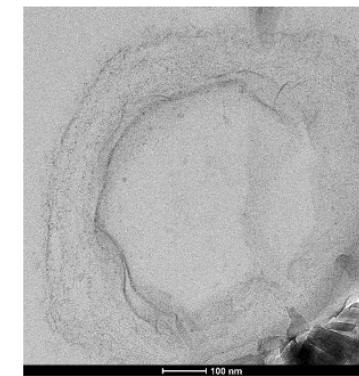
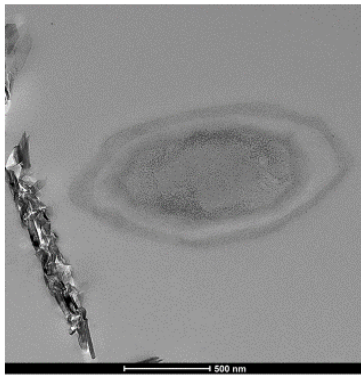
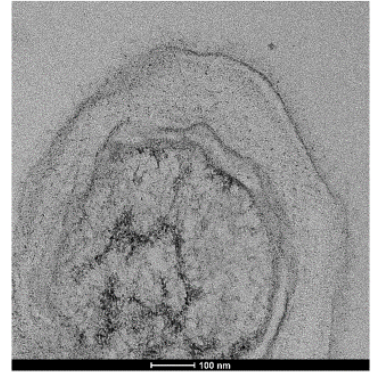
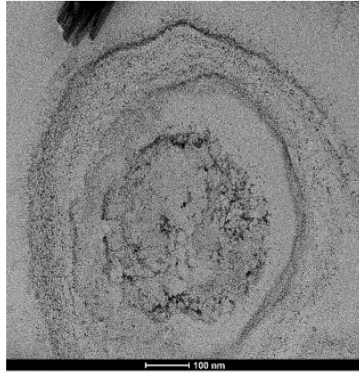
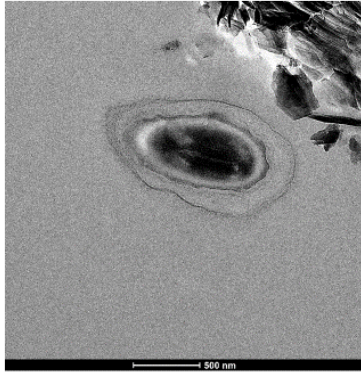
Figure S8 Additional fields of view of *B. thuringiensis* treated with 750 ppm chlorine dioxide for 30 min (24 SEM and 24 TEM fields of view)



Supporting information



Supporting information





Supporting information

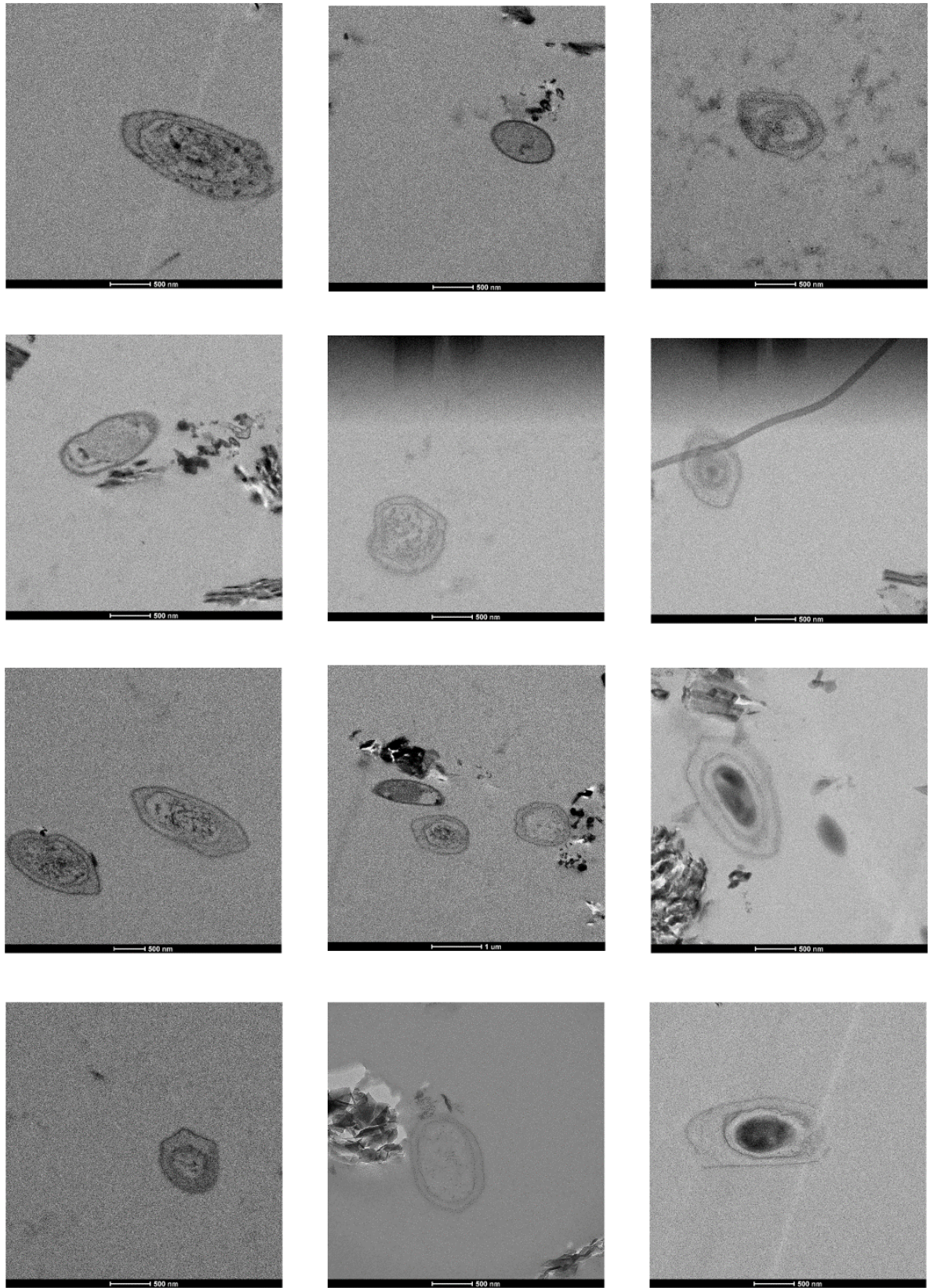
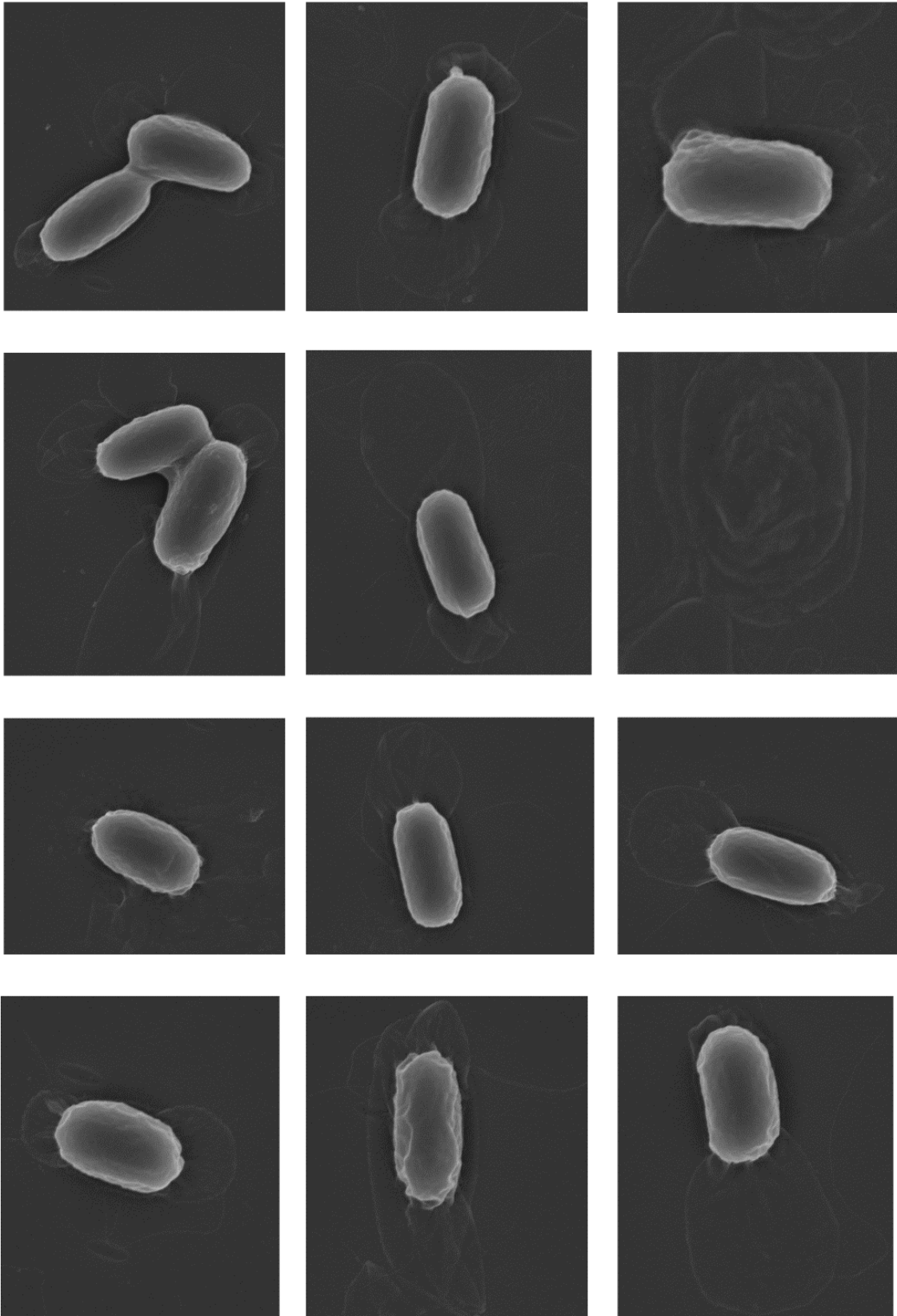
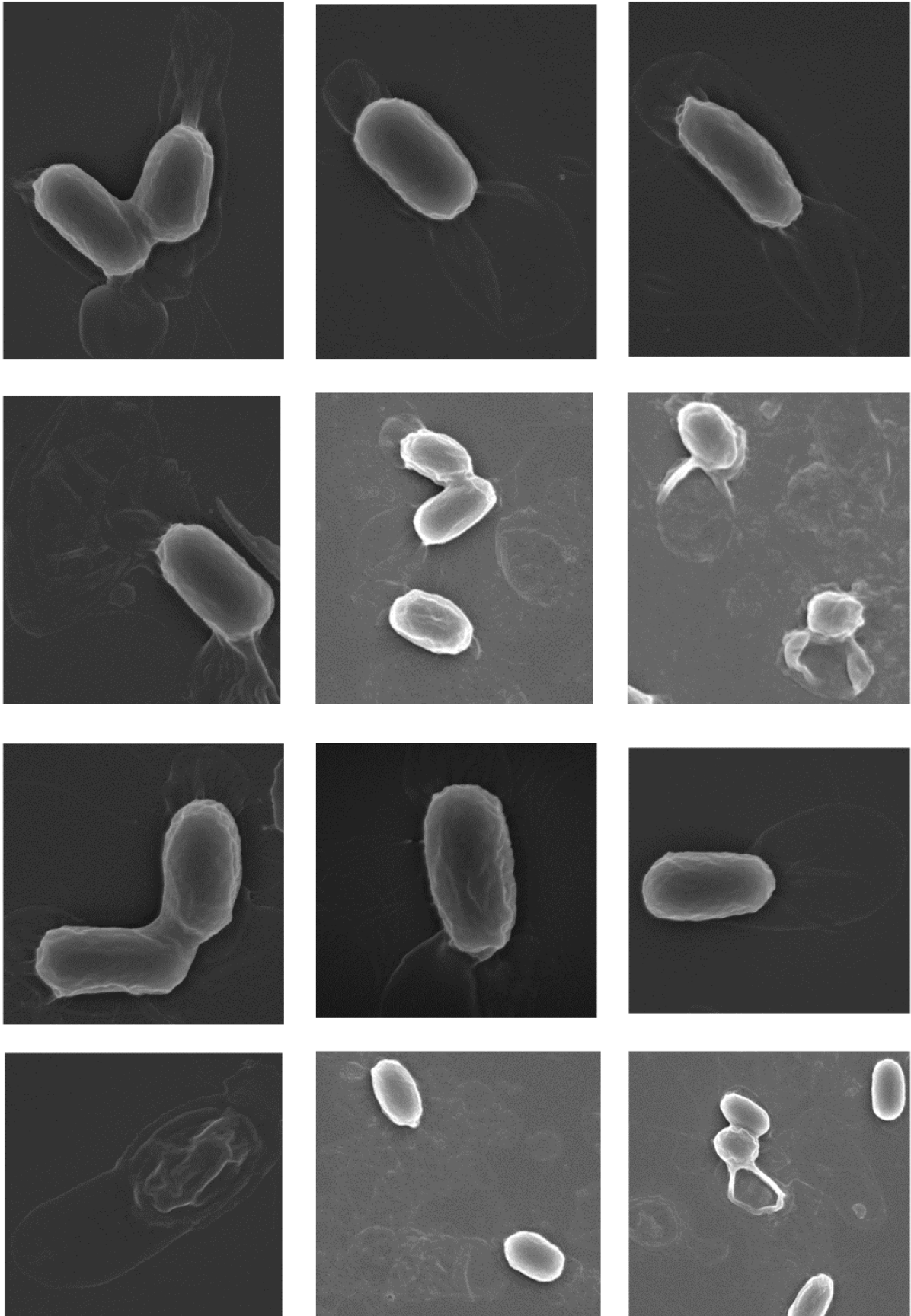


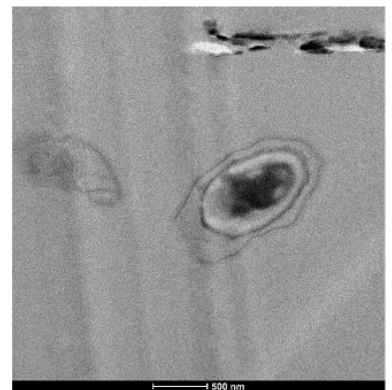
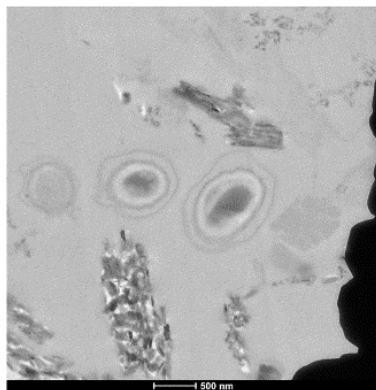
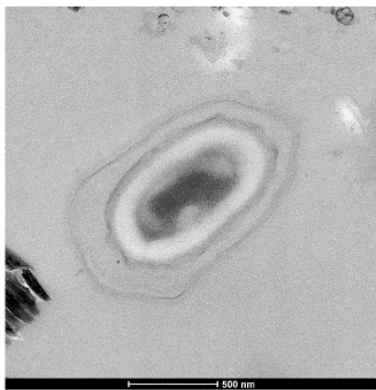
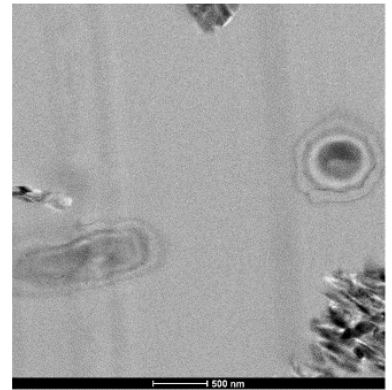
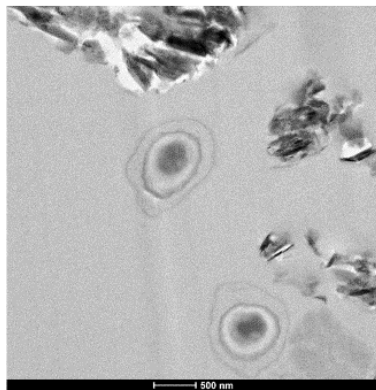
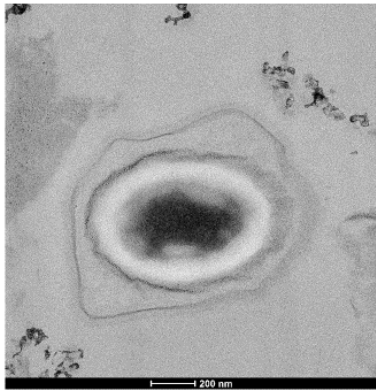
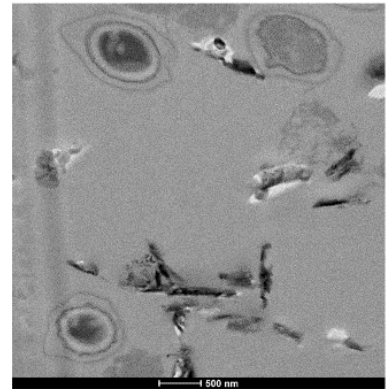
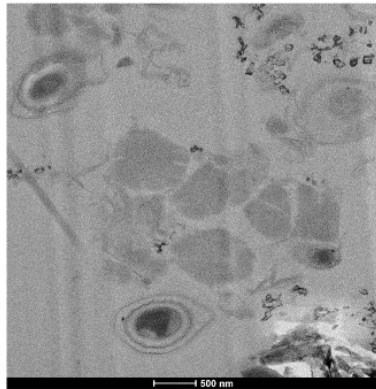
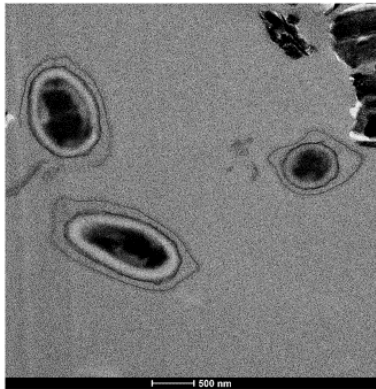
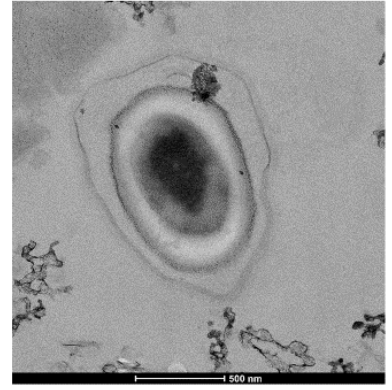
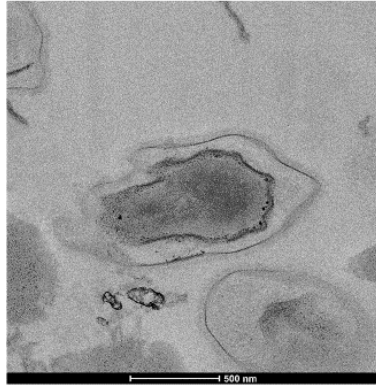
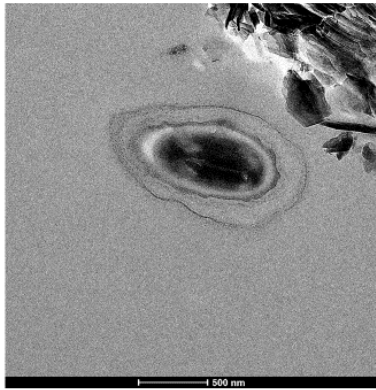
Figure S9 Additional fields of view of *B. thuringiensis* treated with 0.5% sodium hypochlorite for 30 min (24 SEM and 24 TEM fields of view)

Supporting information





Supporting information



Supporting information

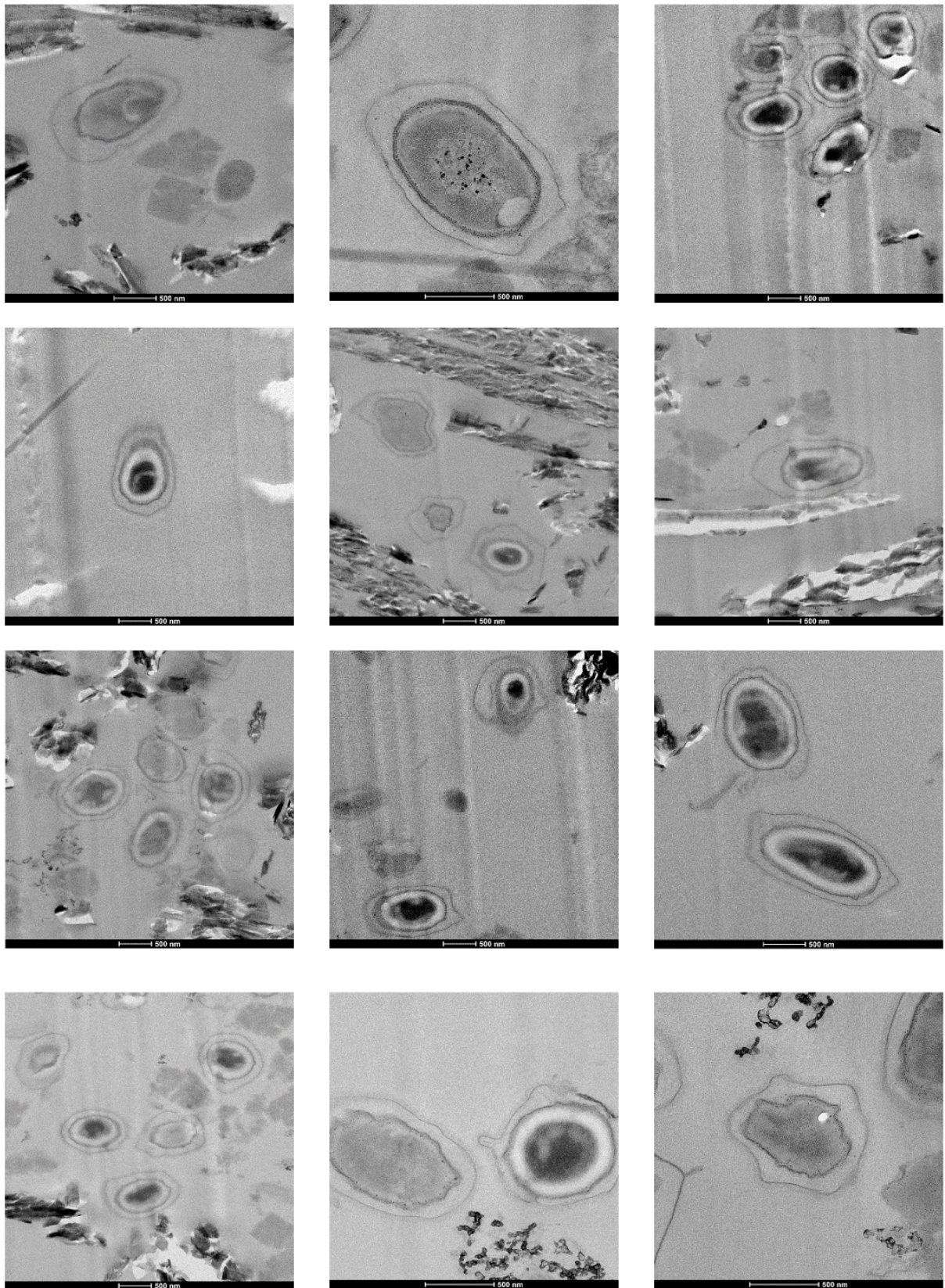


Figure S10 Additional fields of view of *B. thuringiensis* treated with 1% peracetic acid for 30 min (24 SEM and 24 TEM fields of view)