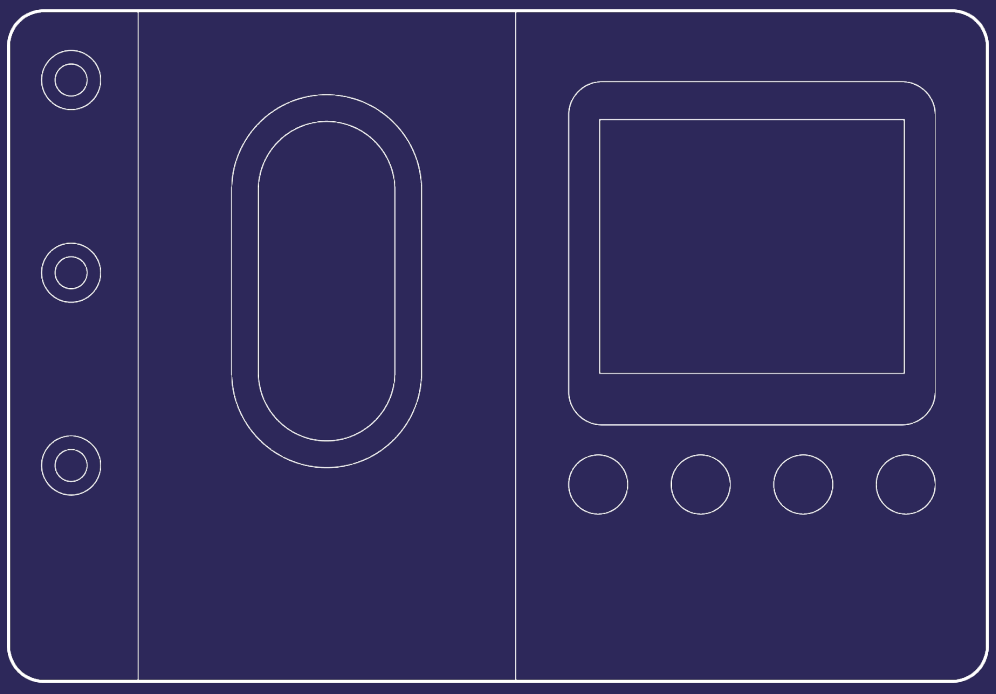
SBT

Application Note



BactoBox: An Essential Tool for Enumerating Spores

Abstract

Due to their rugged properties, spores are gaining increasing interest as a vehicle for delivery of live therapeutic and probiotic bacteria. User-friendly, precise, and reliable tools are needed to optimize yield of spore-forming bacteria. Plate counts are currently the gold standard for enumerating spores. They are, however, laborious, slow, and associated with pitfalls that may lead to underestimation. Recently, several promising fluorescence flow cytometry (FFC) techniques have been published, but these require access to expensive analytical instruments and skilled operators. In this whitepaper we show that BactoBox impedance flow cytometry is an appealing technique for real-time process control and enumeration of the final spore products. With no need for labeling, this technique can provide fast results for both vegetative cells and spore populations in complex samples.

Keywords

Endospore, exospore, sporulation, impedance spectroscopy, impedance flow cytometry, FCM, flow cytometry, sporangium, *Bacillus*, Firmicutes, *Streptomyces*, Actinobacteria, enumeration, In process control, process analytical technologies, PAT, real-time, optimization, staining, fluorescence microscopy, sporulation dynamics.

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Interest for spore-containing products is growing

Bacterial spores have a fascinating ability to endure extremely harsh environments[1]. An example of this is the isolation of viable spores from plant samples dating back to 1640[2]. Some spore-formers, e.g. *Bacillus anthraxis*, are notorious pathogens, but nonetheless, non-pathogenic spore-formers have received growing interest in live biotherapeutic products or as ingredients in probiotic foods and supplements. This interest is based on the fact, that – compared to vegetative cells - bacterial spores show superior survival through the acidic stomach so they can colonize the intestines and confer health protection to the host[3]. Apart from probiotics and live biotherapeutics, benign *Bacillus* spores are also formulated in cleaning products to suppress bacterial pathogens like *Staphylococci* and *Streptococci* on surfaces[4]. Last, but not least, spore-forming bacteria are also finding applications within agribiotech to aid in growth promotion, disease resistance, quality improvements and abiotic stress[5].

Two *Firmicutes* genera are the most exploited organisms for deliberate spore-production, namely the aerobic *Bacillus* species and the anaerobic *Clostridia* species. The present application note focuses primarily on *Bacillus* endospores. The sporulation process has been detailed elsewhere (for an excellent illustration, see McKenney *et. al*, 2012[1]). *Bacillus* initially follows a vegetative growth cycle, but deprivation of nutrients or environmental stressors can trigger the sporulation cycle. Through an asymmetric cell division, the initial sporangium forms and the endospore starts to appear as the smaller part of the cell division, the forespore. Later in the sporulation process, the endospore is engulfed within the mother cell and will only be released upon mother cell lysis. When nutrient conditions are again favorable, the spore can enter the vegetative cycle[1]. Thus, the endospore provides a convenient, well-protected “escape pod” to resist environmental stressors.

Existing methods for spore enumeration

Two overall methodologies exist to enumerate bacterial spores: Cultivation or particle counting by e.g microscopic counts, flow cytometry and particle analyzers like the Coulter counter[6].

Plate counts: The gold standard is regular cultivation by spread-plating, typically following a two-step protocol, where samples are plated before and after heat treatment[6,7]. Before heat (BH) result plate counts will show the total number of viable bacteria, i.e. both spores and vegetative cells, while the after heat (AH) result will only reflect the concentration of heat-stable endospores. While this is a simple and inexpensive method, substantial manual efforts are needed and the turnaround time is several days. This is mainly due to the long germination time for all spores to "hatch". Also, there are pitfalls that can lead to underestimation, for example endospores tend to agglomerate, which can lead to a ten-fold underestimation, and in addition, the early-germinating mucoid colonies of e.g. *Bacillus* can often hide late-sporulating colonies. The hydrophobic spores are also known to adhere to the typical glass or polymer Drigalsky spreaders used for distributing the sample on the agar[6]. Inter-operator variation is an additional well-known shortcoming of the traditional plate counts[8].

Microscopy: The refractive properties of spores make them easy to distinguish from vegetative cells using phase contrast microscopy. Nonetheless, the small size of spores makes them challenging and tedious to enumerate in counting chambers, and the analysis is operator dependent. The main shortcomings with this technique are low throughput and low precision making it unfit for real-time monitoring of sporulation processes[9].

Fluorescence flow cytometry (FFC): Compared to microscopy, FFC offers higher throughput and much higher precision. FFC is therefore regarded as a promising tool for real-time monitoring of sporulation processes and enumeration of spores in final products [8–11]. Despite their advantage FFC instruments are expensive and extensive training is needed to achieve accurate results. In addition, FFCs are bulky and require tidy working environments for staining processes, sample workup and analysis making them unfit for monitoring sporulation processes close to the action.

Particle analyzers: Instruments like the Multisizer have excellent precision and can be exploited to determine the concentration of spores in highly purified spore preparations[6]. On the other hand, it is not straightforward to perform real-time monitoring of active sporulation processes, as it is difficult to distinguish endospores from other cellular debris with similar size. Finally, size-based particle analyzers will likely not reveal endospores contained within mother cells with a size similar to that of vegetative bacteria.

A BactoBox real-time study of sporulation in two *Bacillus* species

As highlighted above, there is a need for a precise, user-friendly, and operator-independent method for characterization of sporulation processes. We propose BactoBox as a robust, label-free tool for simultaneous monitoring of endospores and vegetative cells. The rationale for this is that the electrical properties of endospores and vegetative bacteria are very different. The G^+ vegetative cell of *Bacillus* has a single, isolating lipid membrane and a conductive cytoplasm. On the other hand, the spores have two sets of isolating forespore lipid membranes and relatively little conductive core water content[1]. The measurement principle in BactoBox is based on impedance flow cytometry (for more information, see Bertelsen *et al.*, 2020[12]). Lipid membranes pose a significant hindrance (impedance) to current flow, and with two sets of lipid membranes, endospores are expected to be less conductive than their vegetative counterparts. Also, the differences in size results in smaller signal amplitudes for endospores than for vegetative bacteria when flowing through the electric fields in the BactoBox flow cell.

We therefore set out to exploit BactoBox for investigation of fermentation processes for two spore-producing bacteria, namely *Bacillus thuringiensis* (DSM 6108) and *Bacillus subtilis* subsp*. spizizenii* Bundesgesundheitsamt (BGA, DSM 618).

B. thuringiensis displays a remarkable production of protein crystals (delta toxins) with a potent insecticidal effect on insect larvae, and in fact *B. thurigiensis* spores is the most commonly used biopesticide worldwide[13]. To release these toxins, the mother cells of *B. thuringiensis* must lyse efficiently, leading a concomitant release of endospores[13,14]. *B. subtilis*, on the other hand does not form crystal toxins and therefore the endospore-containing mother cell stage is more long-lived and it can be necessary to exploit various treatments to release the endospores, e.g. heat shock, surfactants, organic solvents, enzymes and ultrasound[15–17].

Tracking of sporulation processes in simple shake flask experiments

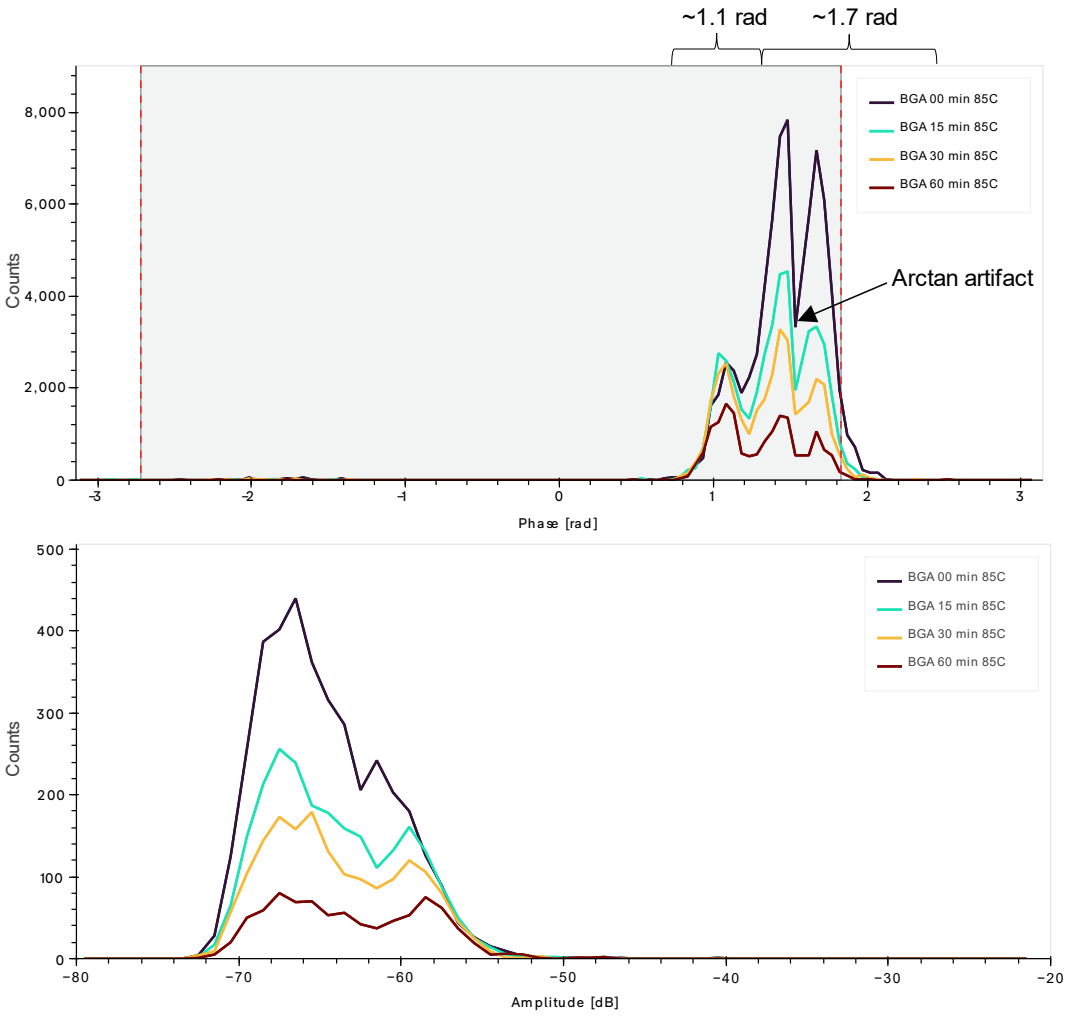
The cornerstone for the study was to initially home in on the optimal BactoBox settings for *Bacillus* endospores using a commercial purified preparation of endospores.

Subsequently the goal was to perform real-time monitoring of sporulating *Bacillus* cultures. In brief, shake flasks were inoculated with a colony from an agar plate and samples were drawn during the early exponential phase and the later sporulation processes. At each time point, 5 mL sample was transferred to a 15 mL vial and homogenized using bead beating with 3 mm glass beads (Bead Genie, speed 4800, 1 min). All samples were diluted in a diluent of ~1850 µS/cm to provide adequate parameters for detection of intact cells and to fit the linear range of BactoBox. Subsequently, samples were measured on a BactoBox in one-plicates. Samples were also inspected with phase contrast and fluorescence microscopy to achieve a qualitative assessment of the transition from vegetative cells to endospores. SYTO-9 was used as a total stain at a final concentration of 10x. TOTO-3 was likewise used at a 10x concentration. Staining was performed in the dark for 15-30 min.

Endospore signature: Low amplitude and minimally conductive properties

The electrical properties of endospores are expected to differ from that of normal, vegetative bacteria. Firstly, they are wrapped in two sets of poorly conductive lipid bilayers, and secondly the low water content in the core deviates from the normal highly conductive cytoplasm of vegetative bacteria.

To find the best BactoBox settings for endospores, BactoBox measurements were performed on commercially available purified spores from *B. subtilis* BGA spores. The suspension was vortexed briefly and diluted 1:100 in standard BactoBox diluent prior to BactoBox measurement. Two populations were observed in the phase shift diagram for untreated purified spores (upper histogram, black curve). The phase shift is a concept from electrical engineering which is best explained as a "delay" of the electrical signal as it passes through objects, e.g. bacteria and other particles. When analyzed with BactoBox, objects with a phase shift of ~1 rad are of an entirely non-conductive nature. The 1.1 rad population is therefore very poor at propagating electricity, while the distribution at ~1.7 rad is slightly more conductive, but still almost exclusively outside the more conductive region of normal intact vegetative cells (white region from 1.83 to 3.14). The amplitude distribution (lower histogram) is mainly reflecting the size of the objects. Two populations are observed with some overlap. The endospores are known to be small (0.5-1.5 µm), ellipsoidal or spherical, and they are therefore expected to belong to the distribution with the lowest amplitudes ranging from -74 to approx. -60.



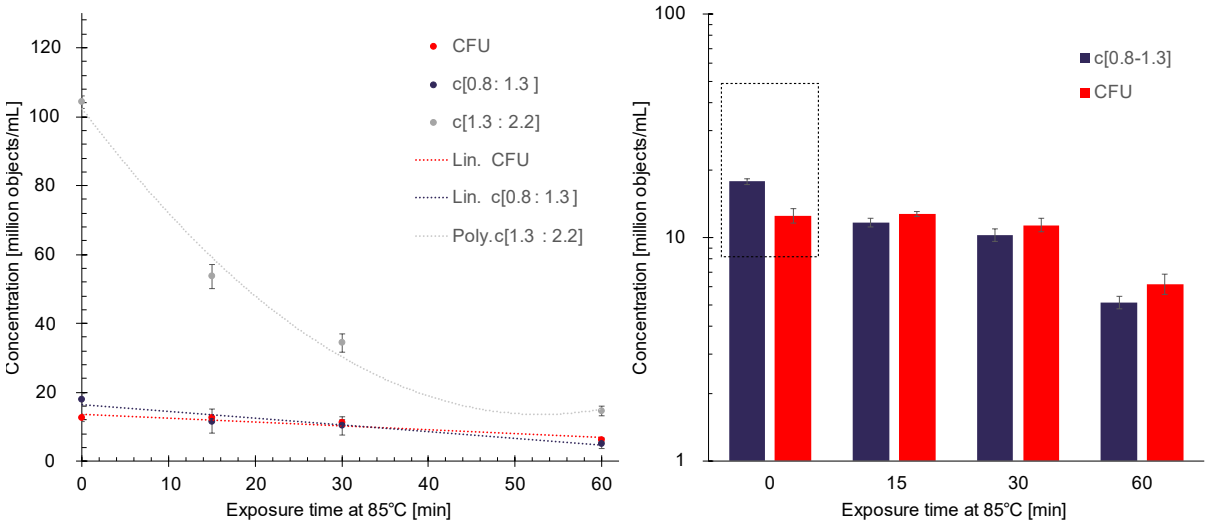
Purified BGA spores reveal two populations with different electrical properties: BactoBox Explorer phase shift histogram. First population is centered at ~1.05 rad, while the second population is centered at ~1.7 rad. The x-axis represents the phase shift properties, while the y-axis displays the number of objects with a given electrical phase shift. The region from
-π to -2 did not contain any relevant data and therefore the range has been set to -2 to 3.14. NB! The valley occurring at 1.57 (π/2), gives the impression that there are a total of three populations, but this dip is an Arctan artifact in the visual representation of data. For more information see page 63 in this SBT PhD thesis.

Due to the presence of the two phase shift populations, it is not yet possible to unambiguously assign endospores to a single population. Curiously, the two populations appear to have different heat tolerance where the ~1.1 rad population is more tolerant to heat (85 °C, 15 min) than the other. In the subsequent plots, specific intervals (gating) for the high frequency (HF) channel have been applied for the two populations. We will later refer to these as impedance fingerprints:

A. ~1.1 rad population: HFphase [0.8 : 1.3] + HFamplitude [-80 : -60]. Dark violet
B. ~1.7 rad population: HFphase [1.3 : 2.2] + HFamplitude [-80 : -20]. Grey

When the concentrations are inspected as a function of heat treatment time, the concentration of ~1.7 rad population (dark purple curve) is halved within 15 minutes, while the ~1.1 population (grey curve) shows a smaller drop in concentration over time. Knowing that endospores have remarkable heat tolerance it is more plausible that the spore population would be the ~1.1 rad population. This is further strengthened by the fact that the ~1.1 rad population shows good correlation to the colony forming units (CFUs) with a close to a 1:1 correlation for the different treatment times (bar chart). The concentration determined from the ~1.1 rad population is in good agreement with the $8x10^6$ to $5x10^7$ CFU/mL range provided by the BGA certificate of analysis, CoA, as indicated with the dashed box over the 0 min bars below.

In summary, from these data, the optimized impedance fingerprint for *Bacillus subtilis* BGA endospores are the HF custom gating parameters HFphase [0.8 : 1.3] and HFamplitude [-80 : -60]. These settings will be used throughout this application note.



B. subtilis spore populations have different tolerance to heat: The x/y chart on the left shows that the population centered at ~1.7 rad has relatively poor heat tolerance, while the population centered at ~1.1 rad has high heat tolerance. The colony forming unit (CFU) concentrations are close to that of the ~1.1 rad population. This relationship is tested further in the bar chart on the right where the ratio between the ~1.1 rad population and the CFUs are relatively close to 100% for the different heat treatment times. The ~1.7 rad population follows a 2nd order polynomial trend for reduction in number of objects as a function of heat treatment time (R^2 = 0.9884), while both the ~1.1 rad population and CFUs follow a linear trend as a function of heat treatment time (R^2 = 0.9312 and 0.8763, respectively). All analyses performed in triplicate dilution series. The CFU/mL accept range from the BGA certificate of analysis, CoA, is shown as a dashed box.

Morphological changes induced by sporulation

After determining the custom gating for purified BGA spores we set out to investigate sporulation over time in more complex cultures of *B. subtilis* and *B. thuringiensis*. To ensure maximal production of spores, an optimized growth medium was kindly provided by Ohly GmbH. Full details of the medium can be accessed here. 100 mL sterile-filtered medium in baffled Erlenmeyer shake flasks were inoculated with a colony from an agar plate and incubated (35 C, 200 RPM). The *B. subtilis* strain was the same as for the initial purified spore experiments (DSM 618). Samples were withdrawn over time and subjected to bead beating with glass beads for 1 min at max speed (bead genie) followed by BactoBox analyses and microscopy on an Etaluma LS620 microscope equipped with a 60x long working distance phase contrast objective and three fluorescence channels. Three visualization principles were used for microscopic evaluation:

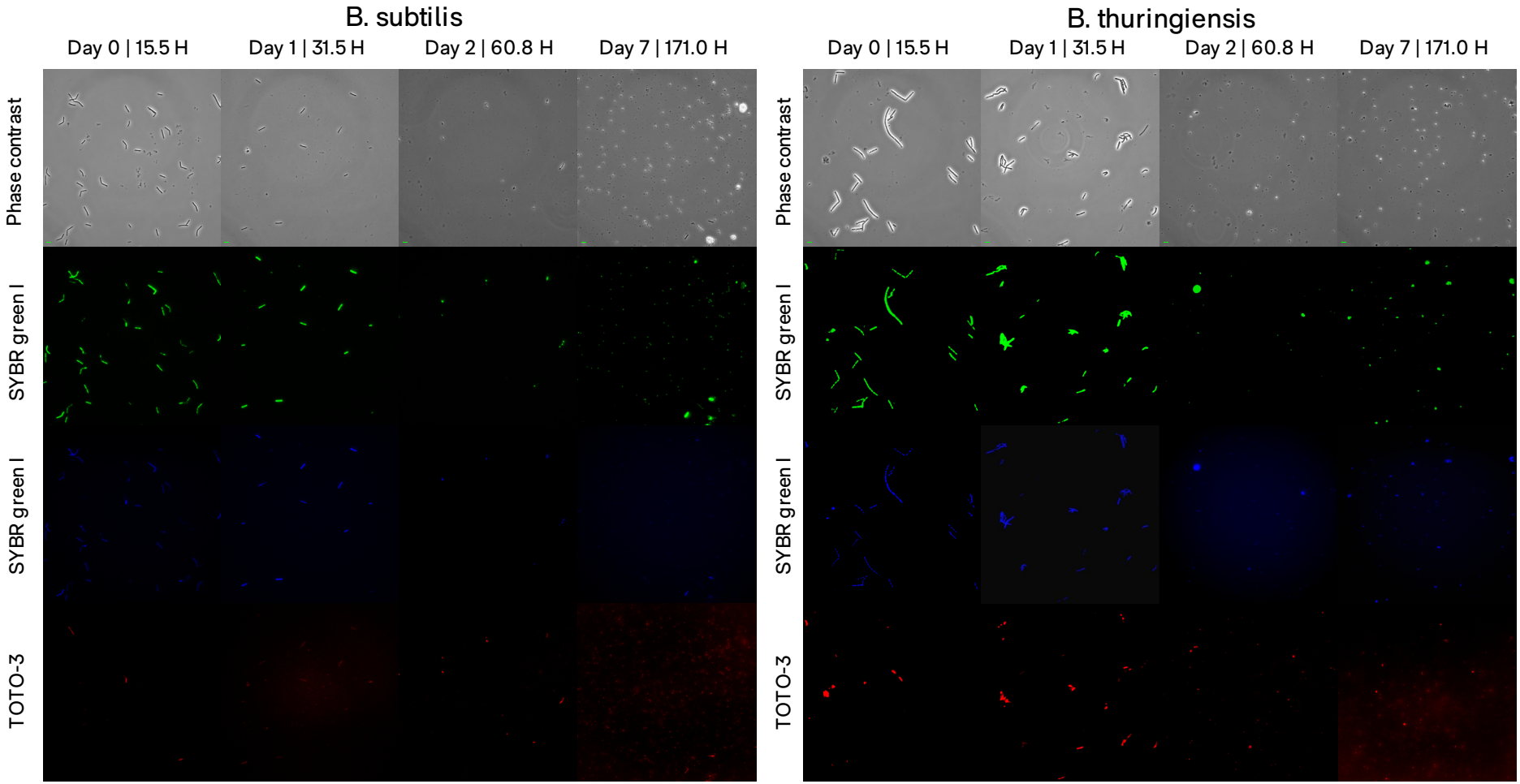
Phase contrast: Vegetative cells appear as black objects. Spores appear as bright, refractive objects.

SYBR green I: This cyanine dye is permeant to membranes and will stain nucleic acids and emit light at 520nm, predominantly in the green fluorescence channel. Some bleed-through is observed in the blue channel. Here, the acquisition settings were set to exploit the green channel for detection of weakly fluorescent objects, e.g. the difficult-to-stain endospores and exploit the blue channel for visualization of vegetative cells.

TOTO-3: This cell-impermeant thiazole red homodimer will stain nucleic acids in cells with compromised membranes and have maximum emission at 661 nm in the red fluorescence channel.

After 10-20 hours, the cells were well within the exponential stage. For *Bacillus subtilis* day 0 and day 1 phase contrast micrographs (upper left row) feature mainly large, dark rod-shaped single cells and chains with intense staining by SYBR and relatively poor staining with TOTO-3. These are likely vegetative cells devoid of pre-spores and endospores. On Day 2, bright, refractive, small objects appear within the rods with distinct staining of a TOTO-3-positive part and a part with less intense TOTO-3 staining. These objects are likely pre-spores and final spores. On day 7 mainly small, refractive endospores are observed with few rod-shaped objects present.

For *B. thuringiensis*, bright, refractive objects appear inside the large rod-shaped cells already on day 1. On day two, no rod-shaped cells are seen and the same goes for day 7, where only small objects are observed. Contrary to the *B. subtilis* endospores the *B. thuringiensis* endospores give relatively intense staining with SYBR. Also note that *B. thuringiensis* is more permeable to TOTO-3 during day 0 and 1 than *B. subtilis* indicating that prespore formation occurs very early for *B. thuringiensis*. In summary, the microscopic data indicates that *B. thuringiensis* forms endospores earlier than *B. subtilis* and that the sporulation appears more complete for *B. thuringiensis* with no detection of rod-shaped objects in the 7-day micrographs.



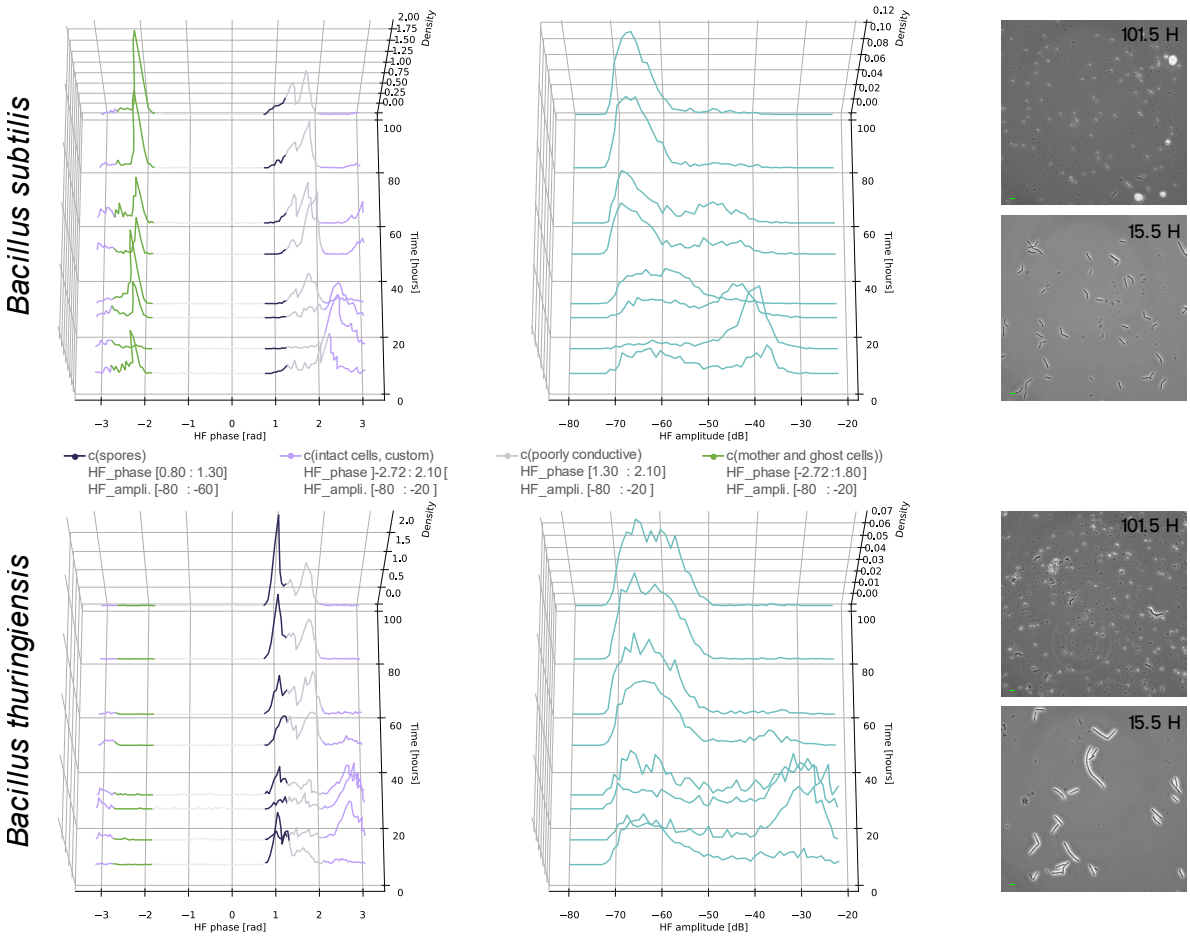
Microscopic progression from vegetative cells to endospores. *Bacillus* shake flasks assayed from the early vegetative cycle to the final sporulation stage. Each column represents the incubation time and each row represents the microscope channel for a fixed x/y position on the microscope slide. The upper row is the phase contrast image. SYBR-green emission mainly occurs in the green channel but also bleeds into the blue channel. The exposure settings for the green channel have been set to enhance detection of weakly fluorescent objects like endospores. The acquisition settings for the blue channel have been balanced for visualization of vegetative cells. The lowest row features fluorescence emission from TOTO-3 that mainly stains bacteria with non-intact membranes. A 5 µm scale-bar is shown in all images. Note that the samples on Day 7 have been concentrated x5 using centrifugal filters with 1,000,000 molar weight cutoff, MWCO.

BactoBox tracks sporulation processes in real time

The microscopic inspection shows that *B. subtilis* is predominantly in a vegetative state at 15.5H and 31.5H. This is also the case when with BactoBox analyses in the upper left part of the figure with the intact cell range of the HF phase distributions (shown in violet). Two additional populations are appearing at 31.5H: a population with conductive objects centered at -2 rad (green part) and a population with poorly conductive properties (grey part). The objects with impedance signatures corresponding to the purified BGA spores mainly appear later than 31.5H (dark purple part).

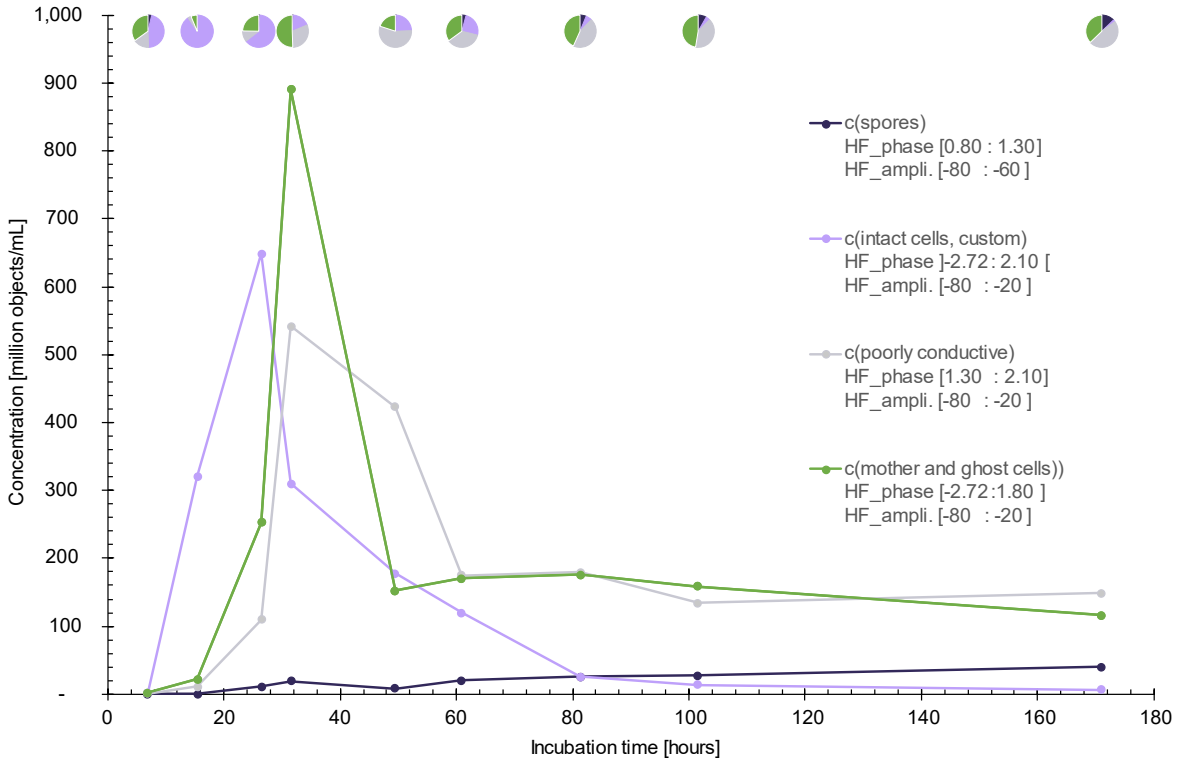
For *B. thuringiensis*, the vegetative cells are also mainly present at 15.5H and 31.5H, but curiously, the conductive objects (green part) are completely absent. The amplitude distributions (turquoise curves) indicate that some of the objects are extremely large, which is consistent with the huge (>20µm) chains in the phase contrast images at 15.5H (right part of the figure). Of note, objects larger than 5µm will typically not enter the measurement channel, and therefore improved homogenization of chain formers must be done before the quantitative results for *B. thuringiensis* at the early time points can be trusted. For *B. subtilis*, the events have lower amplitude, which means that the quantitative results are more reliable.

The highly conductive objects at -2 rad are typically observed when (especially G+) bacteria have compromised membranes, but still have somewhat intact cell peptidoglycan cell walls. As mentioned in the introduction, *B. thuringiensis* needs to efficiently release toxins from the interior of the cell, and therefore the mother cell state with a permeabilized membrane is expected to be very short. This fits well with the absence of the conductive objects at -2 rad. For *B. subtilis*, the -2 rad population appears to be more persistent and somewhat variable over time. In principle, this population could harbor both dead cells (ghosts) as well as endospore-containing mother cells with non-intact membrane. For convenience, this population will be referred to as “mother cells + ghosts”.



BactoBox tracks sporulation in real time. 3D-plot of the phase shift distributions (left), reflecting the electrical properties of *Bacillus* objects. Upper part represents *B. subtilis* while the lower part represents *B. thuringiensis*. X-axis depicts high frequency (HF) phase shift angle in radians, while the probability density function is shown on the y-axis. Z-axis reflects the incubation time. The impedance signatures underlying the grey, green, dark purple and violet coloring are described in the text. HF amplitude distribution is represented on a log scale from -80 to -20 dB. Small objects are seen at high negative values, while larger objects move right towards smaller negative values. The two phase contrast micrographs show the presence of either only vegetative cells (15.5 H) or mainly spores (101.5 H).

Below, the *B. subtilis* data have been subjected to custom classification for the three observed populations when adjusted for dilution factor. Early in the growth curve, the vegetative cells dominate (violet curve) until 31.5 hours where this population drops in concentration. At this time point, two other populations increase in concentration, namely the "mother and ghost cells" (green curve) and the "poorly conductive" population just right of the spore population in the phase shift diagram (grey curve). After the 31.5 H sampling point, these populations drop in concentration to stabilize after ~49.4 H. The region corresponding to the *bona fide* purified spores (black curve) starts to appear when the intact cell concentration drops. The endospore concentration increases at least to the last sampling point. The pie charts in the upper part of the figure indicate the relative abundance of each species. The most striking change over time is the reduction of intact cells from virtually 100% of the detected events at 15.5 H to almost nothing after 101.5 H. Also, the spores with the narrow custom gating constitute an almost negligible part until after the 60.8 H sampling point.



Quantitative *B. subtilis* changes from vegetative to sporulation cycle monitored by BactoBox: The individual populations from the phase shift diagram adjusted for dilution factors and depicted as absolute concentrations. The pie charts show the relative concentrations of the three populations for each sampling point with respect to the incubation time. Open brackets in the legend for the custom gating indicate that the outside limits are used for classification, while closed brackets indicate inside limits. Note that the intact cell region has been narrowed slightly from the default]-2.72:1.83[limits based on comparison with qualitive results from fluorescence microscopy (results not shown). Empirical concentration correction (ECC) factors were not used in the present dataset.

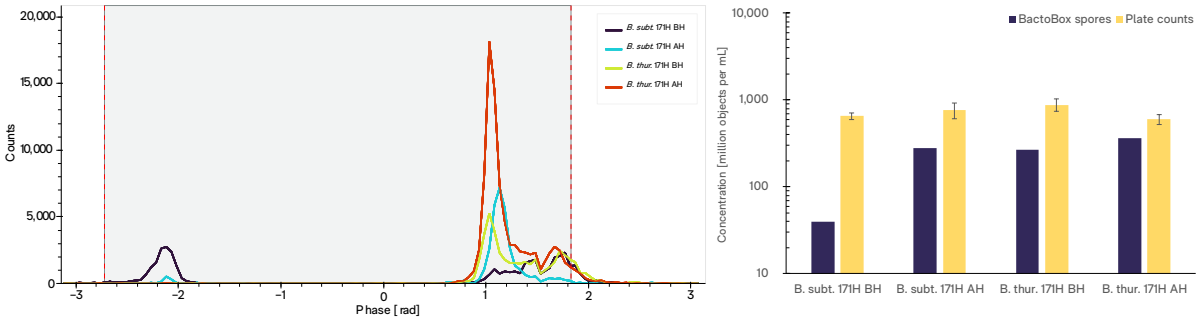
B. subtilis endospores may be trapped within mother cells

For the *B. subtilis* growth curves, the spore concentrations obtained by narrow gating constitutes a surprisingly small proportion (12%) of the total particles, while the proportion is higher for *B. thuringiensis* (18%). It is possible that some of the endospores are still contained within mother cells and that e.g. enzymatic or heat treatment is necessary to release them from the mother cell "cages". The effect of heat treatment is investigated below (phase shift diagram, left part). Initially, before heat (BH), the 171H *B. subtilis* sample shows a complex distribution with the "mother and ghost cells", "poorly conductive" and endospore populations (black curve). After heat treatment (AH), the dominant population is the ~1.1 rad endospore population (cyan). This could indicate that the heat treatment liberates a proportion of the endospores from the mother cells. It is also possible that some states of endospores are found in the poorly conductive region.

For *B. thuringiensis*, the populations are more similar before (green) and after (red) heat treatment, although some left shifting of the populations is observed indicating that the objects become less electrically conductive after heat treatment.

When the endospore-gated populations are compared with plate counts, the AH measurements for *B. subtilis* are closer to plate counts than the BH measurements. For *B. thuringiensis*, the correlation is also improved, but not as dramatically as for *B. subtilis*.

In summary, the *B. thuringiensis* endospores appear to be effectively released from the mother cells during sporulation while e.g. heat treatment is necessary to liberate the endospores from *B. subtilis*.



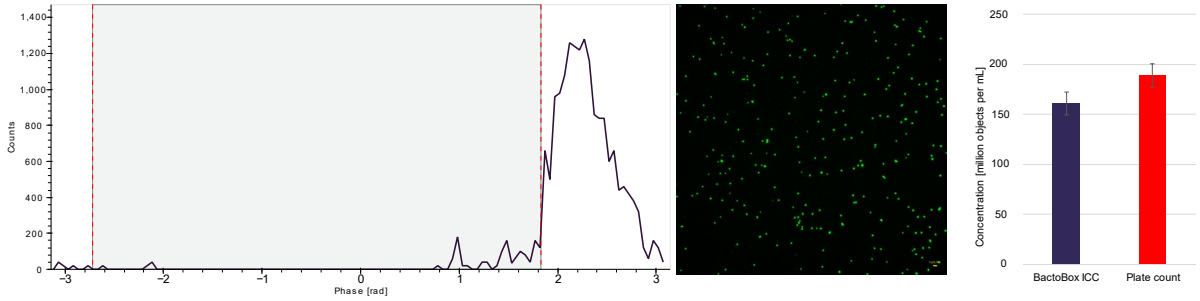
Heat treatment is needed to release B. subtilis but not B. thuringiensis endospores: Left phase shift diagram shows B. subtilis and B. thuringiensis endospores before and after heat treatment at 85 °C for 15 min. The black and green curves are before heat (BH) while the cyan and red curves are after heat treatment (AH). The white region indicates the default intact cell classification limits, while the grey region indicates other objects than intact bacterial cells. Right part of the figure shows the BactoBox and plate count results for the samples from the phase shift diagram. Plate counts were performed in triplicates plating from the same dilution series, while BactoBox measurements were performed in 1-plicates.

Make sure to track the abiotic background in the system

When enumerating endospores, it is important to keep an eye on the background from other objects with poorly conductive properties. This is because the endospore phase shift is relatively close to that of e.g. microplastic particles released from the peristaltic tubing in the BactoBox. Two workarounds are possible: i) Check the background from the system using a sterile filtered "blank" diluent sample and subtract this from the result, or ii) perform measurement at relatively high spore concentrations where the background from the system is negligible, e.g. >1,000,000 total particles/mL.

Exospores are quantified reliably with default measurements

Streptomyces exospores are formed by a completely other mechanism than endospores. Upon starvation, the bacterial chains (filaments) grow into the air and desiccate to form exospores that can be spread to more nutrient-rich environments. Contrary to endospores, the exospores are wrapped in a single cytoplasmic membrane and the cytoplasmic water content is also comparable to normal vegetative bacteria. Therefore - in theory - the exospores should be more electrically conductive than the endospores. When subjected to BactoBox measurements, this indeed turns out to be the case (left part of the figure below). In fact, the exospores have phase shift properties very similar to normal vegetative bacteria. Moreover, with no need for custom gating, the bar chart shows that the exospore concentration determined by BactoBox is in very good agreement with plate counts.



Streptomyces griseus exospores have similar electrical properties as vegetative cells: Phase shift diagram of exospores scraped from a 7-day S. griseus DSM 40855 tryptic soy agar (TSA) plate with 5% sheep's blood. Practically all objects are confined to the intact cell range (white region). The micrograph shows the green fluorescence channel from SYBR green-stained exospores revealing very homogeneous, small exospores (40x objective, 5 µm scalebar shown in yellow). The bar chart shows a head-to-head comparison of BactoBox intact cell concentrations (ICC) and plate counts, all performed in dilution series triplicates.

BactoBox as a Swiss army knife for spore characterization

It is tedious to enumerate endospores with cultivation-based methods and FFC requires expensive analytical instruments and skilled operators. BactoBox represents a promising alternative to plate counts and the impedance signatures for endospores are clear cut: Firstly, the small size leads to low-amplitude populations and secondly, the presence of a double set of membranes results in distributions very close to the entirely non-conducting region of the phase shift diagram.

For purified spores, BactoBox shows good agreement with orthogonal plate counts results also after heat treatment. For complex, sporulating cultures, BactoBox gives real-time, simultaneous, qualitative information on four different populations: Vegetative (intact) cells, released endospores, mother + ghost cells, and objects with poorly conductive properties. Best of all, the BactoBox instrument is affordable, sample workup is fast, and the procedure is easy to standardize.

Here, BactoBox reflected the sporulation landscapes for *B. subtilis* and *B. thuringiensis*. Consistent with established findings, *B. thuringiensis* releases spores efficiently from mother cells, likely concomitant with release of insecticide-like toxins. Endospore release from *B. subtilis* was conversely not as effective and the plausible mother/ghost cell population was present throughout the sporulation stage.

Finally, it was shown that BactoBox works well for exospores from *Streptomyces*. Exospores were associated with the same phase shift properties as vegetative cells. The advantages of using BactoBox for spore enumeration are summarized below:

Fast: While plate count takes several days (or weeks) for all the spores to hatch, BactoBox provides the results within few minutes.

Informative: Enumeration of both vegetative cells and spores

Precise: Coefficient of variation is typically within 5-10% for purified spores

Accurate: Close agreement with plate count results for purified spores

Optimization of fermentation processes for spore-producing bacteria

The advantages above greatly accelerate the learning loops for optimizing spore production. With plate counts several days of incubation would be needed to enumerate vegetative cells and endospores. With BactoBox, you get the results in real time and therefore the subsequent optimization tests can be initiated immediately. For purification of spores, the impedance data can unveil if spores appear to be trapped in certain populations and assess the presence of unwanted particulate impurities. The typical use case is to track fermentation processes for spore-producing bacteria, e.g. to optimize the medium constitution or maximize the yield of the final spore product.

BactoBox Explorer is a must for BactoBox spore enumeration

To harness the power of BactoBox for spore enumeration, you should first establish the proper phase and amplitude limits for your specific species. This can be done easily by post-analysis in the data visualization tool, BactoBox Explorer. Subsequently these settings can be uploaded to BactoBox as a custom program for real-time measurements. Contact info@sbtinstruments.com to get started with BactoBox Explorer.

Inspiration for gating is provided in the table below. It is also possible to download the BactoBox classification files for *Bacillus* endospores and *Streptomyces* exospores.

Spore type	HF phase [rad]	HF amplitude [dB]
B. thuringiensis / *B. subtilis*	[0.80 : 1.30]	[-80 : -60]
Streptomyces exospores	[1.83 : 3.14]	[-80 : -50]

Stay tuned for updates & join us in the quest for more answers

Some questions remain: What is the most effective sample treatment to liberate endospores from mother cells. What does the “poorly conductive” region in the phase shift diagram correspond to. And where do the crystal protein toxins from *B. thuringiensis* occur in the phase shift diagrams. More studies and orthogonal techniques are needed to answer these questions. Also, there are other interesting, sporulating microorganisms out there. This application note will be updated when BactoBox measurements are available on clostridial and fungal spores. We welcome you to join us in the quest for answers. By submitting (anonymized) data on growth curves from sporulating organisms it can be investigated if the impedance fingerprints for BGA spores are representative of all endospores or if the parameters must be optimized for the individual species. Please contact us at info@sbtinstruments.com if you are interested in contributing.

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