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Sensitive quantification of dipicolinic acid from bacterial endospores in soils and sediments

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Summary

Endospore-forming bacteria make up an important and numerically significant component of microbial communities in a range of settings including soils, industry, hospitals and marine sediments extending into the deep subsurface. Bacterial endospores are non-reproductive structures that protect DNA and improve cell survival during periods unfavourable for bacterial growth. An important determinant of endospores withstanding extreme environmental conditions is 2,6-pyridine dicarboxylic acid (i.e. dipicolinic acid, or DPA), which contributes heat resistance. This study presents an improved HPLC-fluorescence method for DPA quantification using a single 10-min run with pre-column Tb^{3+} chelation. Relative to existing DPA quantification methods, specific improvements pertain to sensitivity, detection limit and range, as well as the development of new free DPA and spore-specific DPA proxies. The method distinguishes DPA from intact and recently germinated spores, enabling responses to germinants in natural samples or experiments to be assessed in a new way. DPA-based endospore quantification depends on accurate spore-specific DPA contents, in particular, thermophilic spores are shown to have a higher DPA content, meaning that marine sediments with plentiful thermophilic spores may require spore number estimates to be revisited. This method has a wide range of potential applications for more accurately quantifying bacterial endospores in diverse environmental samples.

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Introduction

Cell dormancy is an effective ecological survival strategy in deep marine environments where nutrients required for cell growth and activity are often sparse or dependent on episodic inputs. Recently, Wörmer *et al*. (2019) quantified dipicolinic acid (DPA) to estimate endospore populations in 331 marine sediments, revealing the presence of 2.5 $\times 10^{28}$ to 1.9 $\times 10^{29}$ endospores in the upper km of marine sediments globally. Remarkably, these numbers are comparable to the number of total microbial cells in these environments. Based on this estimate, the dormant endospore population represents a significant proportion of the global carbon pool at 4.6 to 35 Pg corresponding to $\sim$0.3% of Earth's total living biomass. Despite these large contributions, relatively little is known about the roles of endospores in biogeochemical cycling and microbial ecology.

Bacterial endospores are multilayer structures (Fig. 1A) resistant to chemicals, heat, high vacuum and UV radiation (Nicholson *et al*., 2000; Nicholson *et al*., 2005; Setlow, 2006; Setlow *et al*., 2006; Setlow, 2007). Structural toughness and typically low spore numbers make it challenging to extract DNA from endospores in soils and sediments and thereby accurately estimating endospore abundance using PCR- or sequencing-based approaches (Wunderlin *et al*., 2013, 2014; Kawai *et al*., 2015). Sediment enrichment cultures are useful for characterizing viable endospore populations following germination, e.g. using 16S rRNA gene sequencing and microscopy but lack the capacity to quantify the initial endospore population (Chakraborty *et al*., 2018). An alternative method to determine endospore abundance is to employ the spore-specific biomarker 2,6-pyridine dicarboxylic acid (i.e. DPA) which forms a chelate with calcium during endosporulation and has been proposed to intercalate between DNA bound acid groups within the endospore structure (Lindsay and Murrell, 1986). DPA constitutes 5%–15% of the dry weight of typical bacterial spores (Powell, 1953; Powell and Strange, 1953; Church and Halvorson, 1959; Fichtel *et al*., 2007) and its

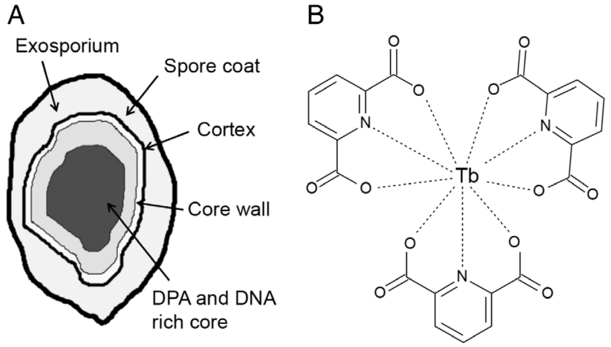

Fig 1. A. Schematic of an endospore cross-section showing the core where DNA is concentrated and intercalated with dipicolinic acid (DPA).
B. DPA complexed with the lanthanide metal terbium (Tb^{3+}) structure, based on the figure by Yang and Ponce (2011).

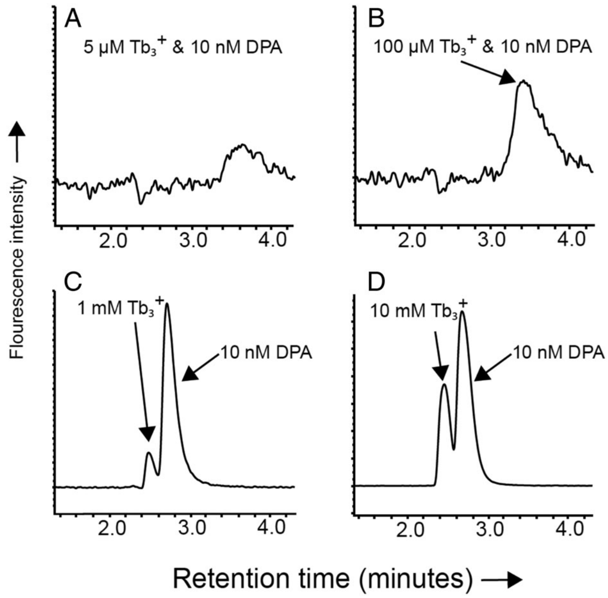

Fig 2. HPLC fluorescence chromatograms showing: A. 5 μM Tb^{3+} complexed with 10 nM DPA standard, resulting in a signal below the limit of detection.
B. Increasing the concentration to 100 μM Tb^{3+} complexed with the 10 nM DPA standard results in a single peak of the complexed mixture of Tb^{3+} and DPA, since there is insufficient excess Tb^{3+}.
C. 1 mM Tb^{3+} complexed with the 10 nM DPA standard. D. 10 mM Tb^{3+} complexed with the 10 nM DPA. The later retention time of 3.5 min in panel B. is likely due to a slight difference in ionic composition at the lower Tb^{3+} concentration.

quantification has been the focus of analytical methods using lanthanide metals (Tb^{3+}, Eu^{3+}, Gd^{3+}) as the fluorescing chelate (Barela and Dean Sherry, 1976; Wilschut *et al*., 1980; Sacks, 1990; Rosen *et al*., 1997; Pellegrino *et al*., 1998; Hindle and Hall, 1999; Fichtel *et al*., 2007; Yang and Ponce, 2009; Ammann *et al*., 2011; Yang and Ponce, 2011; Lomstein and Jørgensen, 2012; Wang *et al*., 2015; Wörmer *et al*., 2019). The Tb^{3+}-DPA chelate depicted in Fig. 1B has specific fluorescence excitation and emission wavelengths (Hindle and Hall, 1999). DPA measurements are also used as an indicator of endospore germination due to the rapid release of DPA prior to spore cortex hydrolysis and cell germination. In experiments that stimulate spore germination, 'free' DPA (i.e. release by endospore lysis) in the experimental medium is measured following direct chelation to Tb^{3+} (Setlow, 2003; Yang and Ponce, 2011; Francis *et al*., 2015), however in sediment enrichment slurries, chelation to Tb^{3+} is conducted after acid hydrolysis of the sample (Volpi *et al*., 2017).

This study constrains endospore numbers in complex matrices (organic-rich soil, various aquatic sediments) through an improved Tb^{3+} detection assay that can differentiate signals from intact endospore DPA and the 'free' DPA from germinated spores.

Results

Chromatography optimization

A variety of analytical methods has already been presented for quantification of DPA using Tb^{3+} chelation (Table S1). The method developed in this study improves and enhances pre-existing methods by changing the column type to a more recently developed Kinetex 2.6 μm EVO C18 100 Å LC column (150 × 4.5 mm, Phenomenex, USA). As described in the HPLC-FLD method of Lomstein and Jørgensen (2012), which uses the predecessor 5 μm Luna HPLC column, a 10 nM DPA standard was complexed with 5 μM Tb^{3+} in 1 M sodium acetate. This concentration of Tb^{3+}-DPA chelate resulted in no peaks measured by fluorescence detection (Fig. 2A). To further investigate and test if 10 nM DPA was below the limit of detection, DPA standards up to 2500 nM were complexed with 5 μM Tb^{3+} in 1 M sodium acetate but these chelate concentrations also gave no peaks (data not shown). It was only after increasing the concentration of Tb^{3+} that a low intensity, single chromatographic peak was observed (100 μM Tb^{3+} complexed with 10 nM DPA, Fig. 2B). In metal chelation chromatography methods, it is essential that two peaks are visible, i.e. one peak being the metal chelated to the target analyte and the second peak being the excess metal. This observation is required in order to show that an overabundance of metal is present and the analyte chelation capacity is reached. The presence of only a single peak at different DPA standard concentrations suggested that the amount of Tb^{3+} added was insufficient both to chelate DPA and leave excess Tb^{3+} visible at the 545 nm emission wavelength, in addition to the peak representing the Tb^{3+}-DPA. After experimentation with increasing Tb^{3+} concentrations 1 mM Tb^{3+} complexed with 10 nM DPA resulted in an excess Tb^{3+} peak at

2.4 min and the chelated Tb^{3+}-DPA peak eluting at 2.7 min (Fig. 2C). A final concentration of 10 mM Tb^{3+} was found to be optimal based on good chromatographic peak shape over a range of DPA concentrations (1 nM–2500 nM DPA) while not exhausting the Tb^{3+} supply (Fig. 2D). Due to the method using metal chelation an injection peak of unretained compounds or interfering solvents is not observed, meaning, either the unretained compounds are not chelated to Tb^{3+} or all the chelated compounds are retained. The column void volume was estimated based on the column volume (2.49 ml) and the porosity of the packing material (60%) to 1.49 ml.

Lowering the limit of detection

DPA standards and samples were combined with Tb^{3+} at a ratio of 1:3 (DPA to Tb^{3+}). In order to improve the limit of detection, the dynamic range of quantification can be expanded by inverting this DPA to Tb^{3+} ratio to 3:1 in specific instances, e.g. for low concentrations of DPA that approach the limit of detection. The 1:3 ratio of DPA to Tb^{3+} (resulting in a final Tb^{3+} concentration of 7.5 mM) increases the range of detection to ~2000 nM DPA, whereas the 3:1 ratio of DPA to Tb^{3+} (2.5 mM Tb^{3+}) lowers the range of detection to 125 nM DPA. The increased dynamic range makes it possible to achieve a 0.14 nM DPA limit of quantification (LOQ) and 0.04 nM DPA limit of detection (LOD), representing improvements on other methods reported in the literature (Table S1). The advantage of the 1:3 ratio of DPA to Tb^{3+} is the broader detectable range, whereas its disadvantage is the less sensitive detection limit. The advantage of the 3:1 ratio of DPA to Tb^{3+} is the sensitive detection limit, whereas its disadvantage is the much smaller dynamic range. Further testing indicated lower concentrations of Tb^{3+} gave lower LODs when using standards, however, in practice interfering organic substances in most environmental samples rapidly exhausted the supply of free Tb^{3+} resulting in unbound DPA in the sample leading to unquantifiable underestimation of DPA. It is recommended when starting analysis of an unknown sample set that some samples are initially processed at both 1:3 and 3:1 dilutions to tailor the analysis to suit the DPA concentrations in the samples.

Total extractable DPA

The complexity of sediment matrices coupled with different strategies for endospore lysis (autoclaving, acid hydrolysis) prompted an investigation into repeatability and recovery of DPA from sediment. Estuarine sediment (see Table S2 for sample information) was ground and homogenized after which 10 nM of DPA standard (Sigma Aldrich) was added. DPA was extracted from sediment using acid hydrolysis following the workflow of Lomstein and Jørgensen (2012) where total DPA was extracted from about 0.1 g freeze-dried sediment and hydrolysed with 6 M HCl in an oven at 95°C or using water-based oven or autoclave extraction protocols (Ammann *et al*., 2011). After correcting for natural DPA concentrations, maximum DPA recovery of 94 ± 3% (n = 33) was obtained using acid hydrolysis in 6 M HCl at 95°C (Table S3). Acid hydrolysis was used for all subsequent DPA extractions.

Differentiating between intact spore DPA and ‘free’ DPA

An estuarine sediment slurry prepared in minimal medium with organic substrates was incubated at 50°C in triplicate to investigate DPA dynamics during endospore germination by thermophilic spore-forming bacteria. Subsamples were removed from this incubation for total spore-contained DPA and free DPA quantification. Free DPA samples were processed in the same way as total DPA, but with the 4 h acid hydrolysis step omitted, i.e. by directly freeze drying the supernatant, filtering and complexing with Tb^{3+} (see Experimental procedures). The simplified method for free DPA analysis offers a rapid proxy for assessing spore germination with the potential for high-throughput processing of samples. Figure 3 shows the change in total DPA (acid extractable), free DPA (without acid hydrolysis), as well as the calculated intact spore DPA (total DPA minus free DPA) in an estuarine sediment incubation at high temperature. Following pasteurization, spore germination and free DPA release occur over 24 h, but increases in concentration are observed at 9 h and after

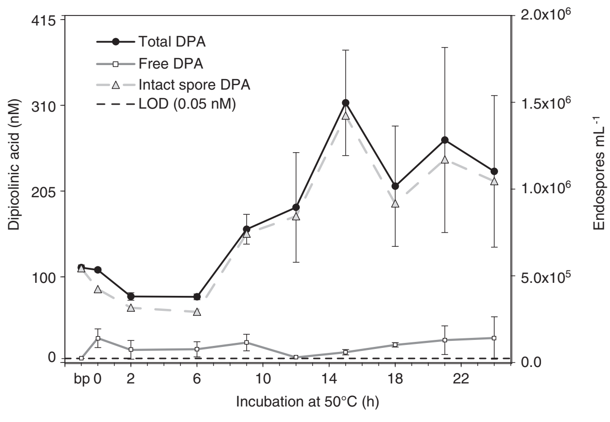

Fig 3. DPA dynamics in sediment slurry during 50°C incubation. The left axis shows the concentration of DPA, the right axis shows calcuated endospores per ml sediment slurry. BP: before pasteurization. Values are the average of triplicate incubations and error bars show the standard deviation. Total extractable DPA (HCl hydrolysed) and free DPA (dissolved organic acid) were measured. Spore contained DPA is the calculated difference between total DPA and free DPA.

15 h. Larger free DPA error bars are observed at 21 and 24 h. For total DPA concentrations, the error bars increased from 11% to 35% relative standard deviation at 9 to 12 h, reaching 40% by 24 h. Replicate samples were taken from three individual bottles of sediment slurry. Due to the large error bars of the total DPA signal in a previous experiment (data not shown), the same experiment (Fig. 3) was repeated and also gave large error bars for total DPA after 12 h. Analytical reproducibility of the method was determined to be <0.01% for repeat injections. During this experiment, the intact spore DPA was never less than 77% of total DPA. Assuming a constant amount of DPA per spore and no losses due to DPA uptake or degradation it is estimated that up to 23% of the overall endospore population had germinated at any given point.

DPA content of endospores from specific bacterial strains

In order to determine strain-specific DPA concentrations, we isolated a new *Desulfallas geothermicus* from a deep sea sediment in the eastern Gulf of Mexico. The culture was maintained on an artificial seawater medium containing 20 mM sulfate and 20 mM lactate and incubated at 50°C. Endospores were enumerated microscopically following malachite green staining and also analysed for DPA. Spore-specific DPA values for the *D. geothermicus* isolates were $14.4 \times 10^{-16} \pm 0.004 \times 10^{-16}$ mol DPA spore^{-1} which are among the highest levels of DPA per endospore reported in the literature (Fig. 4; Table S4). Figure 4 shows *D. geothermicus* (this study) along with spores from a range of different strains and habitats reported in other studies (Fig. 4; Table S4). Thermophilic strains are indicated by shaded bars, including the *D. geothermicus* isolate from this study, which has spore-specific DPA levels similar to other thermophilic spores. The DPA per spore value for *D. geothermicus* in this study ($14.4 \times 10^{-16} \pm 0.004 \times 10^{-16}$ mol DPA spore^{-1}) is considerably higher than the value of 2.2×10^{-16} mol DPA spore^{-1} derived from Fichtel *et al*. (2007) that is routinely used in other studies. This suggests that the conversion factor 2.2×10^{-16} mol DPA spore^{-1} must be revisited to

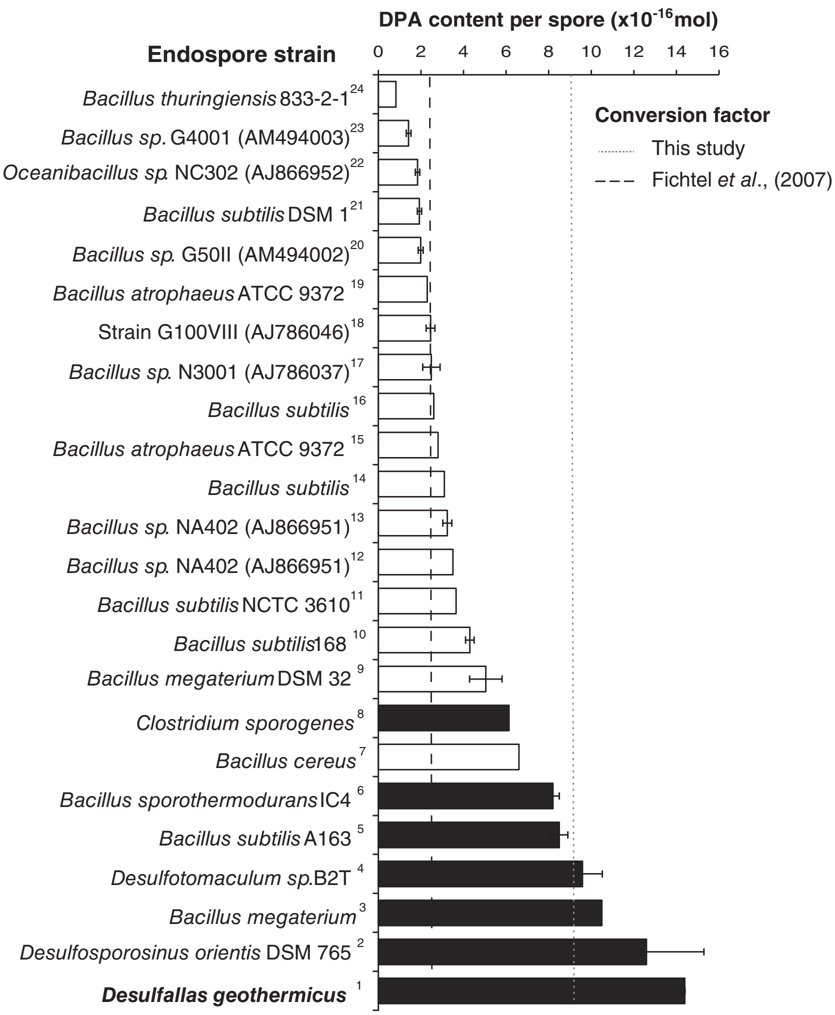

Fig 4. DPA content per endospore for different bacterial strains described in various studies (superscripts correspond to references in supplementary Table S4). Shaded bars indicate thermophilic strains. Dashed lines show the average spore-specific DPA content calculated by Fichtel *et al*. (2007) in wide black dash, and as determined in this study in small grey dash, based on values from literature as described in the text (studies denoted by an asterisk beside the organism name) together with a new strain of thermophilic *Desulfallas geothermicus* isolated in this study (highlighted in bold).

prevent over-estimation of endospore numbers in certain environments.

DPA quantification in soils and sediments

The EVO C18 HPLC column is selective enough to separate between two and four peaks in soil and sediment extracts (Fig. 5A–F). In all sample extracts, peak 1 is an unknown Tb^{3+} chelate eluting at 2.2 min, prior to the Tb^{3+} peak. Due to the high specificity of the fluorescence method for terbium at excitation wavelength 270 nm and emission wavelength 545 nm this is a Tb^{3+} bound compound. Comparison with Tb^{3+}-DPA standard (Fig. 2D) reveals peak 2 is the Tb^{3+} peak, eluting at 2.4 min and is closely followed by Tb^{3+}-DPA complex peak 3 at 2.7 min. Peak 4 is an unknown Tb^{3+} chelation complex, which is so far only observed in deep water marine sediments containing hydrocarbons. In samples containing no peaks at 2.7 min, concentrations of Tb^{3+}-DPA are lower than the LOD. In this case, the method of standard addition is carried out where low concentrations of Tb^{3+}-DPA complex in mobile phase buffer are added to the sample so as to increase the concentration of DPA but not deplete the Tb^{3+} concentrations (e.g. compare Fig. 5 panels A to B, and C to D). Starting concentrations are then calculated after a regression plot is constructed. In natural samples containing high abundances of endospores, quantification by standard addition is not necessary and DPA can be quantified using an external standard curve, as shown in Fig. 5 for River Tyne estuarine sediment (Fig. 5E) and Swedish peat bog soil (Fig. 5F).

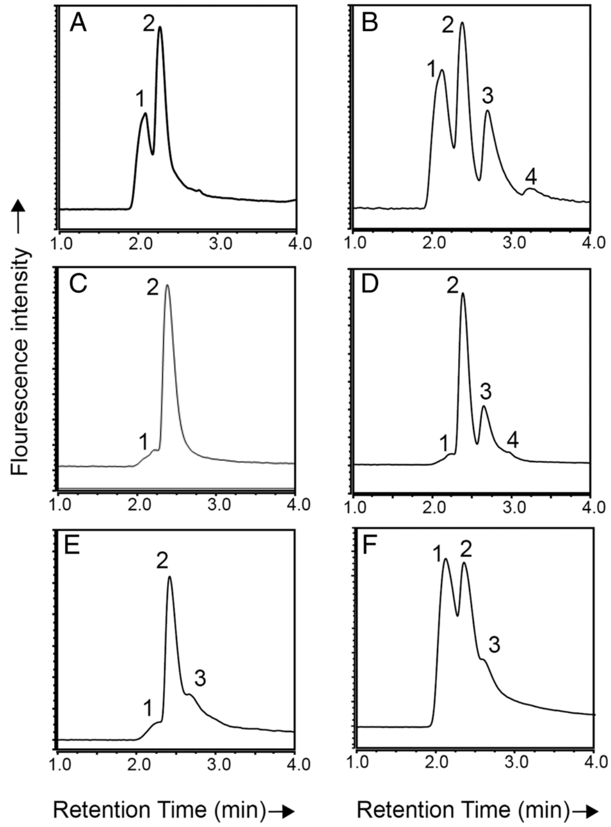

Fig 5. Chromatograms of DPA extracts from A. Deep Gulf of Mexico sediment (without DPA standard addition), B. Deep Gulf of Mexico sediment with 25 nM DPA standard addition, C. Deep NW Atlantic surface sediment (without DPA standard addition), D. Deep NW Atlantic surface sediment with 25 nM DPA standard addition, E. River Tyne estuarine sediment (no DPA spike) and F. Swedish peat bog extract (no DPA spike). Chromatograms show three or four peaks as follows: Peak 1: undefined Tb^{3+} complex A. Peak 2: unchelated Tb^{3+}. Peak 3: Tb^{3+} chelated to DPA, and Peak 4: undefined Tb^{3+} complex B. Dimensionless fluorescence intensity is shown on the *y*-axis.

Discussion

The challenge of accurately quantifying endospores in environmental samples

The method presented here provides an opportunity for better quantitative assessments of endospores in a variety of environmental samples, with particular benefits for soils and sediments. In energy-limited deep sub-seafloor sediments the formation of endospores aids long-term survival and preserves ecological diversity (Jones *et al*., 2010; Kawai *et al*., 2015; Wörmer *et al*., 2019). At the Peru margin site ODP 1229, a biomarker study using DPA in endospores and muramic acid in vegetative cell envelopes reported that numbers of bacterial endospores and vegetative cells were approximately the same (Lomstein *et al*., 2012). Determining the number of conserved single-copy sporulation genes in metagenomes from the same sediment core, however, estimated the frequency of putative endospore-forming bacteria as being <10% of the total cells (Kawai *et al*., 2015). Plausible explanations offered for the discrepancy included the inefficiency of lysis and DNA extraction of subseafloor endospores resulting in their underrepresentation in the metagenomics analysis, or evolutionary divergence in sporulation genes such that not all endospore genotypes were recognized (Kawai *et al*., 2015). In addition, if spore germination results in the accumulation of DPA that is not quickly metabolized by surrounding organisms or otherwise degraded, false-positive spore quantification may result.

Overestimation of endospore numbers can arise if methodological factors complicating accurate DPA quantification are not overcome. Few DPA chelate methods consider the influence of organic matter on the measured DPA signal. When analysing trace amounts of endospores in air samples Li *et al*. (2008) found that chelating organic ligands were particularly interfering and artificially increased Tb^{3+}-chelated DPA measurements. This false signal was postulated as mainly being caused by aromatic acids, which could be washed out from the filters prior to sample chelation with Tb^{3+}. HPLC fluorescence analysis requires good chromatographic peak separation between dissolved organic matter and DPA to avoid DPA overestimation from false signals. The ability to discriminate between DPA and organic matter via good chromatography and peak integration allows for more accurate calculation and a better understanding of DPA and its association with chelatable organic carbon in deep biosphere sediments and other environmental samples. This is critical for preventing false-positive DPA measurement.

Free versus total DPA in the natural environment

Free DPA is typically measured in cultivation experiments studying endospore germination (Yang and Ponce, 2009; Francis *et al*., 2015; Volpi *et al*., 2017). The common activation treatment is sublethal heat shock which catalyses cation release, Ca^{2+}-DPA chelate release and partial core hydration (Setlow, 2003). In environmental soils and sediments, measurement of free DPA has not yet been reported, presumably due to the low rate of germination and rapid uptake of the DPA molecule by microbial communities *in situ* (Arima and Kobayashi, 1962; Seyfried and Schink, 1990). When tracking germination dynamics in incubation experiments, differentiating between free DPA and total/spore DPA can reveal that concentrations are not constant over time as shown here (Fig. 3). Even replicate sediment slurry bottles show dynamic environmental variations of endospore numbers after incubation. Fluctuations suggest that part of the endospore population remains in spore form, but this analysis cannot reveal if those intact spores are viable or not; e.g. the incubation conditions may not be conducive to germinating all spores (e.g. mesophiles may not germinate in high-temperature incubations). It can be reasonably assumed that increases in spore DPA over short timescales correspond to newly formed (and likely still viable) spores. Endospore viability could be determined using a wider variety of incubation conditions (de Rezende *et al*., 2017) or potentially via membrane staining with Live/Dead BacLight dyes as tested using viable and gamma-irradiated endospores (Laflamme *et al*., 2004); although the latter has yet to be reported in complex sediment matrices. The three DPA categories presented here (total DPA, free DPA and spore DPA) can be used to better understand *in situ* endospore ecology and the dynamics of germination and sporulation in complex soil or sediment matrices. Increases in free DPA relate directly to germination and viability, whereas decreases in total DPA can be used to calculate DPA degradation. Increases in total DPA and spore DPA are conversely consistent with vegetative cells initiating sporulation.

Endospore viability and DPA

Viable endospore populations can be assessed by activating spores in sediment incubations and measuring the release of free DPA to estimate the number of germinated spores. In the estuarine sediment incubation experiment presented here ≤77% of the total DPA signal corresponded to non-germinated intact spores. These spores may not have germinated owing to different germination requirements among viable spores, and/or spores that are no longer viable but remain intact and therefore detectable by this approach. Due to sedimentation processes, especially in deep water marine systems, it can be assumed that if non-viable endospores persist as recalcitrant intact structures they will accumulate at depth, which could partially explain observations of spores increasing with depth in relation to total cell counts (Wörmer *et al*., 2019). In environments where both germination and sporulation are occurring simultaneously, e.g. along nutrient or temperature gradients, determining both free DPA and total DPA is likely to promote better understanding of endospore viability and degradation dynamics.

Over-estimation of spore numbers using DPA might arise if the conversion factor for the DPA content per spore is too low. For ease of conversion and comparability between studies, the value of 2.2×10^{-16} mol DPA spore^{-1}, first presented by Fichtel *et al*. (2007) is generally used in environmental studies (Table S4) (Lomstein *et al*., 2012; Lomstein and Jørgensen, 2012; Volpi *et al*., 2017; Wörmer *et al*., 2019). In reality, spore-specific DPA contents vary, with spores from different pure cultures ranging over an order of magnitude in DPA concentration (Aronson and Fitzjames, 1976; Sojka and Ludwig, 1997; He *et al*., 2003; Kort *et al*., 2005; Shafaat and Ponce, 2006; Fichtel *et al*., 2007). Figure 4 summarizes this variability and includes new data from the *D*. *geothermicus* isolate presented here. Like other anaerobic *Clostridia*, spores of *D*. *geothermicus* have a relatively high DPA concentration of 14.4×10^{-16} mol DPA spore^{-1}. Additionally, the high DPA content of *Bacillus megaterium*, reported to have a maximum growth temperature of 63°C (Llarch *et al*., 1997), together with *D*. *geothermicus* are suggestive of higher DPA contents per spore among thermophilic endospore formers. Higher spore-specific DPA levels in *Clostridia* and/or in thermophilic endospore formers have important implications for environmental studies, e.g. in the warm anoxic deep biosphere where anaerobic *Clostridia* are commonly found (Kotelnikova and Pedersen, 1997; Aullo *et al*., 2013). Overestimation of endospore populations could occur when applying a conversion factor not calibrated to the spores found in a given environment.

The original DPA to endospore conversion factor calculated by Fichtel *et al*. (2007) (2.2×10^{-16} mol DPA spore^{-1}) was an average of tidal flat *Bacillus* isolates. Using the data from thermophilic endospore producers and *Clostridia* shown in Fig. 4, an average of 9.1×10^{-16} mol DPA spore^{-1}, approximately fourfold higher, is likely more appropriate for environments containing a majority of thermophilic endospore populations. It is possible that other studies of endospores in natural samples may have over-estimated endospore abundance by a similar factor. DPA is a biomarker for the endospore phenotype in general and is not taxonomically diagnostic, and it remains challenging to accurately determine what strains of endospore are present in environmental samples.

This study addresses the problem of endospore characterization in complex environmental matrices using an improved highly sensitive assay for spore-containing DPA. This is combined with heat shock (pasteurization and incubation) of the same sediment to provoke a portion of the spores to germinate and be estimated using the new ‘free’ DPA proxy. In this way the thermophilic population of spores can be quantified, using a spore-specific DPA conversion factor that is suitable for thermophiles. Despite these advances, unanswered questions remain, including how larger proportions of spores can be germinated, and if they remain viable at all or represent DPA-containing (and therefore quantifiable) relics of life forever relegated to the fossil domain.

Experimental procedures

Sample collection and preparation

River Tyne estuarine sediment was sampled in July 2017 from the River Tyne estuary at Scotswood, Newcastle, UK (see Table S2 for sample metadata). This estuarine sediment was chosen due to its previous characterization in other endospore studies (Bell *et al*., 2018; Bell *et al*., 2020). For sampling, 1 kg of surface sediment (0–20 cm) was transferred into sterile polypropylene plastic bottles and kept at 4°C prior to extraction and analysis.

In 2011 TDI Brooks International conducted sampling in the Eastern Gulf of Mexico at stations EGM 080 and EGM 183 using piston coring. Directly after sampling, sediment cores were sectioned and stored in sterile plastic bags. Subsamples were kept at −20°C until analysis. Surface sediment from 0 to 20 cm depth was used in this study.

Deep NW Atlantic stations were sampled during a 2016 expedition to the Scotian slope (offshore Nova Scotia, NW Atlantic Canada) aboard the CCGS Hudson. Sampling was conducted at stations 2016-0014 and 2016-0044 using a piston corer. Cores were sectioned on board and sediment subsamples were kept at −20°C until analysis.

Analytical standards

Pyridine-2,6-dicarboxylic acid 99% (DPA) was purchased from Sigma, USA. DPA stock standards of 100 mM were diluted to make standards in the range of 1–2500 nM. Stocks were kept at −20°C and dilutions were prepared fresh for each round of analyses.

Acid hydrolysis for determining total DPA in sediment and soil

Soil and sediment samples were prepared in triplicate for analysis using the method of Lomstein and Jørgensen (2012). Briefly, 0.1 g of freeze-dried and homogenized sediment was placed in a 20 ml glass tube to which 1 ml ultrapure water and 1 ml 6 M HCl were added. Tubes were sealed and placed in an oven for 4 h at 95°C and then put directly on ice to stop hydrolysis. The hydrolysate was then removed, freeze-dried, reconstituted in milli-Q water, frozen and freeze-dried again, to reduce HCl in the sample. Samples were then dissolved in 1 M sodium acetate and aluminium chloride was added to bind phosphate to prevent its preferential binding with Tb^{3+}. In samples where low levels of DPA were expected (e.g. environmental samples) 750 μl of sample hydrolysate was added to 250 μl of terbium (Tb^{3+}) prepared in 1 M sodium acetate. In samples anticipated to contain higher concentrations of DPA (e.g. enrichment cultures) the ratio was inverted by adding 250 μl of sample hydrolysate to 750 μl of terbium (Tb^{3+}) prepared in 1 M sodium acetate. Changing the ratios of Tb^{3+} extends the dynamic range of analysis as explained above.

Extraction of free DPA from sediment and soil

Free DPA analysis uses a simplified processing method that offers a rapid proxy with potential for high-throughput processing of samples where endospore germination is of interest.

Free DPA samples were processed by centrifuging a small aliquot of sediment or soil (ca. 2 ml; depending on the water content) in Eppendorf tubes for 5 min at 14 000 rpm. Subsequently, 1 ml of supernatant was removed and directly frozen at −20°C then freeze-dried. The dried residue was then re-constituted in 1 ml 1 M $NaSO_4$, filtered and complexed with Tb^{3+} as described above for the total DPA assay.

Analysis of DPA using HPLC fluorescence

The method developed in this study provides an update based on existing methods using Tb^{3+} chelation for DPA analysis (see Table S1 for a list of other Tb^{3+} based fluorescence methods). DPA separation was performed using a Kinetex 2.6 μm EVO C18 100 Å LC column (150 × 4.5 mm, Phenomenex, USA) fitted with a guard column and connected to a Thermo RS3000 pump. Gradient chromatography was used where solvent A was 1 M sodium acetate amended with 1 M acetic acid to pH 5.6 and solvent B was methanol/water (80%/20%); see Table S5 for details. The sample injection volume was 50 μl and the total run time was 10 min (including flushing). The pump pressure was 185 bar with 100% solvent A. Detection was performed using a Thermo FLD-3000RS fluorescence detector set at excitation wavelength 270 nm and emission 545 nm. Data were processed using Chromeleon version 7 software using valley to valley peak integration algorithms.

Standards and samples were mixed at a 1:3 ratio of DPA to Tb^{3+}. The dynamic range of quantification can be expanded by inverting the DPA to Tb^{3+} ratio to 3:1 in specific instances. The 1:3 ratio of DPA to Tb^{3+} (7.5 mM Tb^{3+}) increases the range of detection to ~2000 nM DPA and the 3:1 ratio of DPA to Tb^{3+} (2.5 mM Tb^{3+}), the limit of detection is 125 nM DPA. This dynamic range makes it possible to achieve a 0.14 nM DPA LOQ and 0.04 nM LOD. It is recommended when starting the analysis of an unknown sample set that some samples are initially processed at both 1:3 and 3:1 dilution, and tailoring the analysis to suit the DPA concentrations.

Enrichment of thermophilic spore-forming bacteria in estuarine sediment used for free versus spore-bound DPA analysis

The estuarine sampling site (Scotswood, River Tyne estuary, UK) and enrichment culturing procedure have both been described previously (Bell *et al*., 2018). Briefly, the brackish medium was prepared and autoclaved in sealed bottles and the headspace was exchanged with N_2:CO_2 (90:10 vol./vol.). Tyne sediment (40 g) was weighed into three sterile bottles (250 ml), sealed and the headspace replaced with N_2:CO_2. Brackish medium (80 ml) was then added to each bottle of sediment followed by vigorous homogenization. The first subsample was taken directly after homogenization before pasteurization was carried out at 80°C for 1 h in a water bath. A second (i.e. 0 h) subsample was taken directly after pasteurization. Bottles were subsequently incubated at 50°C and subsampled at regular intervals for DPA.

Isolation of spore-forming sulfate-reducing bacterium Desulfallas geothermicus

Sediment from the deep Eastern Gulf of Mexico station EGM 080 stored at −20°C was used to inoculate an artificial seawater medium containing 20 mM sulfate. The slurry was pasteurized at 80°C, then amended with 20 mM lactate and incubated at 50°C, to enrich spore-forming thermophilic sulfate-reducing bacteria. A sediment-free enrichment culture was obtained via two successive serial dilutions to extinction in artificial seawater medium (Widdel and Bak, 1992) amended with 20 mM sulfate and 20 mM lactate. A pure culture of a sulfate-reducing bacterium was subsequently obtained by picking single colonies grown on anoxic agar plates followed by re-inoculation into liquid medium. The culture was classified as *Desulfallas geothermicus* based on 100% identity of the near-full-length (>1400 bp) 16S rRNA gene sequence. Prior to spore staining cells were kept in a stationary phase for 3 weeks to promote sporulation.

Endospore quantification using microscopy

Samples of *D. geothermicus* and marine sediment from station EGM080 were fixed using 8% paraformaldehyde in phosphate buffer and endospores were stained using the Schaeffer Fulton technique (Schaeffer, 1933). Spores were counted manually using bright-field microscopy on a Leica microscope at 100× magnification.

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Appendix S1: Supporting Information