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Estimating the Abundance of Endospores of Sulfate-Reducing Bacteria in Environmental Samples by Inducing Germination and Exponential Growth

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ABSTRACT

It is a challenge to quantitatively distinguish active from dormant microbial populations in environmental samples. Here we present an approach for estimating the abundance of dormant sulfate-reducing bacteria (SRB), present as viable endospores in environmental samples. This is achieved by inducing endospores to germinate and grow exponentially. We demonstrate this approach for thermophilic SRB in temperate sediment from Aarhus Bay, Denmark. The approach is based on measuring bulk sulfate reduction rates (SRRs) in pasteurized sediment and calculating associated cell-specific SRRs based on average values for SRB growth yield and cell size known from exponentially growing pure cultures. The method presented is a faster bioassay than most probable number enumerations and has the potential to distinguish between slow- and fast-growing SRB populations in the same sample. This bioassay is attractive given the challenges posed by endospores with respect to cell permeabilization and lysis, which are prerequisite in quantitative microscopy- and nucleic acid extraction-based strategies. These molecular approaches additionally rely on designing target-appropriate oligonucleotide probes, whereas the method presented here considers the trait of interest more broadly. This strategy thus presents a useful complement to other methods in ecological investigations of microbial biogeography and for specific industrial applications such as reservoir souring and corrosion risk assessments in the oil and gas sector.

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Dormancy; Firmicutes; sulfate-reducing bacteria; oil reservoir souring; quantification methods

Introduction

Many bacteria form resting stages, which have a strongly reduced endogenous metabolism and do not contribute to biogeochemical processes in the surrounding environment. Yet, dormant cells can become active again, either in response to environmental cues (Blagodatskaya and Kuzyakov 2013) or randomly (Buerger et al. 2012). Resting cells constitute an environmental reservoir of genetic and functional diversity that can withstand environmental change and stress. Dormant endospores are perhaps the most stress resistant of all living organisms, able to survive temperatures well above what vegetative cells of the same strain can tolerate: ca. 40°C higher for wet heat and ca. 30°C higher for dry heat (Setlow 2006). Endospores of certain thermophilic bacteria even remain viable after triple autoclaving at 121°C (O'Sullivan et al. 2015). The temperature at which endospores are formed, their core water content and DNA repair capability are among the main factors that influence endospore resistance to stress (Setlow 2006).

Dormant microorganisms may comprise a significant proportion of microbial communities in aquatic and terrestrial environments, with estimates varying from 20 to 80% of total cells (Lennon and Jones 2011). For example, it has recently been estimated that endospores comprise half of all cells in subsurface marine sediments (Langerhuus et al. 2012; Lomstein et al. 2012). Despite their ecological potential and apparent numerical importance, little is known about the metabolic and physiological diversity or the abundance of dormant populations within microbial communities due to a paucity of methods to distinguish and study dormant cells (Lomstein et al. 2012; Wunderlin et al. 2013; Wunderlin et al. 2014). One difficulty concerns unambiguously differentiating between active, dormant and dead cells; combinations of methods are required to target these various physiological states (Blagodatskaya and Kuzyakov 2013). It remains however important for many investigations in environmental microbiology to identify and quantify dormant populations in order to understand their role as a seed bank for the persistence of microbial community members in a given environment (Gibbons et al. 2013; Yarwood et al. 2013) and to understand the mechanisms and the role of dispersal in shaping microbial diversity (Hanson et al. 2012; Müller et al. 2014).

Such ecological insights can also be useful for bioengineering strategies aimed at controlling specific microbial populations. For example, a major concern in the oil and gas industry, especially at offshore production platforms, is the control of the sulfate-reducing bacteria (SRB) that cause reservoir souring—the production of toxic and corrosive hydrogen sulfide, which

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poses serious safety risks and leads to significant economic losses (Voordouw et al. 1996; Gittel et al. 2009; Hubert 2010). SRB that can switch between dormant and active states can be an important factor in reservoir souring control (Rosnes et al. 1991; Nilsen et al. 1996). Biocides are often applied in oil fields to combat microbial activity; however experiments with produced water have shown that insufficient doses of biocides lead to microbial dormancy instead of death (Keasler et al. 2013). Additionally, dormant SRB present in seawater, e.g., endospores of thermophilic SRB (de Rezende et al. 2013), may be continuously introduced into offshore hot reservoirs during secondary oil recovery by seawater injection into the reservoir. This may contribute to the observation that Firmicutes, which include all endospore-forming bacteria, are the most commonly detected phylum in fossil fuel reservoirs, especially at high temperature (Hubert et al. 2012), where SRB are often associated to the endospore-forming genus *Desulfotomaculum* (e.g., Rosnes et al. 1991; Nilsen et al. 1996; Gittel et al. 2009; Aüllo et al. 2013; Guan et al. 2014; Frank et al. 2015). Regardless of whether endospore-forming SRB are indigenous to the reservoirs or introduced via injected seawater, the potential for germination and growth of dormant endospores contributes to the ongoing challenge of souring control.

As highlighted by Aüllo and colleagues (2013), monitoring endospore-forming SRB and distinguishing endospores from vegetative cells is important in several contexts, yet difficult because of the technical limitations of quantitative methods, including widely used molecular tools such as quantitative PCR (qPCR) and fluorescence *in situ* hybridization (FISH). These DNA- and RNA-based quantification methods can suffer from the low efficiency with which endospores are permeabilized for accessing their nucleic acid contents and by the challenge of designing oligonucleotide primers or probes that comprehensively and specifically cover environmental SRB populations. Furthermore, microscopy-based methods such as FISH are also challenged by the typically low abundances (0.001%) of these endospore populations against a background of 10^9 cells per cm^3 (Langerhuus et al. 2012; de Rezende et al. 2013) in complex matrices such as sediment.

Here we present an alternative method to estimate the abundance of endospores of thermophilic SRB that germinate and grow in experimentally heated marine sediment. We chose this as an example of a complex environmental sample where distinct populations of endospores become active within few hours of incubation at favorable conditions, resulting in two phases of exponential increase of sulfate reduction in less than 120 h. The method is based on the measurement of each exponential increase in metabolic activity, and calculating cell-specific metabolic rates using average values for growth yield and cell size known from pure cultures. We focus on sulfate reduction, but these principles can be applied to any other dormant population for which resuscitation and growth resulting in an exponential increase in metabolic activity can be induced and monitored.

Materials and methods

Sediment samples

Marine sediment was sampled in January 2009 from 56°06′20″N, 10°27′48″E (station M5, 28 m water depth) in Aarhus Bay (Langerhuus et al. 2012), located on the transition between the Baltic Sea and the North Sea. Surface sediment (0–10 cm) was collected with a box corer (Hessler and Jumars 1974) and stored at 4°C in gas-tight plastic bags (Hansen et al. 2000) for three months before it was used for experiments. The temperature of the surface sediment in Aarhus Bay varies seasonally between 0 and 15°C (Thamdrup et al. 1994).

Sediment slurry incubation with radiotracer at 50°C

Slurries were prepared by homogenizing sediment with sterile anoxic synthetic seawater medium (pH 7.0, 20 mM sulfate) in a ratio of 1:2 as described previously (Isaksen et al. 1994; de Rezende et al. 2013). The anoxic sediment slurry was divided during stirring and under N_2 atmosphere into two separate master slurries of ca. 400 mL in sterile 0.5 L GL-45 bottles sealed with nonhalogenated black butyl rubber stoppers (Glasgerätebau Ochs, Bovenden, Germany). One slurry was amended with a mixture of substrates commonly used by SRB as electron donors: formate, lactate, acetate, succinate, propionate, butyrate and ethanol (each to a final concentration of 1 mM), and the other slurry was left unamended.

Substrate-amended and unamended master slurries were pasteurized at 85°C for 30 min to kill vegetative cells and then immediately incubated at 50°C to select for germination and growth of endospores of thermophilic bacteria. As shown in previous experiments, pasteurization at 80°C or 85°C for 30 min kills vegetative cells of psychrophilic and mesophilic SRB. There are no indications that thermophilic SRB able to survive pasteurization are present as vegetative cells in these cold sediments prior to incubation at higher temperatures (Hubert et al. 2009; de Rezende et al. 2013). During incubation at 50°C in the dark, slurries were subsampled regularly for 120 h. At each subsampling event, duplicate aliquots of 3 mL were transferred under N_2 into Hungate tubes (16 × 125 mm, Glasgerätebau Ochs) containing 150 kBq carrier-free ^{35}S-sulfate (PerkinElmer, Skovlunde, Denmark), and incubated in parallel with the master slurry at 50°C for a further 1–1.5 h. Microbial activity was subsequently terminated and H_2S was fixed by injecting ca. 6 mL 20% (w/v) zinc acetate solution into the Hungate tubes, transferring the mixed contents to 50 mL centrifuge tubes, and immediately freezing at −20°C. Sulfate reduction rates (SRRs) for each incubation in Hungate tubes were determined via single-step cold chromium distillation and liquid scintillation counting of both the reduced ^{35}S compounds and unreacted ^{35}S-sulfate tracer (Røy et al. 2014). Master slurries were also subsampled at 26 and 92 h for DNA extraction and quantitative PCR-based estimation of SRB abundance (see Supplemental Material).

Results and discussion

Slurries of Aarhus Bay surface sediment were incubated at 50°C with and without organic substrate amendment. Under both conditions, two separate exponential increases in SRRs were measured within 120 h (Figure 1A and 1C). This is similar to our observations in previous studies on the presence of endospores of thermophilic SRB in

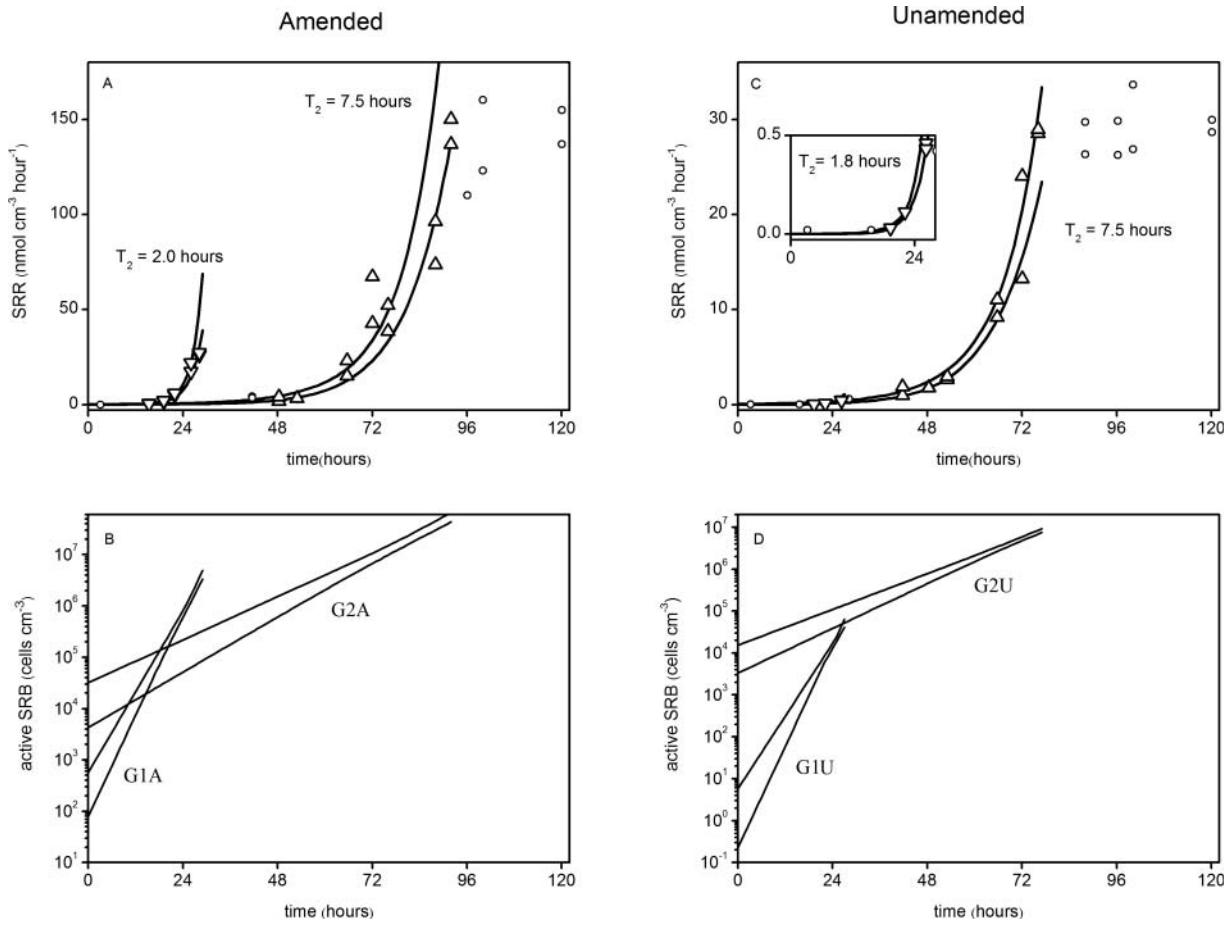

Figure 1. Sediment slurries were amended with organic substrates or left unamended, pasteurized at 85°C and incubated at 50°C for up to 120 h. Panels A and C: sulfate reduction rates (SRRs) measured by ^{35}S technique (triangles and circles). Triangles indicate data from the exponential phase that were used in the model. Parallel curves represent 90% confidence intervals of the exponential fits. Average doubling times (T_2) for the exponential phases are presented. Panels B and D: estimated numbers of faster-growing (G1) and slower-growing (G2) thermophilic SRB (cells cm^{-3} slurry) increasing with time during each of the exponential phases of the amended or unamended incubations, respectively. Therefore, assuming endospore germination at time = 0 h, the estimated initial endospore numbers in the sediment slurry correspond to the intersection of the curves on the y-axis.

sediments from Aarhus Bay, Svalbard (in the Arctic Ocean), and other cold marine sediments (Isaksen et al. 1994; Hubert et al. 2009; Hubert et al. 2010; de Rezende et al. 2013; Müller et al. 2014). Thermophilic Firmicutes were enriched in all previous cases (Isaksen et al. 1994; Hubert et al. 2009; Hubert et al. 2010; Müller et al. 2014) as well as in the present experiment (de Rezende et al. 2013). The exponential increase in SRRs reflects the exponential growth of SRB provided that the amount of sulfate reduced per cell per unit time, i.e. the cell-specific SRR (csSRR), is constant. This is a reasonable assumption at a population level within the strictly exponential phase. For example, batch culture experiments with a marine sulfate-reducing *Desulfovibrio* strain grown on different electron donors have shown little variation of csSRR during exponential growth (Sim et al. 2011).

The number of active sulfate-reducing cells at any given time during the incubations (Figure 1) was estimated by dividing the bulk SRR at that time point by the csSRR. Average csSRRs can be obtained from literature values for pure cultures (Detmers et al. 2001) or estimated for each exponential phase based on literature values of SRB growth yield and cell sizes. The first approach has been applied previously to estimate the abundance of thermophilic SRB endospores in Arctic sediments, and briefly described in the supplementary material of that work (Hubert et al. 2009). The latter approach is presented below, described in detail, and the two approaches are compared.

Estimation of the initial number of thermophilic SRB endospores that germinated at 50°C was thus based on initial SRRs. These are calculated using the first and the second exponential increases in SRRs and extrapolating back to when endospores germinate and start dividing using the corresponding csSRR. For simplicity we assume that the viable endospore population germinates and starts exponential growth immediately upon incubation (time zero); however, as discussed below this assumption is not essential to the approach (later germination times can also be measured and used).

Estimation of initial sulfate reduction rates using an exponential function

Sulfate reduction in both organic substrate-amended and unamended sediment slurries was detectable after 15 h of incubation at 50°C (Figures 1A and 1C, respectively). The

initial phase prior to 15 h when SRRs were below our detection limit includes the time necessary for germination and outgrowth of endospores (Zhang et al. 2010; Kong et al. 2011; Yang and Ponce 2011; Wang et al. 2015; Zabrocka et al. 2015) plus the time needed for growing cells to reach sufficiently high density to produce detectable quantities of reduced ^{35}S compounds (Røy et al. 2014). The occurrence of successive exponential increases in SRR within 120 h of incubation was also observed in similar experiments with Arctic sediments (Hubert et al. 2010) and is explained by the exponential growth of faster- and slower-growing groups of thermophilic SRB populations associated with the consumption of different organic substrates. In these incubations where excess sulfate is provided, available electron donors are the limiting factor for the different exponential growth phases.

An exponential function that fits the measured SRR (Equation 1) allows the SRR for the faster- and the slower-growing groups (G1 and G2, respectively) to be estimated at any time point in the experiment, including during the initial 15 h of incubation when sulfate reduction was below the detection limit:

$$SRR(t) = SRR_0 \times e^{kt} \quad [1]$$

In Equation 1, SRR is the sulfate reduction rate (nmol cm^{-3} h^{-1}), t is time (h), SRR_0 is the SRR at the start of the exponential phase, following germination, and k is the growth rate constant (h^{-1}). The two exponential phases were treated separately in both the amended and unamended incubation (see Figure 1). For the initial analysis we assumed that sulfate reduction always started at time zero, i.e. all endospores germinated at time zero, which gives a minimum estimate of the initial SRR. Other implications of this assumption are considered below.

Estimation of cell-specific sulfate reduction rates

The number of new vegetative SRB cells produced during exponential growth can be calculated from the amount of sulfate reduced during a corresponding time interval, the growth yield ($Y_{sulfate}$; i.e. pg biomass generated per nmol sulfate reduced) and the amount of biomass per cell (bm_{cell}; pg $cell^{-1}$). The accumulated amount of sulfate reduced during a given time interval can be found by integrating Equation 1 over time. The total amount of sulfate reduced multiplied by the growth yield gives the biomass production (pg cm^{-3}). Dividing the amount of biomass produced by the amount of biomass contained in a single SRB cell (bm_{cell}) results in the number of vegetative cells produced per cm^3 during the time interval in question (Equation 2):

$$amount\ of\ sulfate\ reduced = \int_0^t SRR_0 \times e^{kt} = SRR_0 \times \frac{1}{k}e^{kt}$$

$$biomass\ produced = SRR_0 \times \frac{1}{k}e^{kt} \times Y_{sulfate}$$

$$number\ of\ cells\ produced = SRR_0 \times \frac{1}{k}e^{kt} \times Y_{sulfate} \times bm_{cell}{}^{-1} \quad [2]$$

For our experiment, we assumed an average growth yield of 8.2×10^3 pg biomass per nmol sulfate based on values reported for nine pure cultures of SRB, including three endospore-forming species, shown in Table S1 (Supplemental Material). The biomass per cell (bm_{cell}) is estimated by multiplying cell volume, wet density and dry weight. Typical values for SRB cell volumes are also provided in Table S1, including 44 endospore-forming species. The vegetative cells in our incubations are growing exponentially in the presence of excess substrates; therefore, their phenotypes are more likely to resemble those of pure cultures than those of starved environmental populations. A cell volume of 1.0 μm^3 was assumed, as this was the median biovolume among the 68 SRB strains (Table S1). Assuming a cellular wet density of 1.0 pg μm^{-3} and a cellular dry weight of 30% (Habicht et al. 2005) gives a bm_{cell} of 0.3 pg $cell^{-1}$. In the case of the experiments shown in Figure 1, precise assumptions of growth yield and biomass per cell are not critical, as the variation of these parameters (Table S1) is smaller than the uncertainty derived from the extrapolation of SRR toward the start of the exponential phase (Figure 1).

The csSRR of a group of SRB can be calculated by dividing the SRR (Equation 1) by the total number of active cells at a given time point. The total number of vegetative cells present during the incubation corresponds to the number of cells produced by cell division (Equation 2) plus the number of cells present as endospores that germinate at the beginning of an exponential increase in SRR. Considering a time point well into the exponential phase, after the number of cells has increased by many orders of magnitude, the contribution of the original cell number to the total cell number becomes negligible. Therefore csSRR can be calculated from late exponential phase data by dividing the SRR (Equation 1) by the number of new cells

Table 1. Calculated parameters for the faster- and slower-growing SRB groups (G1 and G2, respectively) that grew in amended and unamended incubations (A and U, respectively). Values are presented as averages with 90% confidence intervals in parentheses. SRR_0: sulfate reduction rate at 0 h; k: growth rate constant; T_2: doubling time during exponential growth; csSRR: cell-specific sulfate reduction rate. The number of thermophilic SRB endospores in the sediment slurry is estimated assuming germination at 0 h.

Thermophilic SRB group	SRR_0 (10^{-3} nmol cm^{-3} h^{-1})	k (h^{-1})	T_2 (h)	csSRR (fmol $cell^{-1}$ h^{-1})	Estimated number of thermophilic SRB endospores at t = 0 h (cm^{-3} sediment slurry)
G1A	2.2 (1.1 – 6.2)	0.34 (0.30 – 0.38)	2.0 (1.8 – 2.3)	12.5 (11.0 – 13.9)	1.8×10^2 (7.9×10^1 – 5.6×10^2)
G2A	34.7 (16.4 – 93.9)	0.09 (0.08 – 0.10)	7.5 (6.7 – 8.6)	3.4 (2.9 – 3.8)	1.0×10^4 (4.3×10^3 – 3.2×10^4)
G1U	0.018 (0.004 – 0.071)	0.39 (0.33 – 0.46)	1.8 (1.5 – 2.1)	14.1 (12.1 – 16.9)	1.3×10^0 (2.3×10^{-1} – 5.8×10^0)
G2U	23.1 (12.4 – 45.9)	0.09 (0.08 – 0.10)	7.5 (6.8 – 8.5)	3.4 (3.0 – 3.7)	6.8×10^3 (3.3×10^3 – 1.5×10^4)

generated during the experiment according to Equation 2. The results are presented in Table 1.

We used bootstrapping to quantify the confidence in the estimated SRRs, cell numbers, doubling times and csSRR for both the faster- and the slower-growing SRB. Bootstrapping relies on pseudo datasets generated by drawing randomly with replacement from the original data. Detailed description, applications and limitations have been presented previously (e.g. Efron and Tibshirani 1993; Manly 2006). We generated 10,000 bootstrap samples and performed the entire procedure of fitting an exponential function, calculating cell numbers, csSRR and doubling times for each bootstrap sample. This procedure yields an estimation of the probability density distribution of each parameter. We then calculated 90% confidence limits as the 5th and 95th percentiles of the estimated distributions (Table 1).

Estimation of the initial abundance of endospores of thermophilic SRB

With the estimated mean csSRR for the faster- and slower-growing SRB groups, we calculated the number of vegetative SRB cells at any time point during the incubation by dividing the SRR determined from Equation 1 by csSRR (Table 1). Results are shown in Figures 1B and 1D for the amended and unamended experiment, respectively. At the beginning of each experiment, the total number of vegetative cells corresponds to the number of germinated thermophilic SRB endospores. Although our data cannot confirm that all thermophilic SRB endospores germinated instantaneously at time zero, the determination of exact germination time is difficult to constrain beyond the 0–15 h interval based on the detection limit of the ^{35}S technique, which depends on the amount of radiotracer used in experimental incubations. Assessing germination time by microscopy is also challenging because of the sediment matrix, the difficulty to selectively identify endospores of thermophilic SRB and the low overall abundance of these endospores.

The timing of endospore germination and outgrowth has been studied for *Bacillus* and *Clostridium* species in pure culture. While this can vary between individual endospores within a population, most endospores stimulated to germinate in an experiment typically conclude the process in minutes to less than two hours (e.g., Zhang et al. 2010; Kong et al. 2011; Yang and Ponce 2011; Wang et al. 2015; Zabrocka et al. 2015). We therefore assumed that thermophilic SRB endospores germinated at time zero to yield a conservative abundance estimate, since it results from the lowest SRR (SRR_0) divided by the estimated csSRR. The estimated abundances of the faster- and slower-growing SRB groups at time zero in the amended incubation were 1.8×10^2 and 1.0×10^4 thermophilic SRB endospores per cm^3 sediment slurry, respectively (maximum likelihood estimates; 90% confidence intervals are presented in Table 1). In the unamended incubation, the estimated abundances of these two groups at time zero were 1.3×10^0 and 6.8×10^3 thermophilic SRB endospores per cm^3 sediment slurry, respectively (Table 1). As the slurries were composed of two parts medium and one part sediment, the *in situ* abundance of thermophilic SRB endospores per cm^3 sediment is obtained by multiplying the above values by three. If the mean germination times were 2 h, instead of 0 h, estimates of thermophilic SRB endospore abundance would double for the faster growing group and increase by only 20% for the slower-growing group, which is within the uncertainty of the method.

Validation of the approach

Calculation of csSRR from experimental data in order to estimate the initial number of thermophilic SRB endospores provides an opportunity to compare the resulting csSRR with literature values for pure cultures of SRB including known thermophilic endospore-forming species (Detmers et al. 2001). For the different SRB groups in both substrate-amended and unamended incubations, the calculated csSRRs (Table 1) are within the range reported for pure cultures of SRB, from 0.04 to 18.8 fmol $cell^{-1}$ h^{-1}, with an average of 2.0 fmol $cell^{-1}$ h^{-1} (Detmers et al. 2001). The csSRR of sulfate-reducing populations in surface sediments are reportedly 10 to 100-fold lower than that for exponentially growing pure cultures (Sahm et al. 1999; Hoehler and Jørgensen 2013). However as discussed above our sediment slurry experiments do not reflect conditions *in situ*, where the temperature is between 0 and 15°C and cells are strongly electron donor limited. Germinating thermophilic populations in our slurry experiments grew exponentially in the presence of excess electron donors and nutrients released from the vegetative community killed by the pasteurization, once incubated at 50°C, as reflected by the exponential increases in SRRs (Figure 1). Even in the unamended high-temperature incubation, fermentation products rapidly increase to concentrations orders of magnitude greater than normal *in situ* concentrations (Hubert et al. 2010). Therefore, even our unamended incubations resemble the typical conditions of excess substrate in pure cultures where exponential growth occurs. This is also reflected in the calculated doubling times. The doubling time (T_2) for SRR during an exponential phase is equivalent to the doubling time of the SRB populations catalyzing the sulfate reduction, and can be calculated according to $T_2 = \ln(2) \times k^{-1}$. The T_2 values obtained, 1.8–2.0 h for the faster growing group and 7.5 h for the slower-growing group (Table 1), are similar to pure cultures of thermophilic heterotrophic *Desulfotomaculum* species (Daumas et al. 1988; Min and Zinder 1990; Liu et al. 1997; Vandieken et al. 2006).

We have previously estimated the abundance of thermophilic SRB endospores in Arctic sediments from Smeerenburgfjorden, Svalbard, by performing experiments similar to the ones described here, at the same incubation temperature, and simply assuming the average literature value for csSRR of 2.0 fmol $cell^{-1}$ h^{-1} (Hubert et al. 2009). By applying this new model presented here to the data in that previous report, a csSRR value of 2.3 fmol $cell^{-1}$ h^{-1} is obtained, thus supporting the estimate that there are at least 10^5 thermophilic SRB endospores per cm^3 of Svalbard fjord sediment.

Validation of the estimated *in situ* abundance of thermophilic SRB endospores is obtained by comparing the present results with the abundance estimated for the same Aarhus

Bay sediment by tracer-enhanced most probable number enumerations (T-MPN) as reported recently (de Rezende et al. 2013). T-MPN followed the method of Vester and Ingvorsen (1998) and were performed in similar 50°C incubations, with the same type of medium, pasteurization treatment and organic substrate amendments. T-MPN indicated 4–9 × 10^3 thermophilic SRB endospores cm^{-3} sediment, which is comparable to estimates obtained by the SRR-based approach presented here (Table 1).

Furthermore we have compared the results obtained from the SRR-based approach and the results from quantitative PCR for estimating the abundance of vegetative thermophilic endospore-forming SRB, at the end of each exponential phase of the amended experiment (26 and 92 h; Figure 1A). These results are presented in the Supplemental Material. We designed a qPCR assay targeting the *dsrA* gene of the thermophilic *Desulfotomaculum* lineage phylotype F. According to 16S rRNA and *dsrAB* gene PCR amplicon sequence libraries, phylotype F represents a predominant population within the sulfate-reducing endospore community of temperate marine sediments likely belonging to the group of faster-growing SRB driving the G1A response (Figure 1; see Supplemental Discussion). Our qPCR assay gave an abundance 10 times lower than the estimated abundance of thermophilic SRB for G1A after 26 h of incubation. This difference may be due to factors including (i) limitations of DNA extraction efficiency and (ii) the presence of other thermophilic SRB not targeted by the *dsrAB* primers. The PCR amplicon sequence libraries indicated that phylotype F is overgrown by other SRB populations during the later stages of the incubation period (de Rezende et al. 2013). This explains the >1000-fold difference between qPCR-based abundance of phylotype F and overall thermophilic SRB abundance (SRR-based) later in the experiment, after 92 h of incubation (Table S3). This example illustrates the need for qPCR primer pairs (or, more likely, multiple primer pairs) that encompass the diverse thermophilic endospore-forming SRB community reducing sulfate in each exponential phase. We also analyzed the same two samples (after 26 and 92 h of incubation) using a published 16S rRNA gene-based qPCR assay for Firmicutes (Bacchetti De Gregoris et al. 2011) to determine maximum estimates of thermophilic endospore-forming SRB. This assay also gave considerably lower SRB abundances than the SRR-based approach (six and >100 times lower for G1A and G2A, respectively; Table S3).

We are confident that SRB endospore abundances are underestimated by the qPCR assays, rather than overestimated by the SRR-based approach. The qPCR-based abundances produce unrealistically high csSRR, in the order of 100 to 1000 fmol $cell^{-1}$ h^{-1} (Table S3), whereas the highest csSRR known for pure cultures is 18.8 fmol $cell^{-1}$ h^{-1} (Detmers et al. 2001). In contrast, the csSRR resulting from the SRR-based approach of 3 to 17 fmol $cell^{-1}$ h^{-1} (Table 1) fall within the range known for pure cultures. This highlights the challenges of performing quantitative DNA extractions from a complex matrix such as sediment, quantifying low abundance target populations with qPCR, and designing primers that are appropriate for a target group in question that is of interest on the basis of its physiology (i.e., thermophilic sulfate reduction) rather than its phylogeny. In contrast the SRR-based approach provides rapid results that are in agreement with T-MPN-based estimates of SRB abundance (de Rezende et al. 2013). In light of these results the SRR-based approach offers a quantitative strategy that is a useful complement to other available methods for environmental investigations.

Physiological parameters of thermophilic SRB and the effect of organic substrate amendment

The approach presented estimates the *in situ* abundance of two distinct thermophilic SRB groups growing in the same sediment sample after their endospores germinate. The distinct exponential phases and the difference in abundance and doubling times between the faster-growing (G1) and slower-growing (G2) thermophilic SRB groups suggest that they are composed of distinct populations with different physiological characteristics.

Comparisons can also be made between the two treatments, leading to hypotheses for further investigation: the faster-growing SRB in the unamended incubation (G1U) were the least abundant, on average two orders of magnitude lower than the faster-growing SRB in the amended incubation (G1A; Table 1). This difference could be due to substrate amendment stimulating the germination and growth of a larger proportion of the faster-growing thermophilic SRB endospores present in the sediment. On the other hand, the slower-growing groups in the amended and unamended incubations (G2A and G2U) have similar abundance estimates and doubling times (Table 1), even though SRR are on average three times higher in the amended incubation (Figure 1). This suggests that G2A and G2U groups are physiologically similar, and that substrate amendment promoted higher overall SRRs. This could be explained by a greater accumulation of fermentation products (SRB electron donors) in the substrate-amended experiment. Comparison of these two experiments indicates that the bioassay for endospores of thermophilic SRB should include amendment with a broad spectrum of substrates to stimulate the highest number of endospores to germinate and grow and thereby provide the most complete estimate of the size of the *in situ* community of endospores. This may be especially important in environments that are less organic rich and therefore do not generate large amounts of endogenous fermentation products upon heating (Hubert et al. 2010). These results also highlight the importance of using a constant incubation condition to enable quantitative comparison of SRB numbers between different samples in a given study. In this respect, substrate addition provides a normalization effect that would make many samples more directly comparable with regard to numbers of SRB endospores.

Advantages of the SRR-based approach

The SRR-based assay presented here detects SRB endospores that germinate and grow under the incubation conditions provided, similar to MPN enumeration and other cultivation-dependent methods. The approach is relatively fast compared to MPN testing and presents a simple alternative for estimating the abundance of viable SRB populations. Our physiology-based approach does not require knowledge of the genetic diversity of the target group, and does not depend on accessing

nucleic acids or genetic analyses, which are essential for molecular quantification methods such as qPCR and FISH. This is valuable when quantitative DNA extraction from the target population requires laborious processing, e.g. for endospores (Wunderlin et al. 2013), or when the target population is difficult to access due to low relative abundance and a complex matrix such as sediment. A trait-based approach is also advantageous when probes or primers are not available to fully encompass the diversity of the target group, or when its diversity is simply unknown. Indeed for some investigators, e.g. operators dealing with oil reservoir souring risk, microbial physiology and the potential for H_2S production are a primary concern, rather than phylogenetic diversity. MPN enumeration or the SRR-based approach described here are limited to endospores that germinate and grow, yet they are suitable for estimating the abundance of these potentially active populations, i.e. populations that switch between dormant and active states. These populations may be poorly detected while in a dormant state, yet are still viable and can grow quickly once conditions change to favor germination. Methods that are based on microbial activity and growth can be improved by the refinement of cultivation media, which can be achieved by further understanding of the physiology of the target populations.

The SRR-based approach presented here provides several advantages over the MPN method. First, it allows the estimation of abundance, doubling times and cell-specific SRRs for distinct SRB groups that may proliferate in succession during the incubation. Hence information about the composition and physiology of multiple viable SRB populations that were stimulated to germinate and grow can be inferred. Second, the calculation of csSRR (and its comparison to literature values) provides built-in quality control, and indicates that the estimated abundance of SRB endospores by the SRR-based approach provides a more realistic minimum estimate than MPN enumeration. This is evident from the T-MPN estimates for this sediment being in the lower end of the estimated abundance by the SRR-based approach, even if a conservative germination time (at 0 h) is assumed. Third, results can be obtained in a much shorter time frame of only a few days (Figure 1), while the MPN procedure usually requires incubation times lasting weeks to months. Together with other quantification methods, the approach presented here contributes to a more comprehensive quantitative understanding of dormant and active populations in microbial communities. The advantages of our SRR method are relevant in several other contexts, e.g. the long-term control of SRB in industrial environments such as offshore oil production where an efficient control strategy must consider active SRB as well as dormant populations that can be easily reactivated. The parameters used for the SRR-based approach (germination time, growth yield, cell biovolume, csSRR) can be derived from literature for a fast assessment, or calculated empirically for each system for a more accurate and tailored quantification.

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