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Method Article

An efficient, cost-effective method for determining the growth rate of sulfate-reducing bacteria using spectrophotometry

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A B S T R A C T

The use of sulfate reducing bacteria (SRBs) in laboratory studies is a common approach for investigating microbially influenced corrosion (MIC). The characteristic formation of black iron sulfide precipitates during SRB growth, however, preclude the use of traditional spectrophotometric approaches for capturing growth data instead necessitating labour-intensive or technically specialized approaches. As such, an understanding of SRB growth responses to experimental conditions is often missing from MIC studies. Bernardes and de Andrade Lima (2015) have outlined a spectrophotometric approach for estimating SRB cell mass via the addition of HCl. This method has potential for the study SRB growth however its applicability is currently limited by the use of large aliquot volumes (45 mL), which restrict the number of timepoints that can be sampled from one culture, and the extensive time devoted to cell preparation prior to OD readings.

- We demonstrate an improved method for capturing SRB growth data via spectrophotometry following acidification. We incorporate lower sample volumes and adapt the method described in Bernardes and de Andrade Lima (2015) to a high throughput microtiter plate approach that increases the efficiency of this method and its applicability to growth rate studies.
- Our results allay theoretical concerns that acidification may distort growth rate analysis by impacting cells differently depending on their metabolic state.
- We further demonstrate that this method (acid-amended OD measurements) is more accurate and far more cost efficient than traditional methods (dilution spread-plate counting) and popular molecular methods (quantitative PCR) currently in use in SRB growth research.

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A R T I C L E I N F O

Method name: Spectrophotometric growth analysis of sulfate-reducing bacteria using acid-amendments

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Specifications Table

Subject Area:	Immunology and Microbiology
More specific subject area:	Biocorrosion
Method name:	Spectrophotometric growth analysis of sulfate-reducing bacteria using acid-amendments
Name and reference of original method:	Bernardez L.A., De Andrade Lima L.R.P. (2015) Improved method for enumerating sulfate-reducing bacteria using optical density. MethodsX 2: 249–255.
Resource availability:	n/a

Method details

The protocol for acid-amended OD (aaOD) growth estimation was modified from Bernardez and De Andrade Lima [1] which details the use of relatively large culture volumes (45 mL) to estimate microbial biomass from OD readings following the dissolution of iron precipitates using HCl. We have adapted this method to a high throughput microtiter plate approach that is more time efficient, does not require cell washing steps and, due the incorporation of smaller aliquot volumes (180 μ L), allows for collection of higher resolution time series data (Supplementary Table S1). We validate the use of this method by demonstrating that the addition of acid does not skew growth rate estimations and that the aaOD method is more accurate than traditional (dilution spread-plate counting) and popular molecular methods (quantitative PCR) for capturing SRB growth data. We include a cost analysis comparing aaOD to dilution spread-plating and qPCR to demonstrate the practicability of this method.

Growth and storage conditions of microbial cultures

This study used the SRB *Desulfovibrio desulfuricans* subsp. *desulfuricans* (ATCC 27774) and *Escherichia coli* (ATCC 25922). All experiments were conducted using growth media and conditions that are considered optimal for the growth of each bacterium.

D. desulfuricans was cultured in American Type Culture Collection (ATCC) 1249 Modified Baar's (MB) medium containing 0.1% Ferrous Ammonium Sulfate. The components of the MB medium were as follows: 4.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g NH_4Cl , 1.26 g $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 0.5 g K_2HPO_4 , 5.79 g sodium citrate, 5.83 g sodium DL- lactate (60% solution), and 1.0 g yeast extract were added for each liter of MilliQ water. The pH was adjusted to 7.5 ± 0.1 using a 3.5 M KOH solution, and sterilized by autoclaving at 121 °C and 103 kPa for 20 min. After cooling, 0.1 mL of 5% $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ solution, sterilized by filtration using syringe filters (0.02 μm in pore size), was added for every 5.0 mL of the previously autoclaved medium. *D. desulfuricans* cultures were incubated anaerobically using Compact W-zip seal pouches (AG0060C, Oxoid) with AnaeroGen Compact Sachets (AN0020D, Oxoid) at 37 °C without shaking. *E. coli* was cultured in Nutrient Broth (NB: CM0001, Oxoid) and incubated aerobically at 37 °C with shaking (180 rpm). For growth on solid media, *D. desulfuricans* was grown on Tryptic Soy Agar (45 g L^{-1}) amended with sodium lactate (4 mL L^{-1}), magnesium sulfate (2 g L^{-1}), and ferrous ammonium sulfate (0.05%) [2].

Prior to all experiments isolates were revived from -80 °C storage and used to inoculate 30 mL of fresh medium. *E. coli* and *D. desulfuricans* were allowed to grow for 24 h and 4 days, respectively, in order for cultures to reach stationary phase and ensure physiological homogeneity of cells within the culture.

Measurements of microbial growth following the removal of iron precipitates by acidification

For growth curve measurements, 30 mL cultures in 50-mL centrifuge tubes were used. Stationary phase cultures were diluted into fresh media to commence the growth curves. To commence *E. coli* growth curves, the OD of the stationary phase culture was determined, and a dilution factor was calculated such that the fresh 30 mL culture would have a starting OD_{600} of 0.05. To commence

D. desulfuricans growth curves, 1:200 dilutions of the stationary phase cultures were used. All growth curves were performed in triplicate.

Measurements for each time point on the growth curve were obtained by removing duplicate 180 μL aliquots of each culture and placing them in a clear flat-bottomed 96 well plate. 20 μL of concentrated HCl (32%) was added to each well and plates were incubated at room temperature for 10 min, to ensure complete dissolution of iron sulfides and allow residual H_2S gas to dissipate. Due to the toxic effects of H_2S gas, all culture aliquoting and plate incubations were conducted in a fume hood. After incubation, OD_{600} was recorded on a PolarSTAR Omega plate reader spectrophotometer (BMG Labtech).

For the purpose of methodological validation, replicate growth curves were created whereby 20 μL of distilled H_2O was added in place of HCl to control for the impact of HCl on culture turbidity and to determine whether HCl addition was impacting measured growth rates. Because *E. coli* (ATCC 25922) does not form black precipitate, *E. coli* growth curves allowed for a comparison of growth rates derived from HCl and H_2O addition, in the absence of interference from FeS precipitants.

E. coli growth curves were created by measuring the OD_{600} every 20 min for the first 2 h, every 30 min for the next 2 h and then every hour until $t = 9$ h. A final OD measurement was taken the following day at $t = 21.2$ h. To create *D. desulfuricans* growth curves, OD_{600} measurements were taken twice daily (8 h apart) for six days.

Because the proportionality between OD_{600} and cell density exists only for $\text{OD} \leq 0.4$ [3], cultures approaching this threshold were diluted as necessary, and the dilution factor corrected for, to estimate the true OD_{600} reading.

Statistical procedures

Statistical procedures were carried out in Microsoft Excel 2016. Growth rates (μ) were estimated by the equation:

$$\mu = \frac{\ln \text{OD}_2 - \ln \text{OD}_1}{(t_2 - t_1)}$$

where t_1 and t_2 are time points and OD_1 and OD_2 are the corresponding optical densities calculated using the line of best fit. Doubling times (t_d) were calculated using the relationship:

$$t_d = \frac{\ln 2}{\mu}$$

Method validation

Fig. 1 shows the effectiveness of the acid-amended OD protocol in measuring SRB growth rates. Black precipitate began forming in the cultures after ~ 50 h of growth (see Supplementary Fig. S1) causing H_2O and acid-amended OD readings to diverge (Fig. 1A). Prior to the formation of precipitates, there was no significant difference in OD readings of acid and H_2O -amended samples ($t_{(8)} = 0.071$, $p = 0.306$; Fig. 1A). Acid-amended OD readings stabilized after 110 h. The rate of SRB growth was determined from 6 time points that were within the logarithmic phase of growth. The growth rate was estimated to be 0.03 h^{-1} with a doubling time of 23.1 h (Fig. 1B).

Fig. 2 shows *E. coli* growth curves derived from the addition of either HCl or H_2O . The addition of acid significantly increased OD_{600} values relative to H_2O controls (Bonferroni corrected t -tests, $t_{(30)} = -0.59$, $p = 0.561$; Fig. 2A). Despite the shift in OD values, identical growth rates (μ) of 0.016 min^{-1} and doubling times of 43.3 min were determined for OD measures taken in the presence of HCl and H_2O (Fig. 2B).

We also examined whether HCl addition impacts microbial growth phases (lag, log and stationary phase) differently by comparing the average difference between HCl and H_2O optical densities (ΔOD) for each growth phase. The acid induced ΔOD was significantly less during the lag phase when compared to the log phase ($t_{(10)} = -4.59$, $p \leq 0.01$) but not when compared to stationary phase time ($t_{(2)} = -4.17$, $p = 0.053$). The acid induced ΔOD was the same during the log and stationary phases ($t_{(3)} = -1.61$, $p = 0.205$).

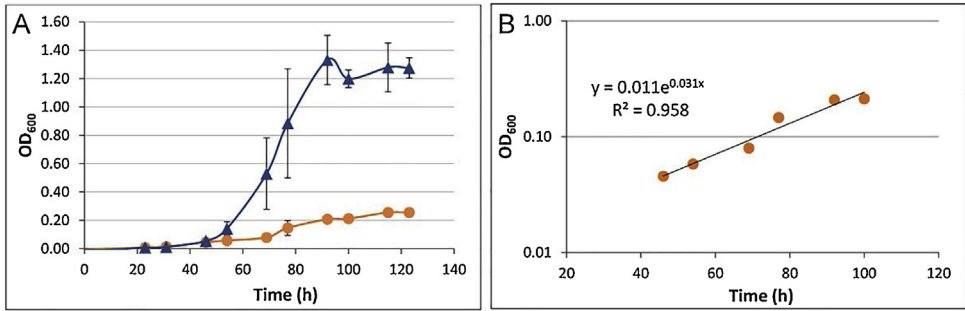


Fig. 1. Growth curves (A) and growth rate estimates (B) for *D. desulfuricans* with OD readings taken either with the addition of 20 μ L concentrated HCl (orange circles) or H₂O (blue triangles). Error bars represent the SE of three biological replicates.

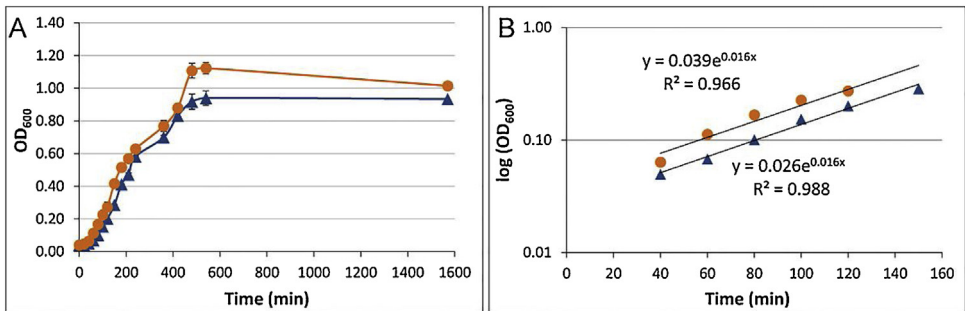


Fig. 2. Growth curves (A) and growth rate estimates (B) for *E. coli* with optical density readings taken either with the addition of 20 μ L concentrated HCl (orange circles) or H₂O (blue triangles). Error bars represent the SE of three biological replicates.

Comparison of method accuracy

To compare the accuracy and detection thresholds of the aaOD method to traditional (dilution spread-plate counting) and molecular (quantitative PCR) methods for capturing SRB growth data, a series of dilutions were created using triplicate stationary phase (grown for 4 days) *D. desulfuricans* cultures. Dilution factors of the cultures, which initially contained 10⁸ CFU mL⁻¹, ranged from undiluted to 1:1000 (Table 1). Because the samples used in this experiment were a dilution series, the relationship between culture dilution factors and the cell density, as estimated via the various

Table 1

Summary of the dilution factors used to evaluate the effectiveness of each technique in estimating the cell density-culture dilution relationship for *D. desulfuricans*. The X symbol indicates the dilutions used to evaluate the listed technique.

Dilution factor	Culture proportion	Acid-amended OD	qPCR	Dilution plates
undiluted	1	X	X	X
1:2	0.5	X	X	
1:5	0.2	X	X	
1:10	0.1	X	X	X
1:50	0.02	X		
1:100	0.01	X	X	X
1:200	0.005	X		
1:500	0.002	X		
1:1000	0.001	X	X	X

methods, should be linear. Deviation from a linear trend is indicative of methodological limitations including detection and saturation limits.

Acid-amended OD

The protocol for estimating OD was the same as described for growth curves without the inclusion of an additional H₂O control. Because only dilutions below 1:100 appeared visually to be clear of black precipitate prior to the addition of HCl, additional low-range dilutions (ranging from 1:100 to 1:1000; Table 1) were included as a control for the acid-amended OD readings to ensure that the relationship between OD and the culture dilution was not due to the presence of residual iron sulfides.

Quantitative PCR

1 mL of culture from 6 dilutions (ranging from 1 to 1:1000; Table 1) was flash frozen in liquid nitrogen and stored at -80°C until DNA could be extracted. DNA was extracted using the Bioline ISOLATE II genomic DNA extract kit, as per the manufacturer's instructions. To enumerate the number of cells in each dilution culture, primer pairs 1114f-1275r, which target a 130 bp of the bacterial 16S rRNA gene were used [4]. The published genome sequence of ATCC 27774 was used to correct for the presence of multiple 16S rRNA copies which can lead to the overestimation of SRBs [5].

qPCR was carried out on a CFX Connect™ real-time PCR detection system (Biorad). Each 5 μL of qPCR reaction contained 400 nm concentrations of each forward and reverse primer, 2.5 μL of SsoAdvanced Universal SYBR® Green Super Mix, sterile DNA-free water and 1 μL of template DNA. Thermocycling conditions were 20 s at 95°C followed by 40 cycles of 95°C for 3 s and 61.5°C for 30 s [6]. Reactions were followed by a melting curve increasing 1°C every 30 s from 60°C to 99°C . Every sample was amplified in duplicate. Bacterial copy number was quantified using the 130 bp 16S rRNA amplicon which had been amplified from *D. desulfuricans*, purified on a 1% (w/v) agarose gel and the DNA concentration determined on a Nanodrop 2000c spectrophotometer (ThermoFisher Scientific). A standard curve was created using triplicate 10-fold dilutions of the gel-purified amplicon. qPCR efficiency was between 98–100%.

Dilution spread-plate

Due to time and consumables constraints, four dilutions were chosen to evaluate the dilution spread-plate technique (Table 1). For each of the four dilutions a 1:10 dilution series (10^{-1} – 10^{-6}) was created in 10 mL volumes. 100 μL from each dilution was plated onto TSA agar amended with salts. All agar plates were incubated anaerobically at 37°C . After 4 days colony counts were performed using plates that contained between 50 and 250 CFUs.

Fig. 3 shows the relationship between dilutions of stationary phase *D. desulfuricans* cultures (expressed as proportion of the undiluted culture) and cell density (as estimated via dilution spread-plate, qPCR or aaOD). The linearity of the relationship gives an indication of the appropriateness of each method, as the relationship should be perfectly linear. Both spread-plate and aaOD techniques reproduced strong, linear cell density-culture dilution relationships ($R^2_{\text{spread-plate}} = 0.84$, $R^2_{\text{aaOD}} = 0.96$). In contrast, qPCR exhibited a weak linear relationship ($R^2_{\text{qPCR}} = 0.34$) over the full range of dilutions tested and the plot of the residuals indicated that 16S rRNA copy number estimates followed a non-linear relationship [7] (Fig. 3).

The poor linear fit in qPCR data appeared to be due to the 16S rRNA copy number being underestimated at high cell densities (Fig. 3C). Examination of the gDNA concentrations, measured using a Nanodrop 2000c spectrophotometer, revealed that DNA concentrations follow similar plateauing trends to the qPCR data with no significant differences between the concentration of undiluted and 1:2 diluted cultures ($t_{(4)} = 1.15$, $p = 0.31$; Fig. 4). We hypothesize that high concentrations of iron precipitate, which are inherent in laboratory cultures of SRBs, impeded gDNA recovery during gDNA extraction and in turn the ability of qPCR to estimate SRB growth responses in laboratory settings. However, qPCR could still be an accurate method for capturing SRB growth curve data provided cultures are diluted prior to gDNA extraction steps. Fig. 5 demonstrates that the expected linear trend was preserved in our qPCR data amongst cultures diluted at least 1:5 ($R^2 = 0.96$, Fig. 5).

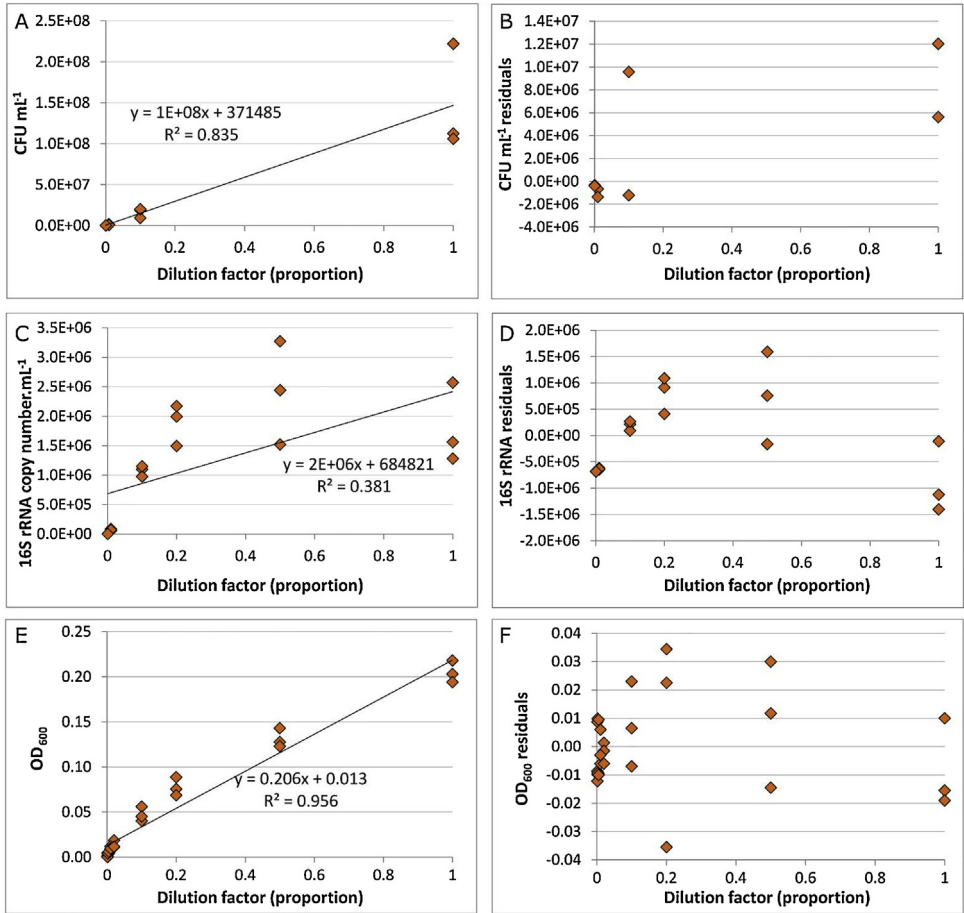


Fig. 3. Linear regression models for cell density against culture dilutions, with cell density estimated by CFU mL⁻¹ count for dilution spread-plating (A), 16S rRNA copy number mL⁻¹ for qPCR (C) and OD₆₀₀ for acid-amended OD (E) techniques. Corresponding residuals plots for dilution spread-plating (B), qPCR (D) and acid-amended OD (F) data.

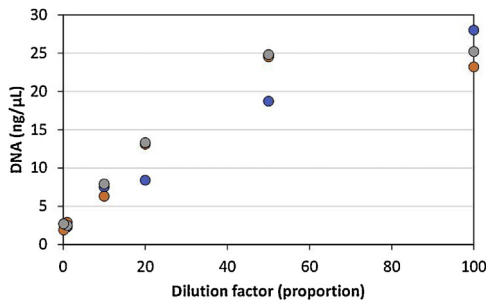


Fig. 4. Concentration of gDNA as determined via a Nanodrop 2000c spectrophotometer, showing that DNA concentrations plateaued at high culture densities. Colours = values associate with three replicate cultures.

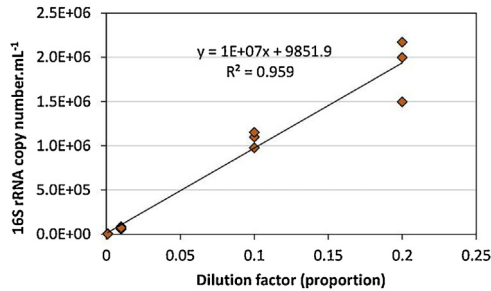


Fig. 5. Linear relationship amongst SRB cultures diluted 1:5 and below as detected by qPCR of 16S rRNA gene.

Whilst both spread-plating and aaOD methods were able to re-create linear cell-density dilution factor relationships, examination of residuals plots revealed heteroscedasticity in the spread-plating data (i.e. the error terms increased with cell density; Fig. 3B). The positive correlation between error and cell density reflects the propagation of error as additional serial dilution steps are incorporated to reach appropriate culture densities (typically cited as 25–250 CFUs per plate) for CFU counting [8]. As such, spread-plating is a sub-optimal method for assessing growth curves because a) growth curves are, by definition, designed to track increasing levels of cell density and error in cell density estimates will increase with time as growth proceeds and b) error propagation due to the need for additional dilutions will be compounded because studies of growth typically use rich media and/or optimal growth conditions which achieve cell densities 2–4 orders of magnitude higher than what is typically found in aqueous environmental samples [9–11]. The same shortfall can be applied to bacterial numbers estimated via the most probable number (MPN) method which similarly requires the use of dilution series but is of lower precision because it does not use direct counts [12]. In contrast to residual plots of spread-plating data, residuals plots for the aaOD data (Fig. 3F) found no trend in the error term distribution indicating these data can be appropriately modelled by a predicted linear relationship [7].

Comparison of method time and cost efficiencies

To demonstrate the practicality of the aaOD method, we compared on the costs associated with personnel time and consumables. Our analysis assumed a minimum of 6 time points to be measured per growth curve with three biological replicates. For each method, costings accounted for method-specific consumables (i.e. not gloves or pipette tips).

For spread-plating, costings accounted for the use of disposable Petri dishes and spreaders and enough media to create a dilution series down to 10^{-6} for each time point. The cost of reagents used to make liquid (MB) and solid (TSA + salts) media was estimated using prices for ASC grade chemicals obtained from Sigma Aldrich.

For qPCR, a minimum of 2 technical replicates were assumed for each time point. Reagents for a triplicate 10-fold standard curve and 3 negative controls were incorporated into the cost of each 96-well plate. Consumable costs were estimated using the cheapest compatible plastics for a CFX Connect™ real-time PCR detection system (Biorad). Reagent costing for qPCR was based those used in the qPCR method described above. The cost of individual gDNA extractions was based on cost of BiolineSololate II gDNA extraction kits.

Time was costed on a per hour basis using a Level 1 Research Assistant wage (AUD\$36.71/h). The amount of time required for each process (spread-plating, CFU counting, aliquoting media etc.) was determined by timing how long it took an experienced user of each protocol to complete a given process five times and then taking the average.

Fig. 6 shows that the major cost for each method was generally associated with personnel and the aaOD technique was always found to be the most time/cost efficient. For the collection of one data point, spread-plating was more efficient than qPCR, largely due to the time associated with DNA

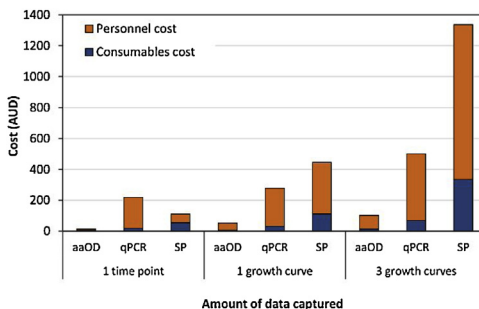


Fig. 6. Cost comparison of three methods for measuring SRB growth data. The cost of each method is broken down by the contribution of consumables and personnel. 6 time points are required to generate 1 growth curve. aaOD = acid-amended OD, qPCR = quantitative PCR, SP = dilution spread-plating.

extractions, however the cost of the spread-plating technique rapidly increases as additional data points are added. The major contributor to cost/time inefficiency for the spread-plating technique stems from the need to establish multiple dilutions (we factored for a dilution series down to 10^{-6}) for plating and from the time associated with counting plate data. The amount of time associated with these activities incurs a cost in excess of AUD\$1300 for the comparison of just 3 growth curves (i.e. 3 different strains or 3 different growing conditions). In contrast the cost of the aaOD method for the same number of data points is just over AUD\$100.

Additional information

Sulfate reducing bacteria (SRB) are an environmentally and industrially important class of microorganism. In the environment, SRB couple the oxidation of organics or H_2 to the reduction of sulfate and sulfur-containing compounds (known as dissimilatory sulfate reduction), making them major drivers of global sulfur cycles with roles in the cycling of carbon and nitrogen and other metals [22]. Whilst the metabolic processes of SRBs can be harnessed for beneficial practices, such as the removal of sulfates and heavy metals from waste water [13], it is their contribution to microbially influenced corrosion (MIC) processes that has generated the most interest in these organisms. MIC can impact key concrete and metallic infrastructure, causing significant economic losses in waste water, petroleum and maritime industries [17,21].

The use of sulfate reducing bacteria (SRBs) in laboratory studies is a common approach for investigating microbially influenced corrosion MIC. Laboratory studies are advantageous in because they offer a high level of control over environmental parameters that can influence MIC. However, the formation of a FeS black precipitate when SRBs are grown in standard laboratory media, such as Modified Baar's medium (MB) and Postgate's medium, make it difficult to incorporate biological growth data. Whilst the active growth (i.e. metabolic activity) of planktonic SRB has been linked to pitting corrosion processes [14] the growth rate of planktonic SRB may not be directly related to anaerobic corrosion processes which are carried out within biofilms such as extracellular electron transport, metabolite-driven MIC or cathodic depolarization [19]. The measurement of acid-amended OD, described in this paper, cannot be used to enumerate sessile bacteria in a biofilm however, if biofilms were first dislodged via sonication as per [18] then this method could be adapted to compare biofilm density.

The benefits of incorporating bacterial growth data into MIC testing go beyond facilitating correlation between planktonic cell growth and rates of MIC. We highlight three key advantages which will benefit the field of MIC research:

- 1) *Improved experimental design.* Understanding SRB growth habits enables researchers to make an informed choice regarding the metabolic status of SRBs used in test systems. For instance

Chen et al. [15] desired stable metabolic activity in their SRB induced corrosion testing and, with knowledge of SRB growth habits, were able to use 8-day old cultures to achieve this aim. In contrast, other studies [26,14] have demonstrated that MIC proceeded whilst SRBs were actively dividing. As such understanding when an SRB is metabolically active is a necessary step for incorporating metabolic activity (or indeed stability) into MIC tests.

- 2) *Improved repeatability through standardization.* Because the length of lag, log and stationary growth phases will vary depending on the microorganism and the experimental parameters (culture volume, temperature, pH, etc.) used, growth curve data can help standardize the microbial component – in terms of its metabolic phase – between MIC tests. Indeed standardization of the microbial component in terms of metabolic activity would also be a step towards comparing and replicating results from different studies. Currently there are no standard method or reporting criteria for the number of bacteria to use in MIC studies [25], consequentially (sub)culturing conditions (inoculation volume, preparation of inoculum, presence/absence of agitation) are often reported in a manner that does not allow replication [14,27].
- 3) *Disentangling biotic and abiotic effects.* An understanding of how bacterial growth proceeds during an experiment can be integral for interpreting outcomes between treatments, for example growth data can help disentangle whether differences in attachment or biofilm formation are due to the manipulated abiotic conditions or due to an intrinsic difference in the number of planktonic cells [25].

Due to black precipitates interfering with OD measurements, SRB studies that do incorporate bacterial growth into their experimental design and controls typically monitor growth using the most probable number (MPN) protocol [26,14] or the spread-plating technique [25]. Whilst these techniques require very little specialist equipment, they are labor intensive and time consuming. Indeed, the intensive nature of these methods has prompted the development of alternative procedures for SRB enumeration which could be co-opted to measure growth rates, including: PCR-based detection [10,24], enzymatic assays [16,23] and microscopy-based counting [20]. Whilst these alternative enumeration techniques are more efficient, they require specialist knowledge or equipment and techniques.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.mex.2019.09.036>.

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