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# Quantification of Gene Expression on Mixed Communities of Sulphate-Reducing Bacterial Biofilms from Crude oil and Produced Water at Laminar Flow

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**ABSTRACT**
*The quantification of in situ levels of Adenosine-5'-phosphosulphate (APS) reductase gene expression to track the activity of sulfate-reducing bacterial (SRB) biofilms in crude oil and produced water at laminar flow was carried out. SRB biofilms indigenous to crude oil (sample A) and produced water (sample B), were grown in square section, glass capillary flow cells at 55 °C. Microscopy image analysis revealed clusters of cells made up of both coccoid and rod-shaped bacteria, ranging in length from 1-3 µm, and some short chain and single cells with a variety of cell sizes demonstrating the variety of consortia in the mixed biofilm. Quantification involved extraction, purification, and DNase treatment of extracted RNA followed by cDNA synthesis by Reverse Transcription (RT) PCR and finally Quantitative-PCR (Q-PCR) analysis with primers designed for APS reductase genes. Biofilm Q-PCR analysis revealed lesser gene expression by sample A, with threshold cycle (Ct) value of 6.81 than sample B with Ct-value of 6.48. The higher Ct-value of sample A showed lesser gene expression than sample B which may have been contaminated by subterranean water. These results have significance in the establishment of quality assessment.*
***Key Words:*** *Gene expression, Biofilm, Laminar flow, Attachment, Quantitative-PCR, Sulphidogens.*

## INTRODUCTION

Since the inception of microbiology, microorganisms have primarily been characterised as planktonic, freely suspended cells and have been described on the basis of their morphological and physiological properties and growth characteristics in nutritionally rich media. However, in most natural environments, microbes are commonly found in close association with surfaces and interfaces in the form of multicellular aggregates glued together with the slime they secrete [1-2]. Microorganisms can grow collectively in adhesive polymers, mainly extracellular polysaccharides (EPS) on biologic or non-biologic surfaces to form biofilms [3]. Microbial biofilms are comprised of both cells and extracellular polymeric substances. EPS are crucial to the structure of biofilms [4] and the composition of EPS from biofilms has been shown to vary widely within SRB [5,6].

SRB form biofilms and studies have shown that gene expression in biofilm cells differs from planktonic stage expression and these differentially expressed genes regulate biofilm formation and development [3]. A number of different molecular 16S rRNA-based methods like PCR systems and probes have been designed and successfully applied to study the SRB community composition in the environment. They include 16S rDNA amplification by PCR [7], fluorescent in situ hybridisation (FISH) [8], rRNA slot blot hybridisation [9-10], PCR-denaturing gradient gel electrophoresis (DGGE), nucleic acid microarrays [11]. More challenging is the measurement of the in situ activity of SRB in environmental samples.

The quantification of functional gene expression may provide the possibility to combine direct measurements of particular microbial activities with a phylogenetic specificity [12]. Messenger RNA (mRNA), the product of gene expression, has a high turnover rate and therefore the amount of mRNA is a direct measure of the transriptional activity in cells. After extracting total RNA from environmental samples, the amount of mRNA may be measured. This would allow to quantify the in situ activity of different physiological groups of microorganisms [12].

Dissimilatory sulfite reductase is the central enzyme in the dissimilatory sulfate reduction and catalyses the final six electron transfer to bisulfite to form sulfide. Dissimilatory sulfite reductase gene sequences of the alpha and beta subunits of the DSR gene, dsrAB, have shown to be a promising and specific approach for investigating the diversity of SRB [13-16]. In addition to dissimilatory sulfite reductase, SRB possess Adenosine-5'-phosphosulphate (APS) reductase [17]. The genes for APS reductase, *apsBA*,

from bacteria and *aprBA* from archaea encode subunits that appear to form a 1:1 alpha beta-heterodimer. Both subunits of the APS reductase are highly conserved, and the APS reductase genes have been proposed as a useful phylogenetic marker [18].

Biofilms accumulating at the injection sites used in enhanced oil recovery adversely affect formation permeability and hence water and oil movement [19,20]. Relevant factors known to alter biofilm formation potential have been studied [21-29]. They include water, temperature and pH, support material, concentration of disinfectant, flow rates, microbiological quality and organic substrata in the water [30].

Current methods to study gene expression such as Northern hybridisation, Ribonuclease protection assay and Competitive reverse transcription PCR have been found to be time-consuming and have a narrow detection range as well as low sensitivity [31]. Real-time RT-PCR assay have been found to be a suitable method for the quantification of the DSR mRNA in the environment and for the quantification of gene expression in experimental studies of other prokaryotes [32]. It has already been applied to quantify 16S rDNA (rRNA) of different groups of microorganisms [33, 34,34,35] and functional genes like *Pseudomonas stutzeri* nitrite reductase or *Pseudomonas sp* carbazole 1,9a-dioxygenase in environmental samples. Real-time RT-PCR has several advantages over conventional methods of mRNA quantification in terms of dynamic range, sensitivity and reproducibility [36-37], hence it is used in this current work for the quantification of gene expression in mixed communities of SRB biofilms at laminar flow regime.

## MATERIALS AND METHODS

**Collection of samples:** Five hundred millilitres each of Bonny Light Crude Oil (Sample A) and Bonny Light produced water (sample B) were collected aseptically from Bonny dispatch tank and oil well respectively in sterile sample bottles from Shell Well 9, Awoba flow station in Degema Local Government Area of Rivers State, Nigeria. They were sealed for laboratory analysis.

**Culture medium:** The culture medium used to grow the biofilm was sea water medium (SWM) prepared by addition of sodium pyruvate (6.0 g/l) and yeast extract (1.0 g/l) to 1 litre of artificial sea water solution ASW, Tropic marin, Aquarientech, Wartenberg, W. Germany, (33 g/l) in deionised water. The pH was adjusted to 6.5 with 1 M HCl/NaOH and autoclaved at 105 ºC for 20 mins to remove dissolved oxygen. Nitrogen sparging was carried out for 20 mins after which the prepared medium was sterilised by autoclaving at 121 ºC for 15 mins.

**Flow cell set-up and experimental procedure:** In this research, a flow cell system according to Pereira *et al*. [38], was developed and modified to anaerobically grow mixed SRB biofilm at laminar flow and to observe the attachment, growth and development of mixed communities of SRB in a flowing system. Growth flow cells were used for this work and the attachment of cells and subsequent growth of cell clusters and biofilms were monitored using image analysis software to determine the rate and extent of biofilm production.

Two flow cells were used in the set-up with oxygen-free nitrogen added into the medium from a nitrogen generator. The flow cell consisted of square section glass tube with internal dimensions 3 x 3 mm (Camlab, Nuffield Road, Cambridge), clamped over an opening in a temperature regulated aluminium and acrylic microscope stage adapter, called platten placed into the flow system set-up. A supply reservoir and waste vessel, both fitted with filtered exhaust ports to allow pressure equilibration to atmosphere were attached. Sterilisation of the entire flow cell apparatus was carried out by autoclaving at 121 ˚C for 15 mins. The temperature of the flow cell was controlled by contact with the supporting aluminium plates beneath and to either side of the flow cell. A microthermocouple attached to the flow cell wall communicated with brass micro-heaters on the aluminium plates through a micro-processor unit. The peristaltic pump maintained a laminar flow. Flow cells were inoculated with 5ml of samples A and B in separate experiments. After three hours inoculation period and restoration of flow of the sterile SWM, cells which had attached were allowed to grow and develop biofilm on the interior glass surfaces. They were monitored for attachment, growth and biofilm development for up to 24 hrs at 55 ºC.

**Flow rate:** The flow rate in this experiment equated a laminar flow rate of 0.5 ml/min (Reynolds number, Re = 3). This was maintained by the peristaltic pump. There was no reduction in the flow rate throughout the course of the experiment.

**Differential Interference Contrast (DIC) Microscopy:** At the end of the experiment, flow cells were allowed to drain and then clamped off and aseptically removed for DIC microscopy using the Confocal Laser Scanning microscope. DIC images of biofilm cells attached to the flow cell glass were taken at different locations of the flow cell.

**Reference strain**

Reference strain *Desulfobacter curvatus* was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), as lyophilised cultures. They were grown in SWM anaerobically according to suppliers' instructions.

**Preparation of samples and reference strain for RNA extraction**

Samples and reference strain were prepared for mechanical disruption using RNAprotect bacterial reagent. One millilitre of RNAprotect was added to 0.5ml of culture, mixed by vortexing and left at room temperature for 5mins. Centrifugation was carried out at 5000rpm for 10mins, after which supernatant was removed and it was allowed to dry for about 5-10mins. Samples were stored at -20˚C until ready for RNA extraction.

**RNA extraction**

RNA extraction was carried out so as to get mRNA of APS reductase genes. This was done using the RNeasy Mini kit according to manufacturer's instructions. Three hundred and fifty microlitres of buffer RLT was added to each sample mixed by vortexing strongly for 5 - 10s to ensure that the pellets are thoroughly resuspended in buffer RLT. The suspension was transfered into sterile 2ml safe-lock tubes containing acid-washed beads and disrupted in a TissueLyser for 5mins at maximum speed. Centrifugation was carried out for 10s at maximum speed and supernatant was then tranferred into new sterile tubes. Three hundred and fifty microlitres of 70% ethanol was added and mixed well by pipetting.

**Purification of RNA**

This was carried out as follows: 700µl of the lysate including any precipitate that may have formed was transferred into an RNeasy Mini-spin column placed in a 2ml collection tube. Centrifugation was carried out at >10,000 rpm for 10s and flow through discarded. The collection tube was reused. Seven hundred microlitres of buffer RW1 was added to the spin column, lid closed gently and centrifugation was carried out at >10,000 rpm for 15s. Flow-through was discarded with the collection tube. The RNeasy column was placed in a new 2ml collection tube and 500µl of buffer RPE was added to mini spin column, lid closed gently and centrifuged for 15s at >10,000 rpm. Flow-through was discarded and collection tube reused. Five hundred microlitres of buffer RPE was added to the column and centrifuged for 2mins at >10,000 rpm. The spin column was gently removed so that the spin column did not touch the flow-through. The spin column was placed in a new 1.5ml collection tube and 50µl of RNase-free water added directly to the spin column membrane. Centrifugation was carried out for 1 min at >10,000 rpm. The eluted RNA was then collected in RNA tubes and stored at -80˚C until needed for DNase treatment.

**DNase treatment of purified RNA**

This was carried out prior to Reverse Transcription-Polymerase Chain Reaction (RT-PCR) carried out to synthesize cDNA. The digestion reaction was set with 8µl of RNA, 1µl of RQ1 RNase-free DNase 10X reaction buffer, 0.5µl of RQ1 RNase-free DNase and 0.5µl of nuclease-free water to make a volume of 10µl. Incubation was carried out at 37˚C for 30mins. One microlitre of RQ1 DNase Stop solution was added to terminate the reaction and incubated at 65˚C for 10mins to inactivate the DNase and RT-PCR carried out to synthesize cDNA.

**Reverse Transcription-Polymerase Chain Reaction**

cDNA of mRNA for APS reductase genes was generated with *apsA*-specific primers with 5µl of extracted DNase-treated RNA serving as template. For RT reactions, Thermoscript Reverse Transcriptase, invitrogen kit (Invitrogen Technologies) was used according to manufacturers instructions. The reaction mixture consisted of 2µl of 10mM dNTP mix, 3µl of DEPC-treated water and 5µl of DNase-treated RNA to make a total volume of 12µl. Incubation was carried out at 65˚C for 5 mins and 7µl mixture of 5x cDNA synthesis buffer (4µl), DTT (1µl), RNaseOUT (1µl), and DEPC-treated water (1µl) added. One hundred microlitres RT enzyme later was added and incubation at 25˚C for 10mins was carried out, followed by gentle mixing and brief centrifugation. This was followed by incubation at 50˚C for 60 mins and at 85˚C for 5mins. The cDNA samples were stored at -80˚C before Q-PCR analysis was

carried out. Control experiments to check DNA contamination was performed by preparing reactions in which no Reverse Transcriptase was added to the extracted RNA.
DNA contamination was checked with agarose gel electrophoresis following RT-PCR by performing control experiments in which no reverse transcriptase was added to extracted RNA before the PCR step. RNA concentration was determined by absorption at 260 nm with a Biophotometer (Eppendorf, Hamburg, Germany). Purified RNA was stored at −80 °C.

**Primer design**

Primers were designed for Q-PCR analysis of apsA genes using the Genscript software. To determine whether the primers were suitable, gene-specific qualitative PCR was performed before Q-PCR. Primers were evaluated for Q-PCR [39].

**Preparation of standard curve**

Standard curve for the Q-PCR quantification was prepared from dilution series of purified RT-PCR products of the reference strain. The cDNA was amplified with *apsA* gene-specific primers and the resulting amplicon was purified. PCR conditions were: 95˚C (5mins); 40 cycles of 95˚C (40s); 58˚C (1min); 72˚C (1min), followed by a final extension at 72˚C for 7mins. The purified RT-PCR product was quantified and five serial dilutions made. Q-PCR analysis was then carried out with the dilutions. Standard curve of the *apsA* RNA copies was obtained by plotting the Threshold cycle (Ct) values of each dilution against the log copy number. The copy number was calculated on the basis of the measured *apsA* RNA concentration (ng/µL), an average molecular mass of 330 for a base in a single-stranded RNA and the *apsA* RNA size which was 115 bases. The lower detection limit was about 3-8 copies of target mRNA.

**Quantitative-PCR**

Quantification of *apsA* expression was performed in an MJ Mini Thermal Cycler and detected by a MiniOpticon system for Real-time PCR detection (Biorad Laboratories, Inc.), using iQ SYBR Green Supermix (Biorad). Q-PCR was undertaken using designed *apsA* specific primers shown in Table 1 and obtained from MWG Biotech (Germany). Quantification of samples was carried out by determining the threshold cycle value and by comparing results to a standard curve. The PCR reaction consisted of 12.5µl of SYBR Green Supermix, 0.5µl of each 10 pM/µL forward and reverse primers, and 1.0 µl of each cDNA template, in a total volume of 25µl. Thermal cycling conditions were started at 95˚C for 3mins, followed by 40 cycles of denaturation (95˚C for 20s) and annealing (60˚C for 1 min) and a final extension (60˚C for 5min). No template-negative controls were included in each Real-time PCR.

## RESULTS AND DISCUSSION

It was observed that laminar flow regime encouraged attachment, growth and establishment of SRB biofilms. This observation was similar to those observed by Cheung and Beech [40] who found that sulphidogens (sulphide-producing microorganisms e.g SRB) have affinity for surfaces. Studies on the attachment of SRB in rock cores to oil reservoir matrix materials by Chen *et al*. [41] also supports this observation. Attachment of SRB biofilms at laminar flow under anaerobic conditions occurred successfully at 55ºC. This temperature was observed to encourage SRB growth in this research. This observation was found to be consistent with the report of Bass [42]who found that attachment and growth of SRB can occur at 55ºC.
The mixed communities of SRB in crude oil and produced water used in this research attached and grew on surfaces as biofilms. Successful biofilm formation was observed in both samples. Figures 1 - 2 represent series of biofilm images of samples A and B displaying development of the entire biofilm structure and SRB populations *in situ*. It was clearly observed that heterogenous biofilm structure was developed, which increased in surface cover with time. These observations were found to be consistent with earlier findings of Stoodley *et al.,* [29], who confirmed the heterogenous structure of biofilms.
*In situ* image microscopy analysis revealed predominantly clusters of cells made up of both coccoid and rod-shaped bacteria, ranging in length from 1-3µm, and some short chains and single cells with a variety of cell sizes demonstrating the varied number of consortium in the mixed biofilm. Differences in structure of biofilms from both samples were observed using both continuous *in situ* microscopy and stationary DIC microscopy. More clusters of round and rod-shaped cells were observed in sample A than sample B, with sample B having more single rod-shaped cells. These findings were similar to those of Stoodley *et al.* [43] who reported that biofilms were complex differentiated communities made up of varying sizes.

Statistical analysis of the means of DIC images of biofilms from both samples was determined and a p-value of less than 0.05 was considered significant. Results from statistical analysis showed that there was significant difference between biofilms of both samples, while there was no significant difference between biofilm within each sample. A significant difference in biofilm cover and gene expression between both samples at 55°C during laminar flow was therefore implied from the statistical results. These findings were similar to those by Stoodley *et al.* [43] who reported that biofilms from the same sample were similar but differ from biofilms of other samples.

Successful quantification of *apsA* expression was observed in both sample. Biofilm Q-PCR analysis for quantification of gene expression revealed less gene expression by sample A, with Ct values of 6.81 than sample B with Ct values of 6.48. The higher Ct-value of sample A showed lesser gene expression than sample B which may have been contaminated by subterranean water. These findings were consistent with those of Singh *et al.* [3] who discovered that differentially expressed genes regulate biofilm formatiom and development.

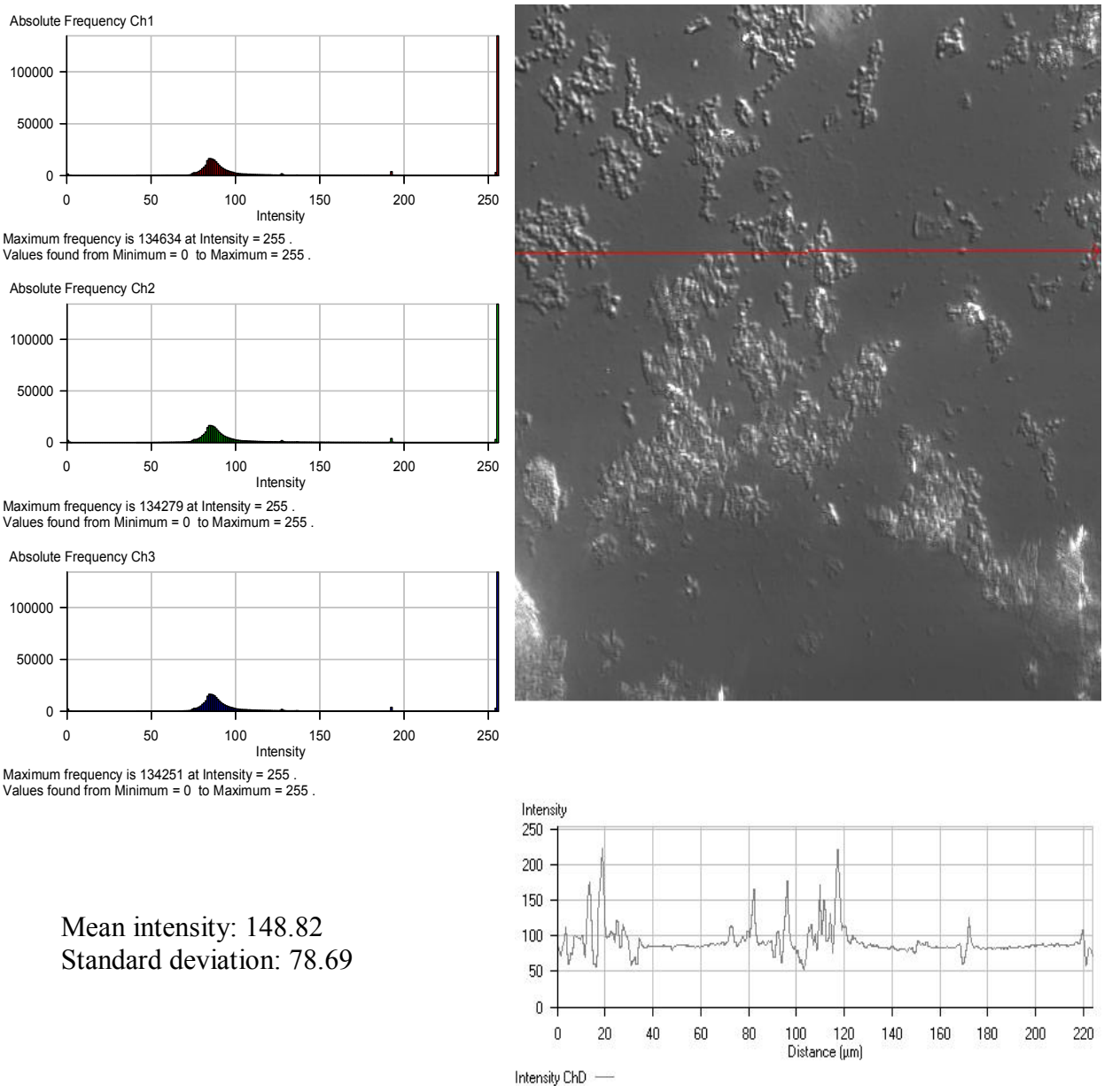
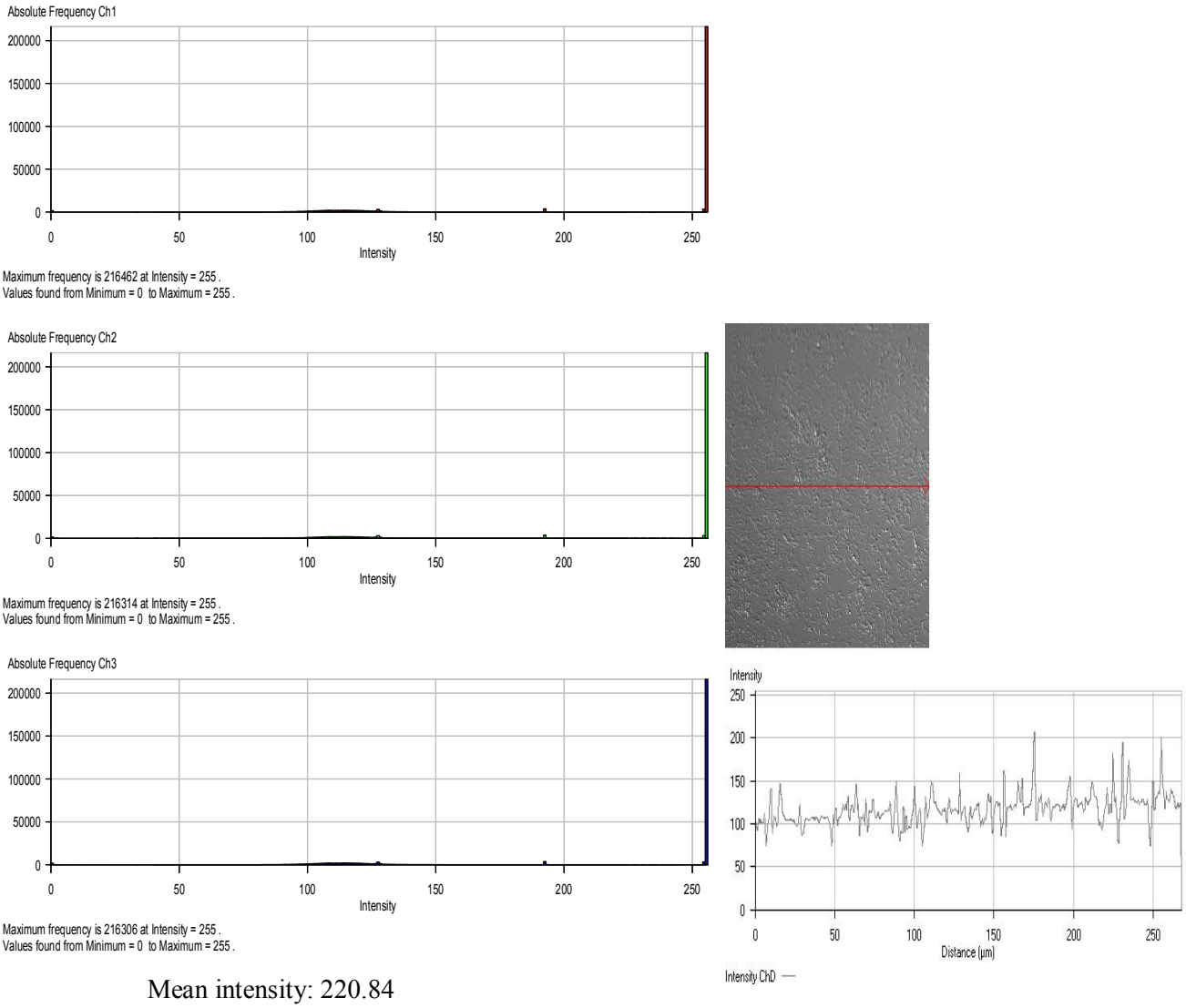



**Figure 1:** Cross-sectional intensity and distance apart of A3 illustrated by peaks.




Mean intensity: 220.84
Standard deviation: 61.53

**Figure 2:** Cross-sectional intensity and distance apart of B1 illustrated by peaks.

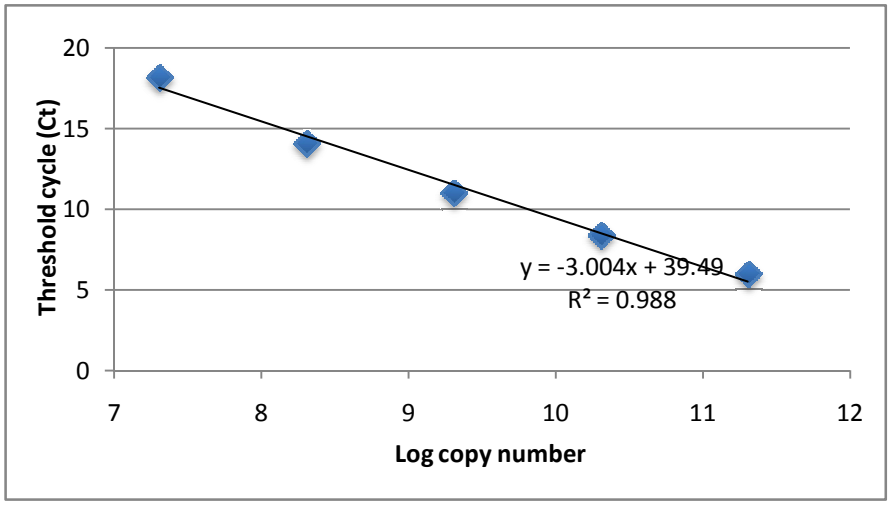



**Fig. 3:** Standard curve for apsA genes. Data points represent the average of triplicate measurements.

## Quantification of Gene Expression

Q-PCR analysis for biofilm gene expression as illustrated by Figure 3 revealed the following:

- Sample A gave Ct-values of 6.81 while sample B had Ct-values of 6.48.
- The lower Ct-values of sample B showed more gene expression than sample A.

## CONCLUSION

Data shows the quantification of apsA genes responsible for hydrogen sulphide production in SRB biofilms. Sources of contamination of these bacteria such as the injection of sea water or other water, containing sulphate, with an indigenous population of viable SRB, a common practice used to increase oil recovery beyond primary production by maintaining reservoir pressure and sweeping oil towards production wells, should be treated in order to prevent microbially influenced corrosion, souring of crude oil and other consequences caused by sessile SRB.

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