

# Enumeration of sulphate reducing bacteria for assessing potential for hydrogen sulphide production in urban drainage systems

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## ABSTRACT

Urban drainage structures have increasing demands which can lead to increasing hydrogen sulphide related problems forming in places where they have not previously been prevalent. This puts pressure on the methods currently used to monitor and diagnose these problems and more sophisticated methods may be needed for identifying the origin of the problems. Molecular microbiological techniques, such as quantitative polymerase chain reaction, offer a potential alternative for identifying and quantifying bacteria likely to be causing the production of hydrogen sulphide, information that when combined with an appropriate sampling programme, can then be used to identify the potentially most effective remediation technique. The application of these methods in urban drainage systems is however, not always simple, but good results can be achieved. In this study hydrogen sulphide producing bacteria were quantified in three small combined sewer overflow storage tanks. Bacterial counts were compared between wastewater, biofilms and sediments. Similar numbers were found in the wastewater and biofilms, with the numbers in the sediments being lower. If remediation methods for hydrogen sulphide is deemed necessary in the tanks, methods that target both the wastewater and the biofilms should therefore be considered.

**Key words** | combined sewer overflow, hydrogen sulphide, qPCR, sulfate reducing bacteria, urban drainage

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## INTRODUCTION

Hydrogen sulphide, formed by sulphate reducing bacteria (SRB) under anaerobic conditions, is a compound often of interest in relation to wastewater management decisions as it is odorous, causes concrete corrosion and is toxic in concentrations not infrequently detected in sewers (Hvitved-Jacobsen *et al.* 2013). Because the hydrogen sulphide formation takes place under anaerobic conditions, a lot of the research on hydrogen sulphide formation and sulphate reduction in urban drainage systems has focused on rising mains and the gravity sewers directly downstream of rising mains, which is where the hydrogen sulphide related

problems are often found (Okabe *et al.* 2003; Mohanakrishnan *et al.* 2009b; Hvitved-Jacobsen *et al.* 2013). However with the pressures on the drainage systems, problems caused by hydrogen sulphide, such as odour, are starting to be detected in other parts of the drainage systems or cause concerns when designing new elements in the systems (Bachmann *et al.* 2007; Sun *et al.* 2013). In rising mains, SRB are generally found in biofilms rather than the flowing wastewater, probably due to their growth rate being low in comparison to the normal hydraulic residence time in the rising mains (Hvitved-Jacobsen *et al.* 2013). However other structures in urban drainage systems have different residence times and therefore differences in the distribution of SRB between sewer biofilms, bulk water and sediments (Sun *et al.* 2013; Lange & Wichern 2013). Understanding where in the urban drainage structures hydrogen sulphide is formed will

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be important in choosing efficient control and prevention strategies. Detection of the presence of SRB may provide one way of diagnosing potential problematic areas as well as giving early opportunity for implementing the most promising remediation techniques. Quantitative polymerase chain reaction (qPCR) also known as real time PCR offers an attractive methodology for quantifying bacteria in samples from the drainage structures of interest or concern and qPCR based methods are already used for microbial source tracking in the environment (Ahmed *et al.* 2015). Using qPCR in urban drainage systems, however, comes with some challenges, that are manageable, but need addressing in order to provide meaningful results. This paper highlights a couple of these challenges and possible solutions.

## Background

qPCR is based on the general principle of PCR, where DNA sequences in samples are multiplied in a reaction mimicking that used for replicating DNA in living cells. This is a very powerful technique as it can detect DNA present in low concentrations. In the qPCR technique, a DNA sequence of interest (target DNA sequence) is amplified in the PCR reaction with the addition of a dye that fluoresces when bound to DNA. As the concentration of the target DNA sequence increases, this fluorescence signal increases and the initial concentration of the targeted gene sequence in the sample can be calculated based on a standard curve (Smith 2005). The challenge for applying qPCR for quantification of a group of bacteria carrying out a specific function such as the production of hydrogen sulphide is the possibility of identifying a gene sequence that will be uniquely present in all bacteria capable of carrying out this function. In sewers, multiple species of SRB have been identified, including *Desulfovibrio*, *Desulfobacter*, *Desulfobulbus*, *Desulfomicrobium* and *Desulfotomaculum* (Okabe *et al.* 1999; Mohanakrishnan *et al.* 2009a; Mohanakrishnan *et al.* 2009b). Of these genera, all but *Desulfotomaculum*, belong to the group of Gram negative mesophilic SRB, whereas *Desulfotomaculum* is a Gram positive, spore-forming SRB (Castro *et al.* 2000). The diversity of the SRB is important when applying PCR based techniques for identifying and quantifying the microbial communities as the techniques rely on the possibility of identifying one gene sequence that will selectively capture all SRB in a sample for reliable detection. Within the literature there are generally two strategies for targeting the SRB; either through the gene 16S or the gene *dsrAB*. With the increasing availability of 16S rDNA sequence information, however, it was found that

the primers that target the 16S rDNA of SRB could also amplify 16S rDNA from *Chlorobium*, *Campylobacter* and *Clostridium* species (Amann *et al.* 1992). Therefore, multiple variations of primers based on the *dsrAB* gene have been tested in different studies, with samples from different environments. Wagner *et al.* (1998) demonstrated that primers DSR1F and DSR4R could selectively amplify the *dsrAB* genes from 22 reference eubacterial SRBs. They also demonstrated that these primers could not amplify the version of *dsrAB* known to be present in sulphur oxidising bacteria (Wagner *et al.* 1998). Other studies have developed variations of these primers which increase the coverage of the SRB detected. Primers that target the *dsrAB* gene have also been developed for qPCR and competitive PCR (Ben Dov *et al.* 2007; Kondo *et al.* 2008; Pereyra *et al.* 2010).

The aim of this paper was to highlight the challenges, along with possible solutions, associated with enumeration of SRBs via quantitative real time PCR to assess the potential for hydrogen sulphide production in CSO storage tanks.

## METHODS

### Sampling and sampling site

In this study, a field site with three pilot scale storage tanks for CSO spillage (combined sewage consisting of foul sewage and rainfall derived runoff from urban areas) was used as model system for the SRB quantification. CSO storage facilities (tanks and tunnels) have been designed to part of a comprehensive solution for controlling CSO discharges to meet more stringent water quality standards and to meet requirements of the EU Urban Waste Water Treatment Directives. qPCR was applied to assess the potential for hydrogen sulphide generation in storage tanks. For these trials the CSO spillage (referred to here as wastewater) after a rain event was kept in the storage tanks for the extended period of 7 days to simulate 'worst case scenario' – i.e. storage time significantly higher than the intended storage time for the system. On the 7th day wastewater samples were collected. The tanks were then drained and samples of biofilms and sediments were collected. The sediments samples were collected from sediment traps that were placed at the bottom of the tanks. The sediment traps were round, each with a diameter of 200 mm. For sediment sampling, all the sediments caught in each sediment trap were collected in sterile containers. Upon return to the laboratory, the water was removed from the sediment sample by centrifuging the sample at 2,500 rpm for 6 minutes and

then decanting the water from the sample. The sample from each trap was then weighed for quantification.

The biofilms needed to be sampled in such a way that the SRB content is relatable to the submerged surface area (and hence assumed biofilms covered area) of the tanks. Samplers for biofilms samples were constructed to provide this surface specific sample. The samplers consisted of a layer of thin sponge attached to a plastic disk with a diameter of 31 mm. These were then autoclaved at 121 °C for 15 minutes for sterilisation. The biofilm samplers were attached to a pole to reach the sides of the storage tanks. Once the sponge had complete contact with the tank wall, it was carefully twisted on the spot to collect the biofilms within the sponge structure. Two separate sampling campaigns were carried out: one in June and one in July.

### Sample preparation

Upon return to the lab, 50 ml of wastewater was filtered onto filters with a pore size of 0.22 µm. The filter was frozen at -20 °C until further analysis. The sponges with biofilm sample were frozen at -20 °C until further analysis and the sediments were likewise frozen after water decantation and weighing. For the wastewater samples, half a filter was used in each DNA extraction, for the biofilm samples a quarter of each sponge was used, and for the sediments 0.21 g ± 0.01 g was used in the DNA extraction. All samples were collected in triplicates.

### DNA extraction

#### Cell lysis

The samples were suspended in 720 µl buffer, consisting of 40 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris-HCl pH 9 and 0.75 M sucrose. 81 µl lysozyme at a concentration of 10 mg/ml was added and the samples were incubated at 37 °C for 30 min. After incubation 90 µl of 10% sodium dodecyl sulphate (SDS) and 25 µl of proteinase K at a concentration of 20 mg/ml was added and the samples were incubated for 2 hours at 55 °C. The samples were centrifuged at 5,000 g for 5 min, the supernatants were withdrawn into a different tube.

#### Cetyltrimethylammonium bromide method

To the supernatant from the cell lysis step, 137 µl of 5 M sodium chloride (NaCl) and 115 µl of cetyltrimethylammonium bromide (CTAB)/NaCl solution was added and the

mixture was incubated for 1 hour at 65 °C. The CTAB/NaCl solution consisted of 4.1 g NaCl and 10 g CTAB in 100 ml distilled water (final volume). After incubation, 838 µl of chloroform was added to the clarified lysate. The mixture was centrifuged at 14,000 g for 5 minutes. The aqueous layer was removed to a different tube and mixed with 838 µl of chloroform. The mixture was centrifuged again and the aqueous layer was moved to a different tube. The aqueous suspension was mixed with at least 3 volumes of isopropanol and incubated at -20 °C overnight. The suspension was then centrifuged at maximum speed (21,000 g) for 10 minutes at 4 °C. The supernatant was decanted. The tube with the DNA pellet was rinsed with 70% ethanol, centrifuged at maximum speed again and decanted. This was repeated. The pellet containing the DNA was air-dried and resuspended in 100 µl of sterile water.

#### Phenol-chloroform method

To the supernatant from the cell lysis step, an equal volume of phenol:chloroform (1:1) at pH 8 was added and mixed. The mixture was centrifuged at 21,000 g for 2 minutes. The aqueous phase was removed to a new tube and an equal volume of ice-cold chloroform was added. The mixture was centrifuged at 21,000 g for 2 minutes. The supernatant was removed to a new tube and 5 M NaCl was added to make a final concentration of 500 mM NaCl in the mixture. An equal amount of ice-cold absolute ethanol was added and incubated at -20 °C overnight. The mixture was then centrifuged at 21,000 g for 30 minutes at 4 °C. The supernatant was discarded. The tube with the DNA pellet was rinsed with 70% ethanol and centrifuged at 21,000 g for 20 minutes at 4 °C. The ethanol rinse was repeated. The pellet containing the DNA was air-dried and resuspended in 100 µl of sterile water.

#### DNA clean up

A portion of the extracted DNA was purified using the QIAquick gel extraction kit (QIAGEN, UK). The DNA was electrophoresed on a 1% agarose gel and then visualised under UV transillumination with ethidium bromide. The DNA was excised from the gels and the QIAGEN protocol was followed. The DNA was eluted from the silica column using 50 µl of buffer EB (provided in the QIAquick gel extraction kit), heated to 50 °C. The purified DNA was quantified using a UV spectrophotometer. The quality of the DNA was evaluated based on the level of DNA shearing seen as the spread of sizes of the DNA on the agarose gel

and from the ratio of the absorbances of the extracted DNA at 260 nm and 280 nm (A<sub>260</sub>/A<sub>280</sub>) on a UV spectrophotometer. The DNA should demonstrate a low amount of shearing and should exhibit an A<sub>260</sub>/A<sub>280</sub> between 1.8 and 2.

### Growth of *Desulfovibrio vulgaris* and calculation of DNA copy number

In order to quantify the SRB in the samples, a pure culture of *Desulfovibrio vulgaris* (*D. vulgaris*) was grown to provide DNA for a standard curve. *D. vulgaris* have previously been detected in samples from sewers (e.g. Mohanakrishnan *et al.* 2009a). A freeze dried culture of *D. vulgaris* (DSM 644) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The culture was inoculated anaerobically into a media containing 0.5 g/l dibasic potassium phosphate, 1 g/l ammonium chloride, 1 g/l sodium sulphate, 0.1 g/l calcium chloride di hydrate, 2 g/l magnesium sulphate hepta hydrate, 2 g/l sodium lactate, 1 g/l yeast extract, 1 mg/l resazurin, 0.5 g/l iron sulphate hepta hydrate, 0.1 g/l sodium thioglycolate, and 0.1 g/l ascorbic acid. The culture was then incubated at 30 °C for 4 days before the cells were harvested by centrifuging 20 ml cell culture at 5,000 g for 10 minutes at 4 °C. DNA extraction was hereafter performed using the CTAB method, but without the gel clean up step. The number of DNA molecules per microlitre in the extract were quantified using the formula,

Number of DNA molecules per microlitre

$$= \frac{\text{Concentration (g/}\mu\text{l)} * 6.022 * 10^{23} \text{ (molecules/mole)}}{\text{Length of the genome (bp)} * 660 \text{ (g/mole)}}$$

using 3570858 base pairs as the length of the genome of *D. vulgaris* (Heidelberg *et al.* 2004).

### PCR amplification of 16S rDNA

The 16S rDNA was amplified from the samples using the universal 16S primers, 27F (5'-AGAGTTTGTATCMTGGCT-CAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), using a Veriti 96 well Thermal Cycler (Applied Biosystems, UK). All reactions were carried out in a final volume of 20  $\mu$ l. Each reaction contained 2  $\mu$ l of the 10X PCR buffer with magnesium chloride at a final concentration of 1.5 mM per reaction, 10 mM of each deoxynucleoside triphosphate, 8  $\mu$ M of the reverse and forward primer, 0.1  $\mu$ l of Taq polymerase, 2  $\mu$ l of the sample and water to bring the final volume to 20  $\mu$ l. An initial denaturation was carried

out for 5 minutes at 94 °C, followed by 35 cycles of the following steps: 94 °C for 1 minute, 57 °C for 1 minute and 72 °C for 2 minutes. A final elongation step was included in the setup for 15 minutes at 72 °C.

### Real time PCR

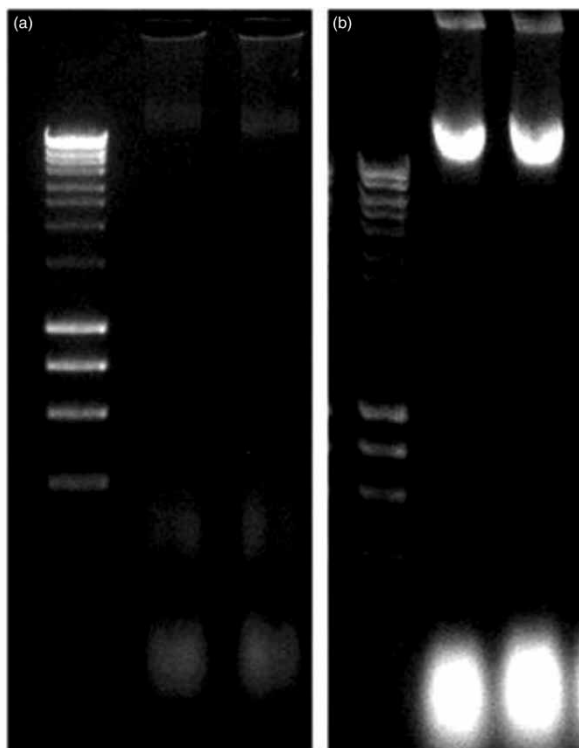
Real time PCR was carried out using primers, DSRIF (5'-ACSCACTGGAAGCACG) and RH3-dsr-R (5'-gGTGGAGCCGTGCATGTT-3') (BenDov *et al.* 2007). The experiment was carried out in an Applied Biosystems 7500 Fast Real Time PCR machine using Power SYBR Green PCR Master Mix containing SYBR Green I dye (Applied Biosystems, UK) in a 96-well optical plate. The PCR reaction was carried out in a final volume of 20  $\mu$ l with 10  $\mu$ l of Power SYBR Green PCR Master Mix, 150 nM each of the forward and reverse primers and 1  $\mu$ l of the template DNA. The thermal cycling conditions were set as follows: 10 min at 95 °C, followed by 40 rounds of 15 s at 95 °C and 1 min at 60 °C. A melting curve between 60 and 95 °C was done to confirm if a single amplicon was produced. All runs included a no template control. The standard curve was obtained by plotting the real-time threshold cycle against the calculated dilutions of the *D. vulgaris* DNA molecules. The standards and samples were assessed simultaneously in triplicates.

## RESULTS AND DISCUSSION

### DNA extraction

Due to a lack of consensus regarding the best, common method for extracting DNA from three different sample types, i.e. wastewater, biofilm and sediment, the initial choice for DNA extraction method was the phenol-chloroform method of DNA extraction – a routine technique often applied for DNA extraction. Cell lysis was achieved by the combined action of lysozyme, an enzyme that hydrolyses the polysaccharides in the bacterial cell wall and SDS – a detergent that solubilises the lipid bi-layers in bacterial membranes.

Whilst the phenol-chloroform method did extract usable DNA from most wastewater samples, a few wastewater samples, as well as all samples from the biofilm and sediments, repeatedly produced a dark pink colouration during the extraction process, which resulted in a very low DNA yield. In addition, agarose gel electrophoresis of these problematic samples demonstrated extensive DNA shearing (Figure 1(a)). It is known that oxidation of phenol



**Figure 1** | Agarose gel electrophoresis of genomic DNA extracted from wastewater by (a) phenol-chloroform method, and (b) CTAB method.

produces coloured quinines and phenoxide radicals (Maloy 1990). It is likely that some wastewater samples, and all samples of the biofilm and sediments, contained strong oxidising agents that react with phenol and subsequently denature the DNA. Hence it was concluded that phenol based DNA extraction methods would be potentially problematic with this type of samples.

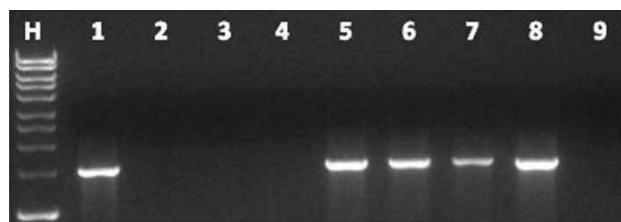
The modification of the DNA extraction protocol was done by replacing phenol-based part of the method with CTAB, DNA from all samples, including the wastewater samples which did not discolour the phenol, were extracted again using CTAB. No interferences was observed with this method and good quality DNA was extracted from all samples. Agarose gel electrophoresis of the extracted DNA indicated a low amount of sheared DNA all the biofilm and sediment samples (data not shown) but not in the wastewater samples (Figure 1(b)). The A260/280 of all the samples were higher than 1.8. In addition, a brown discolouration was evident during agarose gel electrophoresis which co-electrophoresed with the loading dye bands. Under the UV light, this brown discolouration fluoresced brightly and can be seen near the bottom of gel in Figure 1(b). It is likely that this brown discolouration arises from humic substances found in the samples.

## PCR inhibitors

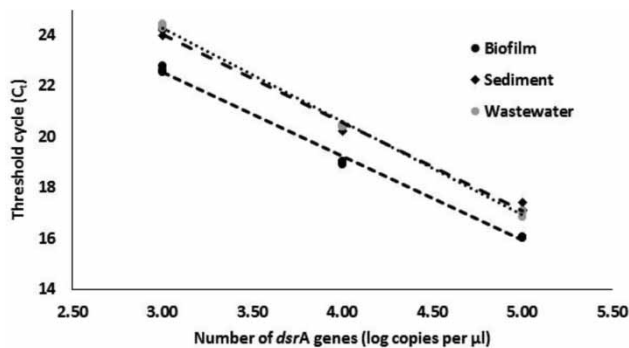
A PCR reaction was setup with primers (27F and 1492R) targeting conserved regions in 16S genes to confirm the presence of genomic DNA from bacteria and archaea in the DNA extract as well as to confirm that the DNA quality did not inhibit the PCR reaction. DNA extracted from a laboratory culture of *E. coli* MG1655 was set up as positive control. Agarose gel electrophoresis of PCR products demonstrated no amplification in the samples containing DNA extracted from wastewater, biofilm and sediment, although positive amplification was seen for DNA extracted from *E. coli*. This suggested the presence of PCR inhibitors in the DNA extract. The presence of any components that inhibit the Taq polymerase, such as EDTA, phenol, ethanol or humic substances in the DNA extracts are likely to result in PCR inhibition. In order to remove impurities from the extracted DNA, the DNA clean-up procedure was applied. The 16S PCR was performed again on the gel extracted genomic DNA and successful PCR amplification was demonstrated (Figure 2) indicating the presence of bacterial and archaeal populations in the samples.

## Real time PCR

In order to quantify the SRB amongst the bacterial and archaeal populations present in the samples, quantitative real time PCR was carried out. The DSR1F and RH3-dsr-R primer pair (Ben Dov *et al.* 2007) was chosen because they generate a short amplicon suitable for qPCR and target a conserved region within the *dsrAB* genes. The use of primers targeting single copy, functional genes may serve to reduce but not completely abolish the problem of overestimation because bacteria are increasingly recognised to harbour multiple copies of their entire genomes (Hansen 1978, Tobiasson & Seifert 2006). In the qPCR reactions, the standards



**Figure 2** | Agarose gel electrophoresis of 16S PCR products. H: Hyperladder IV.1 and 5: positive control. 2-4: PCR reactions contain DNA before 'clean up'. 2: PCR product from wastewater, 3: PCR product from biofilm, 4: PCR product from sediment. 6-8: PCR reactions contain DNA after 'clean up'. 6: PCR product from wastewater, 7: PCR product from biofilm, 8: PCR product from sediment. 9: negative control; sterile distilled water was substituted for DNA template.



**Figure 3** | Standard curves for *dsrAB* genes generated from genomic DNA of *D. vulgaris* and amplified simultaneously along with wastewater, biofilm and sediment samples.

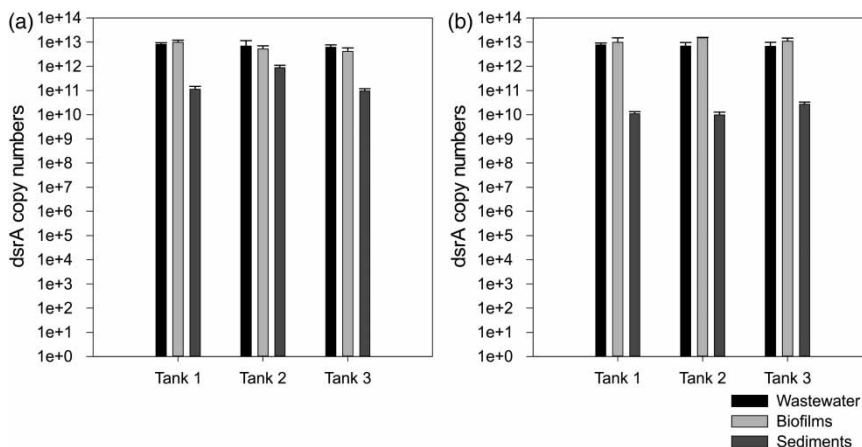
curves from *D. vulgaris* DNA were performed every time alongside enumeration of samples from wastewater, biofilm and sediments. The reproducibility and linearity of the standard curves can be seen in Figure 3. The standard curves had a linear range between  $7.5 \times 10^5$  and  $7.5 \times 10^5$  with an  $R^2 > 0.99$ . The slopes of the standard curves were  $-3.67$ ,  $-3.31$  and  $-3.46$  for quantification of SRBs from wastewater, biofilm and sediment samples; which implies a PCR efficiency of  $>87\%$  across all samples, suggesting that more than  $87\%$  of the target sequences in the template genomic DNA were amplified in every cycle during the PCR.

The results indicate that a higher number of SRB were present in the wastewater and biofilms in the both tanks across June and July when compared to the sediments (Figure 4). Again, this result should be interpreted with caution, because real time PCR does not differentiate between both metabolically active and dormant bacteria, or between live and dead or lysed cells. This is a limitation inherent in the technique.

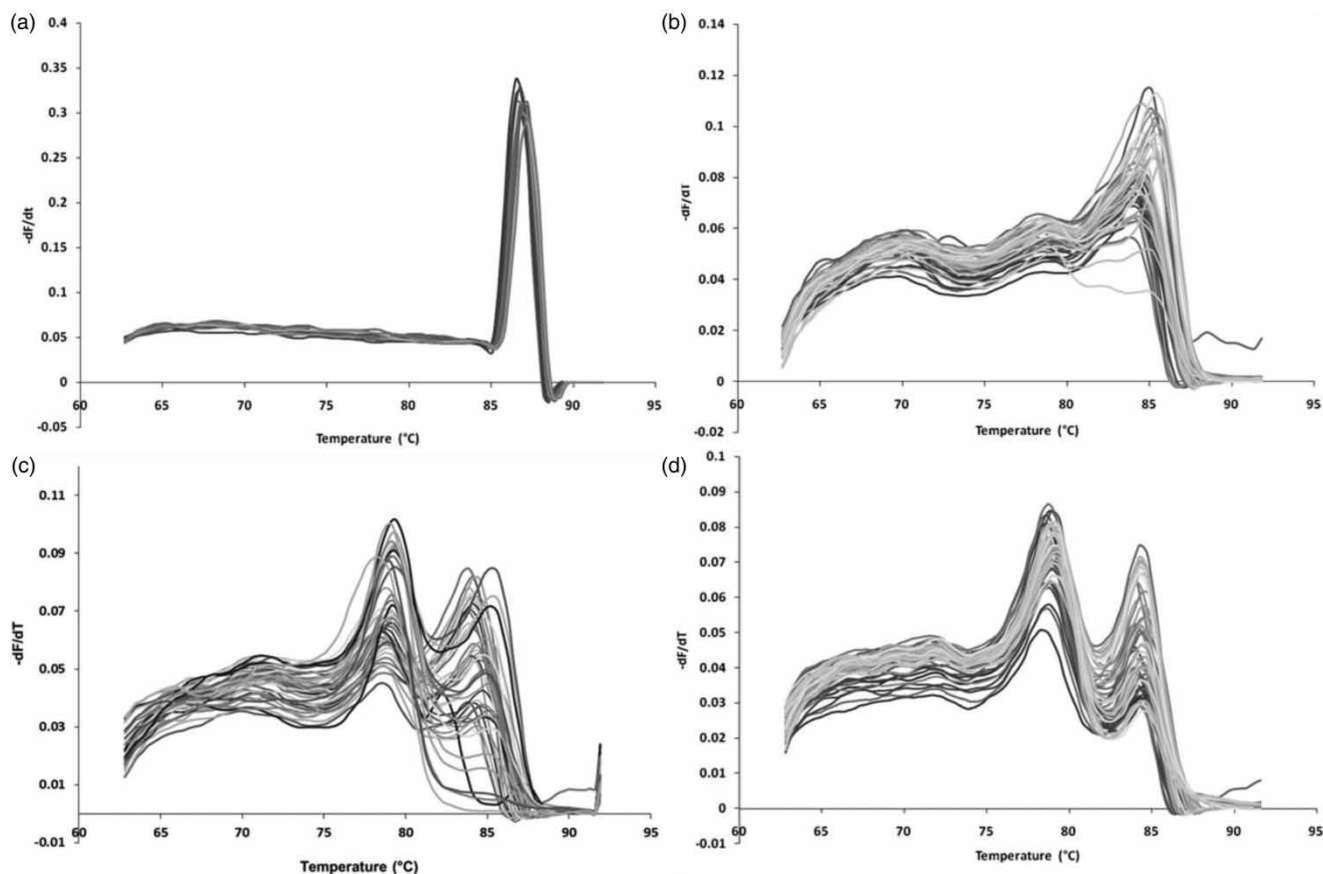
The specificity of the PCR amplification was checked by analysing the melting curves. The melting curve measures the decrease in the fluorescence of the dye as the double stranded PCR product melts with a rise in temperature. The negative first derivative of the decrease in fluorescence is called the melting curve. A sharp peak in the melting curve, as seen with the *D. vulgaris* standards (Figure 5(a)), indicates the specific amplification of a PCR product with identical amplicon length and sequence. On the other hand, the melting curves obtained for the wastewater, biofilm and sediment samples (Figure 5(b)–5(d)) deviate from the ideal and seem to indicate non-specific amplification. However, this is an expected result when running a real time PCR analysis on samples containing mixed populations using degenerate primers such as the ones used in this study. The sharp single peak found in all the standards, run simultaneously alongside the samples, indicates that the samples were not contaminated during experimental setup nor was there a tendency to form primer dimers. It is likely that the multiple peaks seen in the melting curve correspond to different species of SRB giving rise to amplicons of slightly different length and sequence. It is also likely from the melting curves that a higher diversity of SRB is found in the biofilm and sediment phases when compared to the wastewater phase.

## CONCLUSION

The analysis of the samples suggested that phenol based DNA extraction methods may be problematic for some of these samples, however the CTAB based method produced better quality DNA extracts. For these extracts, however,



**Figure 4** | Number of copies of *dsrAB* detected across the three phases, (a) June sampling round, (b) July sampling round.



**Figure 5** | Results of the melting curve analysis carried out after real time PCR. (a) Standards of *D. vulgaris* (b) Wastewater (c) Biofilm (d) Sediment.

additional methods for purification of the DNA extracts was needed to avoid inhibition of the sensitive PCR reactions. The results suggest that the wastewater and biofilm phases in a CSO storage tank harbour a significantly larger population of SRB compared to the sediments. If hydrogen sulphide remediation were to be applied to these CSO systems it would be important to use methods targeting both the wastewater as well as the biofilms. Analysis of the melting curves of the real time PCR products suggested that it is also likely that the diversity of SRBs present in the biofilm and sediment phases is higher than that in the wastewater phase.

This study showed that qPCR does have the potential to identify where in a system there is potential for hydrogen sulphide generation. This study would not only enable the development of a standardised procedure to aid the extraction of high quality DNA for qPCR across different environmental matrices such as biofilms, sediments and wastewater but also aid the practitioner in understanding the caveats that surround the interpretation of the results

obtained via qPCR. Once careful standards and training are established, this technique has immense potential for routine use in the water industry to identify the origin of hydrogen sulphide generation as well as aid the formulation of targeted remedial measures. This is particular of use where operation of a system is changing, for example in the application of increased real time control, or is the addition of new structures to a system as the storage tanks are an example of in this case.

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