Variations in activities of sewer biofilms due to ferrous and ferric iron dosing

Bruno Kiilerich, Pia Kiilerich, Asbjørn H. Nielsen and Jes Vollertsen

ABSTRACT

Addition of ferrous and ferric iron salts to wastewater is a commonly used practice for sulfide abatement in sewer force mains. When iron is added to wastewater where sulfate respiration takes place, it produces ferrous sulfide precipitates with the formed sulfide. The effect of iron addition has traditionally been focused on solely from the perspective of reaction stoichiometry. Possible influences on the microbial communities in biofilms growing in force mains have largely been neglected. In this study the activity and microbiome was examined in three pilot scale force mains conveying real wastewater, two subjected to iron treatment and one operated as an untreated control. Activity was measured on suspended biofilm samples extracted from the experimental setup. The microbiome of the biofilm was analyzed by V3 + V4 16S rDNA sequencing. Correlation analysis of chemical composition of the biofilms and activity measurements for operational taxonomic units of relevance to sulfide and methane production were performed. In conclusion, it was found that both ferrous and ferric treatment reduced sulfate reduction and methane production, and that both iron salts induced significant changes to force main biofilm microbiomes.

Key words | 16S rDNA sequencing, methanogens, microbiome, sulfate-reducing bacteria, sulfide abatement, wastewater

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INTRODUCTION

During wastewater conveyance, available organic and inorganic compounds are somewhat degraded or transformed by suspended wastewater biomass and the microbial biofilm communities growing on the surfaces of the pipelines (Hvitved-Jacobsen *et al.* 2013). Because of topographical differences, the wastewater must sometimes be pumped through force mains. Here the pipeline runs full of wastewater, thus obstructing the reaeration that otherwise would have occurred, had the wastewater been conveyed in a gravity sewer (Boon 1995). The lack of reaeration and the ongoing microbial processes result in available oxygen being quickly depleted. When oxygen and, if present, nitrate and ferric iron are depleted, anaerobic conditions prevail and sulfate is the preferred electron acceptor used for respiration by the microorganisms (Hvitved-Jacobsen *et al.* 2013). Respiration using sulfate results in the formation of sulfides, which has detrimental effects when wastewater is discharged from force mains. These effects concern odor nuisances when sulfide accumulates in the sewer atmosphere, microbial mediated corrosion of sewer assets with derived cost for rehabilitation, and health and safety issues for utility workers due to its toxicity (Boon 1995). If sulfate ultimately gets depleted in the biofilms, anaerobic respiration will result in formation of methane, which, when escaping the wastewater, can act as a greenhouse gas (Guisasola *et al.* 2008).

Due to the immediate detrimental effects of sulfides, abatement of these are widely implemented. Precipitation of the formed sulfides with iron salts of the ferrous (Fe(II)) or ferric (Fe(III)) type is one of the most widespread methods (Ganigue *et al.* 2011). Fe(II) reacts directly with sulfides to form insoluble ferrous sulfides (FeS). Fe(III) on the other hand must first be reduced either biotically or abiotically to Fe(II) which then precipitates available sulfides. During the initial reduction of Fe(III), sulfides are oxidized to elemental sulfur. FeS precipitation is easily recognized in natural environments by a blackening of the water, biofilms, and sediments (Barton 1995). This distinct blackening is also observed in sludge, which indicates that FeS accumulates here (Hao *et al.* 1996). The amorphous iron

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sulfide that forms will under anaerobic conditions then rearrange to more crystalline structures of iron sulfides (Rickard & Luther 2007). Anaerobic biofilms treated with iron salts become black and have often a grainy-like texture, whereas untreated sewer biofilms have slimy textures and, as also observed by Mohanakrishnan *et al.* (2009), brownish colors. These physical differences of biofilms treated with iron salts may cause differences in transport of substrates, electron acceptors etc. in the biofilms. This could consequently contribute to an altered microbial community as metabolic activities depend on substrate availability and interspecies interaction with neighboring cells (Nadell *et al.* 2009).

Specific genera of the microbial biofilm community, as analyzed by denaturing gradient gel electrophoresis and fluorescence *in situ* hybridization, have been found to change along the length of a simulated force main (Mohanakrishnan *et al.* 2009). In a combined sewer system under normal operational conditions a spatial variability in biofilm microbial community was likewise found (Jensen *et al.* 2016). In the study by Mohanakrishnan *et al.* (2009) the changes were ascribed to the changes in substrate type and availability in the force main, which, as shown by Rudelle *et al.* (2016), may be considerable.

Using the same techniques, short term exposure to nitrate was demonstrated to have genus-specific effects on bacteria, in a biofilm enriched in sulfate reducers (Mohanakrishnan *et al.* 2011). Even not linked to specific bacterial genera, Fe(III) addition to wastewater has previously been shown to influence sulfate reduction and methane production in sewer biofilms at laboratory scale (Zhang *et al.* 2009). The cause for this was not clear, but the authors discussed the inhibition of activity due to metal sulfide deposits on the cells, or taking place at a molecular level, with deactivation of enzymes, denaturing of proteins or competition with other essential cations used by the microorganisms. The authors furthermore rejected the hypothesis that Fe(III) is used preferentially to sulfate as electron acceptor, which otherwise has been shown in sediments where electron donors are limited, but as they point out, electron donors are not limited in sewers (Zhang *et al.* 2009).

There are hence many unresolved issues regarding the microbial communities of force main biofilms. The objective of this study is to examine whether sulfide abatement in force mains with salts of Fe(II) and Fe(III) influence the activity of sewer biofilms operated at realistic conditions and how this correlates to the microbiome as analyzed by 16S rDNA sequencing. These changes are looked at with an emphasis on sulfate-reducing bacteria and methane-producing archaea.

MATERIALS AND METHODS

Force mains setup and operation

Three pilot scale force mains were constructed, consisting of three identical PE 80 pipes of pressure grade PN 10 (Uponor, Glostrup, Denmark) (Table 1 and Figure 1). Each main had a length of 300 m and an outside diameter of 50 mm. At distances of 0, 100, and 200 m, the force mains had valves to extract wastewater samples and could be dismantled to allow biofilm sampling. The starting position (0 m) was defined as five pipe diameters downstream of the point of chemical injection, where prior computational fluid dynamics modelling had shown that high turbulence ensured rapid and complete mixing.

The setup was installed in a sewer research and monitoring station in Frejlev, Denmark. The station receives domestic wastewater from a combined sewer system of approximately 2,000 population equivalents. At regular intervals, a pneumatically controlled seat valve let wastewater from the sewer system into a 0.150 m^3 settling tank. From the settling tank, the wastewater overflowed into a Grundfos sololift CWC-3 pump (Grundfos A/S, Bjerringbro, Denmark) which transferred it to a reservoir with an active volume of 0.175 m^3. This reservoir served as a common pump well for the three force mains, which was further connected to a Grundfos SEV.65.65.40.2 centrifugal wastewater pump (Grundfos A/S, Bjerringbro, Denmark) speed-

Table 1 | Characteristic dimensions and flow conditions for the force mains

Number of force mains	3	–
Length per force main	300	m
Inside diameter of force main	40.8	mm
Operation time	5.3–6.5	min (pump cycle)$^{-1}$
Number of pump cycles per day	7	–
Hydraulic retention time at force main discharge	7.7	h
Distance pumped per cycle	133	m
Flow velocity in force main	0.53–0.65	m s^{-1}
Wall shear stress	1.4–1.9	N m^{-2}
Average pH	8.2 ± 0.1	–

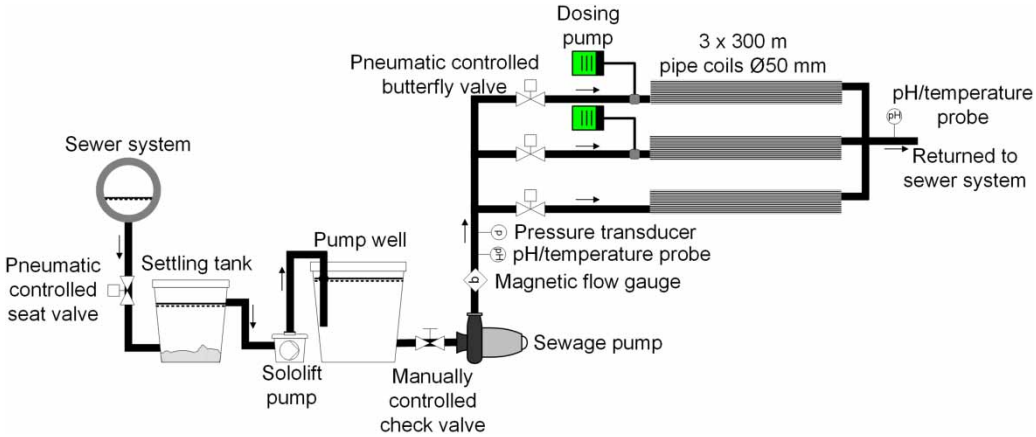

Figure 1 | Schematic illustration of the force mains with three lines receiving different treatments.

controlled by an Invertek Optidrive E2 variable frequency converter (Invertek Drives Ltd, Welshpool, UK). The pump was operated in on–off mode, controlled by impulses from two float switches. Time proportional chemical dosing was performed using Grundfos DDC 15-4 digital dosing pumps (Grundfos A/S, Bjerringbro, Denmark). Dosing with 5.15 w/w% ferrous sulfate (Dankalk, Løgstør, Denmark) and 14 w/w% ferric chloride ($FeCl_3$) (Brenntag Nordic A/S, Ballerup, Denmark) was performed on line 2 (0.53 L $FeSO_4$-solution m^{-3} wastewater) and line 3 (0.15 L $FeCl_3$-solution m^{-3} wastewater) respectively, while line 1 was operated as a non-treated reference. Wastewater was pumped through one line at a time. After a pumping sequence where all three lines had been active, a three-hour intermission was applied before the pumping sequence was repeated, allowing for seven pumping cycles per day which were run at regular intervals. Iron dosages were estimated based on the empirical equation for sulfide production proposed by Nielsen *et al.* (1998).

Design of measuring campaign

Prior to the measuring campaign, the force mains had been operated for half a year where a mature biofilm was allowed to develop on the interior pipe walls. Dosing was initiated 2 months prior to sampling, to allow the biofilms to adapt to these conditions. The measuring campaign was intended to characterize mature biofilms in terms of their activity to consume and form key substances and map the corresponding microbial community. A destructive sampling was performed to achieve this, where biofilms were scraped out of the pipe and one part analyzed for activity and another part for its microbiome. The amount of biofilm that could be obtained was small compared to the amount required for the various tests and the number of repetitions per location was hence limited to one.

Biofilm and wastewater sampling

Wastewater was collected in the settling tank before the force mains and filtered through a 1.2 μm Labsolute® glass fiber filter (Th.Geyer, Renningen, Germany). The pH was subsequently adjusted to 7.5 using hydrochloric acid or sodium hydroxide, and the filtrate was kept overnight at 4 °C. Biofilm samples were collected by dismantling the force mains and scraping it off from the inside of the pipes with a rubber spatula. The collected biofilms were covered with filtered wastewater and kept overnight at 4 °C.

Biofilm and wastewater characterisation

Iron, sulfur, copper, zinc, and calcium in the biofilms were analyzed by inductively coupled plasma optical emission spectroscopy (Thermo iCap 6300 Duo operated in radial view mode; Thermo Fisher Scientific Inc., MA, USA). Biofilm samples were digested in 33.5% HNO_3 for 3 days at room temperature. Before measurements, samples were diluted to 10% HNO_3, and 1 ppm yttrium was added as internal standard. Iron was measured at 238.204, 239.562, and 259.940 nm, sulfur at 180.731, 182.034, and 182.624 nm, copper at 224.700, 324.754, and 327.396 nm, zinc at 202.548, 206.200, and 334.502 nm, calcium at 393.366, 396.847, and 422.673 nm and yttrium at 371.030 nm. Standards and nitric acid were of high purity and traceable to the National Institute of Standards and Technology (NIST) (SCP-Science, Courtaboeuf, France).

Soluble chemical oxygen demand (COD) and nitrate were measured in wastewater from all lines at 0, 100, and 200 m. Wastewater samples were drawn off the force mains into serum bottles immediately after a pumping event. Samples for COD were filtered through a 0.45 μm nylon syringe filter, and measured spectrophotometrically using a Hach Lange COD cuvette test kit (Hach Lange Gmbh, Düsseldorf, Germany). Samples for nitrate were filtered through a 0.22 μm cellulose acetate 25 mm syringe filter and measured using ion chromatography as described by Rudelle *et al.* (2016).

Activity measurement of suspended biofilms

For activity measurements, 8 mL of wet biofilm was homogenized in a tissue grinder. Following homogenization, biofilms were suspended in temperate filtered wastewater in a 500 mL serum bottle flushed with high purity nitrogen gas (5.0). A volume corresponding to 15.3% of the total volume in the bottle was left as headspace. Yeast extract and molasses were added to a final concentration of 0.5 g L^{-1} each. The bottle was placed on a magnetic stirrer in a water bath kept at room temperature to minimize temperature variations. Samples for measuring volatile fatty acid (VFA) and sulfate were drawn from the liquid phase every 30 min. for a duration of 12 hours. The samples for analysis of VFA and sulfate were filtered through a 0.22 μm CA 25 mm syringe filter. Samples for methane quantification were taken from the headspace with an air-tight syringe and transferred to a vacuum vial. The volume lost during sampling was compensated for with injection of high purity nitrogen gas (5.0). At termination of the experiment, pH was measured and a sample was taken for determination of total solids (TS) and volatile solids (VS) (APHA 1995). Sulfate, VFA and methane were measured as described by Rudelle *et al.* (2016).

For characterizing the activity of the suspended biofilms, the overall consumption rate of sulfate and the overall formation rate of VFA and methane were calculated and normalized to VS (mol substance $(g\ VS)^{-1}\ minute^{-1}$).

Correlation analysis with biofilm microbiome

A portion of the freshly sampled biofilms from each line at each of the three positions was used for microbiome analysis and prepared directly after collection. Biofilms were centrifuged to remove excess wastewater and stored in DNA/RNA ShieldTM (Zymo Research, Irvine, CA, USA) at −18 °C until DNA extraction in triplicates using the Quick-DNA™ Fecal/Soil Microbe Microprep kit (Zymo Research, Irvine, CA, USA). The 16S rDNA V3 + V4 regions were amplified and subsequently subjected to Illumina MiSeq 250 base pair paired-end sequencing. Demultiplexed datafiles from the MiSeq were processed using the DADA2 pipeline (Callahan *et al.* 2016). A sequence identity of 97% was used for clustering into OTUs (operational taxonomic units). Chimeras were removed and the resulting sequences were classified against the RDP_train_set_16 database. Before analysis OTUs with a relative abundance at ≤0.005% across all samples were filtered away to reduce background noise in the dataset. Analysis of microbiome composition and Spearman rank correlations between chemical parameters and microbiome data of selected OTUs related to sulfide and methane production were performed in R (version 3.3.2 (R Core Team 2016)) and R studio (version 1.1.383 (RStudio Team 2015)) using the phyloSeq v.1.19.1 (McMurdie & Holmes 2013), metagenomeSeq v.1.16.0 (Paulson *et al.* 2013), ggcorplot v. 0.1.1, and ggplot v.2 2.2.1 (Wickham 2009) packages.

RESULTS AND DISCUSSION

Chemical composition of the biofilms

Iron treatment of the force mains changed the chemical composition of the biofilms compared to the untreated control (Figure 2). Compared to the untreated line, iron was found in high concentration in the treated lines, and so was sulfur, indicating that significant sulfide production and hence precipitation took place in the mains. Similar accumulations of these elements were observed for Fe(III) treated biofilms grown on carriers in a laboratory reactor setup using wastewater (Zhang *et al.* 2012). Visual inspection during biofilm sampling of the iron treated lines suggested that FeS must have accounted for at least some of the iron and sulfur, as the biofilms were completely black. Of the three lines the highest iron and sulfur concentrations were found in the Fe(II) treated line. However, despite the difference in concentrations the iron to sulfur ratio was approximately equal for the Fe(II) and Fe(III) treated lines, with a value around 1.4 mol Fe $(mol\ S)^{-1}$ at all distances. This implies that in the iron treated lines, iron accumulated in the biofilms to an extent exceeding the stoichiometric ratio which FeS would suggest. On which forms these elements more specifically were present was not investigated and thus their chemical availability unknown. This additional binding of iron agrees with

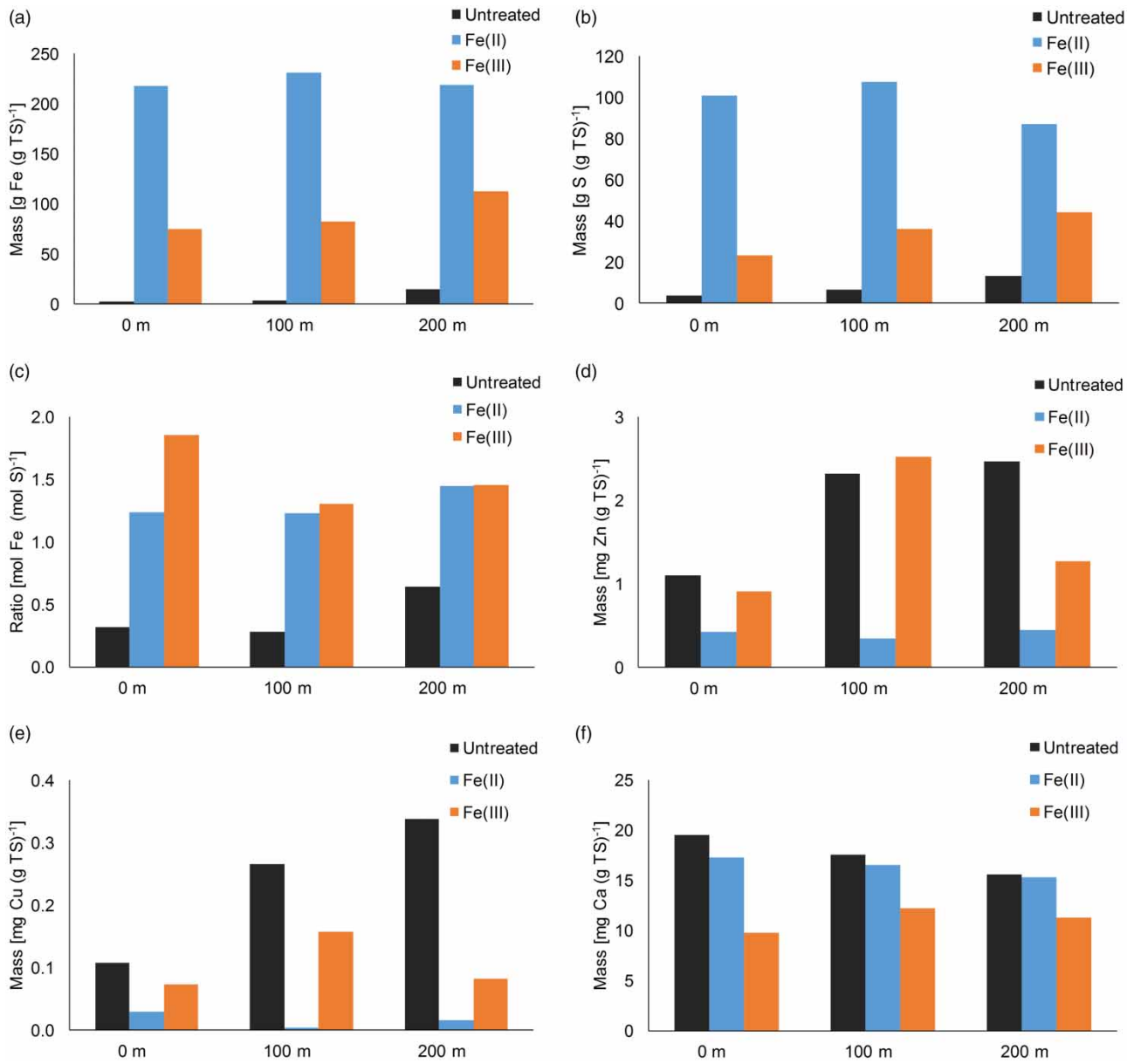

Figure 2 | Elements in biofilms normalized to TS. (a) Iron; (b) sulfur; (d) zinc; (e) copper; (f) calcium. (c) The calculated ratio of iron to sulfur.

previous studies, which have shown that extracellular polymeric substances (EPS) of biofilms could cause biosorption of cations from the solution. Bindings of various cations thus showed differences for an *Escherichia coli* biofilm, where Fe(III) showed a higher affinity for the EPS than did Cd(II), Ni(II), and Cr(VI) (Quintelas *et al.* 2009). EPS structures of biofilms from other bacterial strains have also been shown to have different metal adsorption properties, which may be due to differences in functional groups but also other factors such as adsorption affinity and metal ion concentration (Li & Yu 2014). Compared to the iron treated lines, iron and sulfur levels in the untreated line were significantly lower, as was the ratio between the two elements. Values ranged from around 0.25 mol Fe $(\text{mol S})^{-1}$ at 0 and 100 m to 0.5 mol Fe $(\text{mol S})^{-1}$ at 200 m, suggesting that the produced sulfide primarily was released to the bulk water phase in contrast to accumulating within the biofilms as iron sulfide.

In the treated lines, the levels of zinc and copper were significantly lower than those of iron: 0.0018 mg Zn $(\text{mg Fe})^{-1}$ and 0.00007 mg Cu $(\text{mg Fe})^{-1}$ in the Fe(II) treated line and 0.0175 mg Zn $(\text{mg Fe})^{-1}$ and 0.0012 mg

Cu (mg Fe)$^{-1}$ in the Fe(III) treated line. In the untreated line, levels of zinc and iron were comparable while copper was 1–2 magnitudes lower. Only zinc and iron could hence have played a role for metal sulfides in the untreated line. Zinc and copper concentrations were both lower in the Fe(II) treated line compared to the untreated and Fe(III) treated lines. Calcium was markedly lower in the biofilm of the Fe(III) treated line, whereas the calcium contents in the Fe(II) and untreated lines were similar.

Among other cations, calcium and Fe(III) are both known to be associated with EPS for stability of biofilms and sludge flocs in wastewater treatment (Park *et al.* 2006; Turakhia *et al.* 1983). The interactions of cations with EPS can be in the form of metal ligand complexation or ion exchange with anionic functional groups, but also surface precipitation of metal hydroxides may play a role (Li & Yu 2014). Addition of Cu(II) or Mg(II) to activated sludge showed a release of Ca(II) from sludge flocs indicating competition for binding sites (Bruus *et al.* 1992). However, the low copper content measured in the biofilms does not alone seem to be able to explain the reduced Ca content in the Fe(III) treated biofilms. Due to a higher charge valence, contribution of trivalent cations to floc stability is thought to be of great importance (Park *et al.* 2006) and it has furthermore been shown that Fe(III) forms stronger bonds with EPS than Ca(II) (Li *et al.* 2012; Nielsen & Keiding 1998). In activated sludge this can additionally be assumed to take place for low concentrations of Fe(III) and it has been shown that Ca(II) could not substitute the Fe(III) binding sites before Fe(III) was selectively removed from the flocs (Nielsen & Keiding 1998). This is further substantiated by the finding that an increase in $FeCl_3$ dosing can lead to an increase of sludge floc EPS iron content while the content of monovalent and divalent cations decreases (Li 2005).

In the Fe(II) treated biofilms, the Ca content was equal to that of the untreated biofilms. This might be because Fe(II) has the same valence as Ca(II) and does not have a higher affinity for EPS, which is in line with findings of cation exchange in groundwater (Appelo & Postma 2005). Thus Fe(II) could not substitute Ca(II) in the EPS matrix. Furthermore it has been shown that Fe(II) can have a destabilizing effect on sludge floc stability, at least in cases where Fe(III) is reduced biotically or abiotically within activated sludge flocs (Nielsen & Keiding 1998). For the abiotic reduction, destabilization was explained by FeS colloids being negatively charged (Nielsen & Keiding 1998), which is also the case for the functional groups of the EPS at neutral pH (Li & Yu 2014).

Wastewater composition

Soluble COD in the untreated and Fe(II) treated lines was around 200 mg L^{-1} at 0 m, decreasing to 175 and 165 mg L^{-1} at 100 and 200 m, respectively. Soluble COD in the Fe(III) treated line was 140 mg L^{-1} at all distances and thus significantly lower than in the other lines (one-way analysis of variance, significance level 0.05). This reduction of COD corresponded well to the fact that Fe(III) is a common coagulant in water treatment for removal of dissolved and soluble COD (Duan & Gregory 2003). Treatment with Fe(III) did hence change the substrate availability for the microbial community of the biofilms.

Oxygen was measured in the pump well over the course of 2 days to 0.122 ± 0.004 mg L^{-1} in the inlet to the pump. Nitrate was found in the Fe(III) treated line at 0 m (1.88 ± 0.22 mg L^{-1}) and in trace concentration at 100 (0.11 ± 0.01 mg L^{-1}) and 200 m (0.18 ± 0.01 mg L^{-1}), whereas it could not be detected in the other two lines.

The reason for the presence of nitrate in the Fe(III) treated line is uncertain. The most likely explanation is that the used PIX111 contained beside $FeCl_3$ some nitrate without it being stated in the data sheet. Another, less likely, explanation could be that the batch of wastewater pumped into the Fe(III) treated line contained nitrate, while the batches pumped into the other lines did not. While this is theoretically possible as wastewater constituents can change rapidly (Gudjonsson *et al.* 2002), it does not seem very likely. Furthermore, nitrate is normally only found in wastewater at low concentrations (<1 g m^{-3}) (Henze & Comeau 2008; Hvitved-Jacobsen *et al.* 2013).

Assuming that the nitrate was present in the PIX111 used, this could affect the bacterial community in the first part of the force main. The energy gained by using Fe(III) as electron acceptor is lower than when coupling degradation of organic matter to nitrate (Hvitved-Jacobsen *et al.* 2013). However, microbial systems do not always behave as a simple function of available energy. For example in a study of competition for electron donors in anoxic paddy soils, concomitant usage of the electron acceptors nitrate and Fe(III) was demonstrated when electron donors were in excess, which also is the case in sewers (Zhang *et al.* 2009). In contrast to this, inhibition of nitrate reduction has been reported for a variety of ferric (hydro)oxides (Coby & Picardal 2005) and Liu *et al.* (2014) demonstrated that a stronger inhibitory effect took place with higher availability of Fe(III), attributing it to larger ferric oxide surface areas and less crystallinity. Nitrate reduction by the non-iron-reducing bacterium *Paracoccus denitrificans* has also

been shown to be inhibited in cells treated with Fe(II). This was argued to be due to an Fe-enriched coating of the cells (Coby & Picardal 2005).

Microbiome composition and correlation with chemical parameters

Sequencing of bacterial DNA of biofilms from the three lines led to identification of 581 unique OTUs after filtration for low abundant OTUs. These OTUs roughly correspond to bacterial species, where one or more of the identified OTUs possibly can relate to the same genera. Within these OTUs, 16 (21) phyla, 24 (56) classes, 33 (68) orders, 59 (135) families, and 97 (236) known (unknown) genera were represented. Both ferric and ferrous treatment of the force mains affected the microbiome composition of the biofilm compared to the untreated line (Figure 3(a)) and among the 97 identified genera, 49 were found to be significantly different between the lines. As sulfide and methane formation are processes of concern in force mains, specific

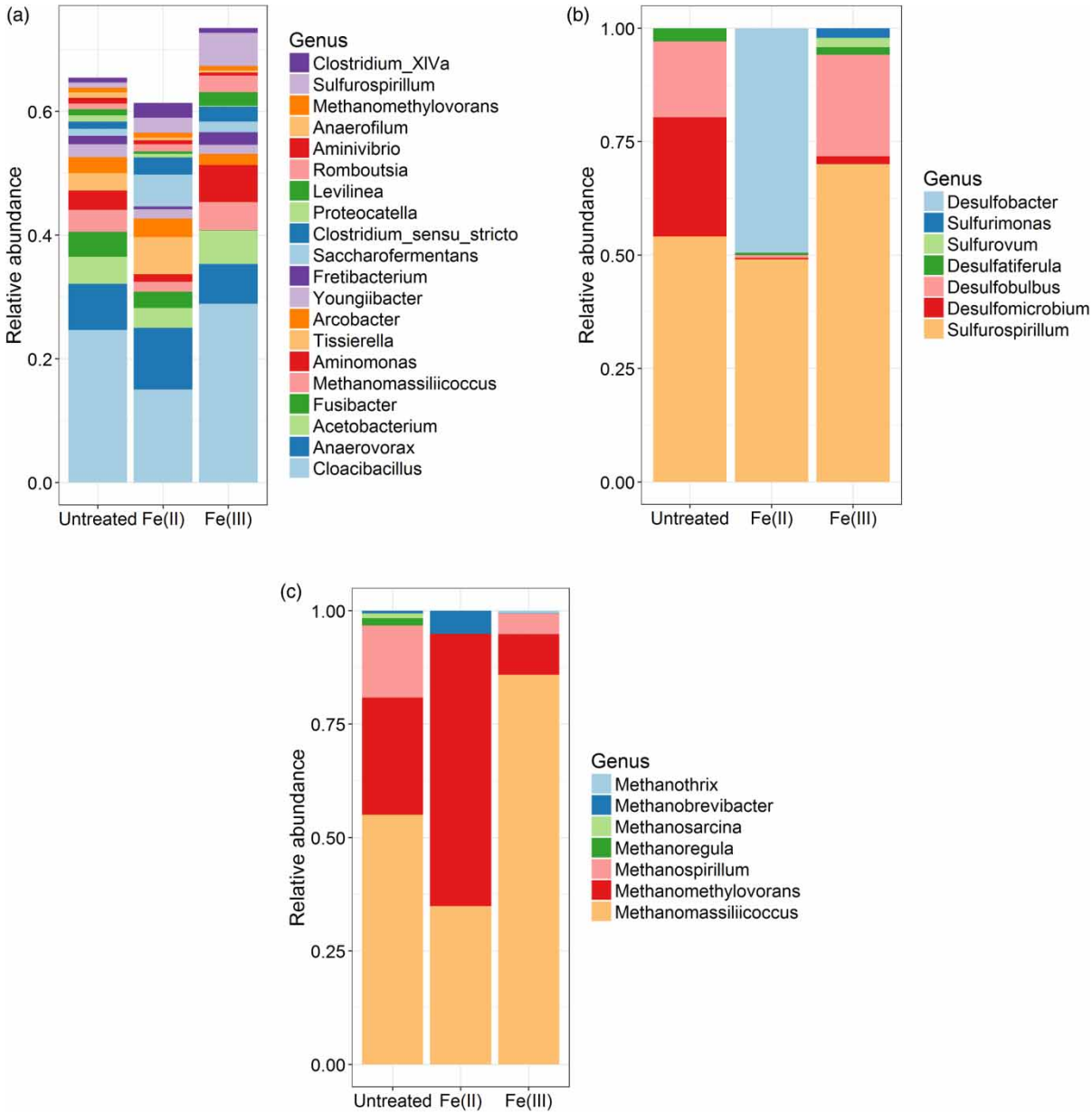

Figure 3 | Taxonomic summary plot showing relative abundance of (a) the 20 most abundant genera, and genera related to sulfide (b) and methane (c) production for the treatments. The abundance of each genus is calculated as the sum of OTUs at all distances. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wst.2018.261.

emphasis was given to OTUs related to these issues. OTUs with the prefix *desulfo-* and *sulfu-* in the genus name – i.e. bacteria capable of sulfate or elemental sulfur reduction – were chosen as representatives for sulfide-producing organisms. The OTUs with the prefix *methano* – i.e. methanogens – were selected as methane-forming organisms.

In the untreated line, the OTU-counts related to sulfide producers were lower than the counts for methane producers, whereas in the iron treated lines this relationship was reversed (3.04; 0.71; 0.76 (*methano-* OTUs (sulfide-related OTUs)$^{-1}$)) for the untreated, Fe(II) and Fe(III) lines respectively). The total counts for OTUs related to sulfide production were found to be in the same range for the untreated and Fe(II) treated lines, whereas the number of counts in the Fe(III) treated line was three times higher. The total counts of OTUs related to methane production in the Fe(II) treated line was around one-third of the counts found in the untreated and Fe(III) treated lines. In the two latter lines the counts for OTUs related to methane production were almost similar.

The distribution of bacterial genera related to sulfide production showed great variations between lines (Figure 3(b)), where *Sulfurospirillum*, *Desulfomicrobium* and *Desulfobulbus* dominated the untreated biofilms. The Fe(II) treated biofilms were dominated by *Sulfurospirillum* and *Desulfobacter*, whereas *Sulfurospirillum* and *Desulfobulbus* dominated in the biofilms of Fe(III) treated line. Figure 3(c) shows that OTUs related to *Methanomassiliicoccus*, *Methanospirillum* and *Methanomethylovorans* dominated the untreated and Fe(III) treated biofilms, whereas *Methanomassiliicoccus, Methanomethylovorans*, and *Methanobrevibacter* dominated the Fe(II) treated biofilms. For the sulfide and methane producers, *Desulfobacter*, *Desulfobulbus, Sulfurospirillum, Methanomethylovorans*, *Methanomassiliicoccus*, *Methanospirillum*, and *Methanoregula* were among the genera that were significantly different between the lines.

The measured chemical parameters were correlated with the abundance of the OTUs related to sulfide and methane production identified in the biofilms from all treatments and at all positions (Figure 4).

For the genera related to sulfide production, the abundances of most of the *Sulfurospirillum* OTUs, together with a group of three *Desulfobulbus* OTUs, were positively correlated to nitrate and negatively correlated with calcium, which indicates that these OTUs would be present in the Fe(III) treated line (Figure 4(a)). Two of these *Desulfobulbus* and the OTU of *Sulfurimonas* were furthermore negatively correlated with COD. *Desulfobacter* OTU_98 correlated positively with COD as the only OTU of this genus and additionally it exhibited a positive correlation with iron and sulfur. A positive correlation with these elements was also observed for OTU_584, OTU_290 and OTU_113. *Desulfobacter* OTU_98 and OTU_584 had negative correlation with copper and zinc, and only zinc, respectively.

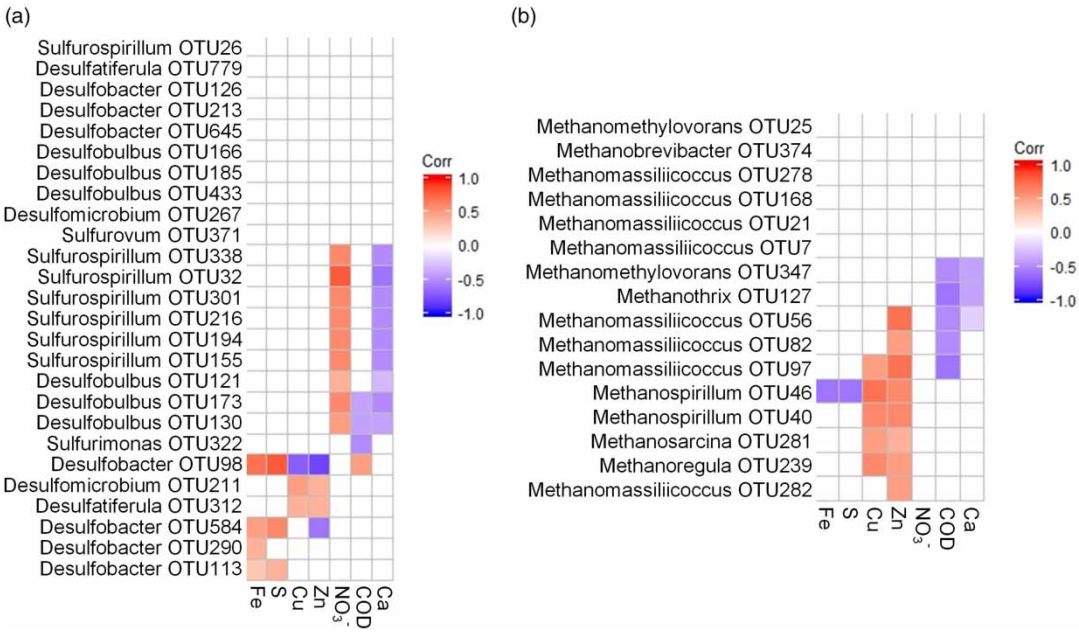

Figure 4 | Spearman Rank correlations of OTUs related to sulfide (a) and methane (b) with chemical parameters of the biofilms and wastewater. The Spearman rank correlation coefficients are color-coded from perfect negative correlation (blue) to perfect positive correlation (red). Only correlations with a p-value <0.05 are shown. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wst.2018.261.

This is opposite to the OTUs representing *Desulfomicrobium* and *Desulfatiferula*, which exhibited positive correlation with these elements.

A group of the OTUs of *Desulfobacter* correlated positively with iron and sulfur, which were present in high concentrations in the biofilms of the Fe(II) treated line compared to the other two lines. However, elevated concentrations of these two elements were also present in the Fe (III) treated line. But the presence of *Desulfobacter* was not detected here. This pattern agrees with the fact that *Desulfobacter* is inhibited by elemental sulfur (Garrity 2005), which could be a product of sulfide precipitation using Fe(III).

Genus-specific effects of sulfate reducers due to nitrate availability in wastewater have previously been demonstrated by Mohanakrishnan *et al.* (2011), and are also observed in this study. Here there is a positive correlation of most *Sulfurospirillum* and some of *Desulfobulbus* OTUs towards nitrate, which makes sense for *Sulfurospirillum* as different species have the ability to utilize nitrate as electron acceptor (Garrity 2005). As nitrate was solely present in the Fe(III) treated line, this might explain the positive correlation. Beside conserving energy from nitrate reduction, *Sulfurospirillum barnesii* is known to also conserve energy from Fe(III) reduction. Different *Sulfurospirillum* species can additionally use elemental sulfur as electron acceptor (Dworkin 2006). *Desulfobulbus* species mostly use sulfate as electron acceptor but have moreover been shown to disproportionate sulfur in the presence of Fe(III) (Rosenberg 2014a). *Sulfurospirillum* exhibited a significant upregulation in the Fe(III) treated line compared to the untreated control, which was most pronounced at 0 m (data not shown). *Desulfobulbus* OTUs fell into two groups. Some OTUs exhibited correlation patterns matching conditions found in the Fe(III) treated line, whereas other OTUs were uncorrelated to any of the measured parameters. This suggests that the latter *Desulfobulbus* OTUs could be present in all three lines. This shows that treatment with Fe(III) or the presence of nitrate might favor different *Desulfobulbus* species and change the community towards species more likely to use higher energetic electron acceptors.

The difference in correlation and thus the presence of different OTUs in the lines correspond to earlier findings where the diversity of biofilm communities grown on waste tire rubber carriers changed under varying regimes of Fe(III) dosing (Sharafat *et al.* 2018). Additionally, Ca(II) addition has been shown to change the microbial community in activated sludge flocs, with a resulting change in EPS production (Ye *et al.* 2016). A change in EPS structures due to variability of the microbial community could also serve as a part explanation for the lower content of Ca(II) in the Fe(III) treated biofilms, where other OTUs seemed to be favored, compared to the untreated and Fe(II) treated line.

Correlation of OTUs related to methane production (Figure 4(b)) showed that *Methanomassiliicoccus* for most of the OTUs was positively correlated with zinc. One of these OTUs furthermore showed a positive correlation with copper, as did the three genera *Methanospirillum*, *Methanosarcina*, and *Methanoregula*, all belonging to the class of *Methanomicrobia*. *Methanospirillum* OTU_46 showed a strong negative correlation with iron and sulfur, which agrees well with the fact that this genus was not observed in the Fe(II) treated line (Figure 3) and that this specific OTU had a lower abundance in the Fe(III) treated line compared to the untreated line. A negative correlation with COD and calcium was observed for a group of *Methanomassiliicoccus*, the OTU related to *Methanothrix* and *Methanomethylovorans* OTU_347.

Activity potential of the biofilms

The activity potential of the biofilms was investigated in batch cultures. Such conditions are different from bacterial cells living in biofilms where substrate diffusion and interspecies cooperation affect which cells are active. In a planktonic state, interactions with other cells are transitory and interspecies cooperation is lacking (Costerton *et al.* 1994). Suspension of the biofilms for activity measurements hence provided the bacterial cells with altered conditions. OTUs found in great numbers in the biofilms may ultimately not have been active in the batch experiments, if these were dependent on interspecies transfer of metabolic products for optimal growth. Additionally, the carbon sources used for the batch experiments, yeast extract and molasses, may have favored some bacteria over others (Nielsen & Hvitved-Jacobsen 1988). With these caveats in mind, the activity measurements are hence only indicative of which processes actually took place in the *in situ* biofilms.

The activity potential for fermentation was the most significant of the addressed microbial processes. This was followed by sulfate respiration and lastly by methane formation (Figure 5). Looking at the overall trend of the measured parameters for the sampling positions on the force mains, biofilms from the untreated line showed a greater growth potential than the two iron treated lines.

In most cases, VFA formation was lower in the iron treated lines than in the untreated (Figure 5). Production of

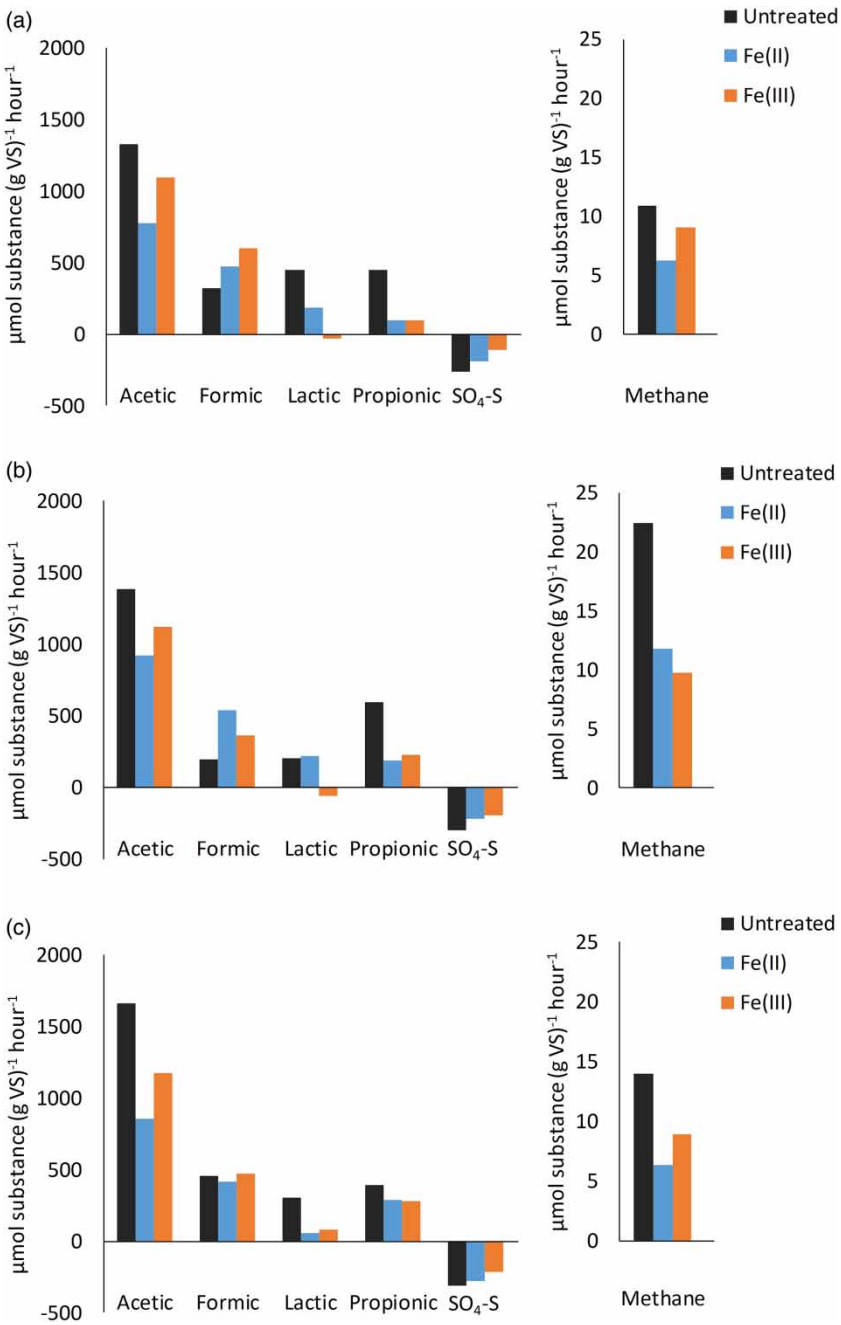

Figure 5 | Net production of volatile fatty acids, sulfate and methane from suspended growth of biofilms in batch experiments at (a) 0 m, (b) 100 m, (c) 200 m from start of the force main. Methane is shown in its own graph as the rates were 1–2 orders of magnitude lower than those of fermentation and sulfate respiration.

lactic and propionic acids was most affected, while production of formic acid was the least affected. The net production of methane in the biofilms from the Fe(III) and Fe(II) treated lines was lower than that of the untreated control. Sulfate reduction was affected negatively in the Fe(II) and Fe(III) treated biofilms compared to the untreated control. In the Fe(III) treated line this could be because the microbial community had adapted to using either Fe(III) or nitrate as electron acceptor and that sulfate-reducing organisms hence were outcompeted in the biofilms. The inhibition of sulfate reduction by Fe(III) is in line with findings of Zhang *et al.* (2009), who observed a similar effect in a laboratory-scale biofilm reactor. However, OTU-counts of bacteria related to sulfide were found in the Fe(III) treated

biofilms in great numbers. Another explanation could be that the OTUs related to sulfide production in the Fe(III) treated line needed to adapt their metabolism from using Fe(III) or nitrate as electron acceptor to using sulfate. This shift may have caused a delay in sulfate reduction activity compared to the other two lines.

Parameters measured in the batch growth experiments of the suspended biofilms were correlated to the microbiome of the biofilms from the force mains. Correlations with OTUs related to sulfide and methane production can be seen in Figure 6.

Figure 6(a) shows that all *Sulfurospirillum* OTUs except OTU_26 correlated negatively with lactic acid and exhibited a positive correlation with formic acid. *Desulfobulbus* OTU_173 also showed a negative correlation towards lactic acid formation, whereas *Desulfobulbus* OTU_433 correlated positively herewith, indicating that different species were favored by the different treatments. *Desulfobacter* (OTU_98 and OTU_113) exhibited reverse correlation with acetic acid formation compared to *Desulfatiferula* OTU_312 and *Desulfomicrobium* OTU_211, with the first being negatively correlated and the latter two being positively correlated.

This pattern corresponds well to *Desulfobacter*, which performs complete oxidation of acetate to CO_2, which is used as sole electron donor for this genus (Garrity 2005). It would therefore be expected that acetic acid levels will be low in environments where *Desulfobacter* dominates. *Sulfurospirillum barnesii*, which performs incomplete oxidation of lactic acid using, for example, Fe(III), nitrate or S^0 as electron acceptors (Dworkin 2006), sits well with the observed activity measurements and correlation patterns. In the presence of *Sulfurospirillum*, lactic acid concentrations would be low and consequently elevated levels of formic acid would be expected, unless it is quickly used as substrate by, for example, methanogenic archaea.

For the genera related to methane production, the correlation pattern for *Methanomassiliicoccus* was very diverse for the different OTUs (Figure 6(b)). Some OTUs showed negative correlation with lactic acid (OTU_82, OTU_282), others with formic acid (OTU_7, OTU_168), and yet others showed positive correlation with acetic acid (OTU_97, OTU_21). OTU_21 and OTU_168 furthermore exhibited positive correlation with propionic acid, and the latter together with OTU_7 showed mixed weak positive and weak negative correlation with sulfate reduction and

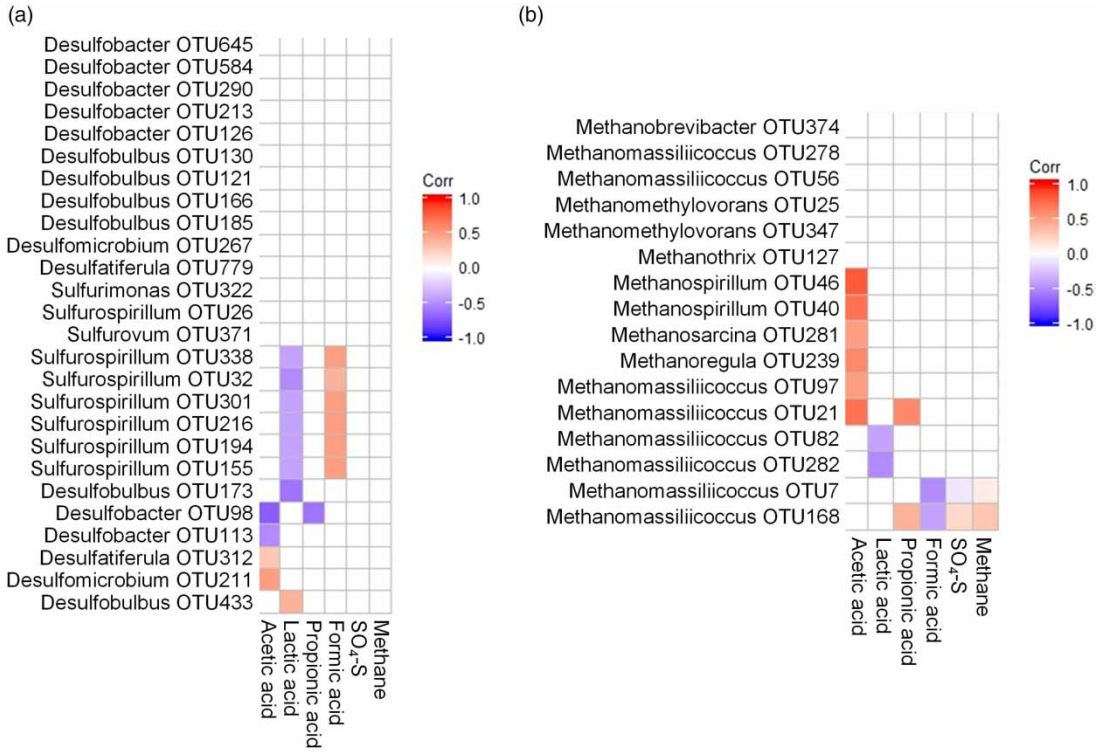

Figure 6 | Spearman Rank correlations of OTUs related to sulfide (a) and methane (b) with parameters measured in the batch experiment. The Spearman rank correlation coefficients are color-coded from perfect negative correlation (blue) to perfect positive correlation (red). Only correlations with a *p*-value <0.05 are shown. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wst.2018.261.

methane formation. Even though *Methanomassiliicoccus* was abundantly present in all lines (Figure 3), it indicates that the different treatments favored diverse species of this genus. As seen previously (Figure 4(b)), genera of the class *Methanomicrobia* exhibited correlation towards the same compounds. In the batch experiments *Methanospirillum* (OTU_46, OTU_40), *Methanosarcina* (OTU_281), and *Methanoregula* (OTU_239) all showed positive correlation with acetic acid.

Coexistence of sulfate-reducing and methane-producing microorganisms in the same sewer biofilms has previously been demonstrated by Guisasola *et al.* (2008) and the difference observed for the methanogens might be coupled to the differences within the sulfate-reducing communities. The dominant species in the Fe(II) treated biofilms (Figure 3(b)) were *Methanomethylovorans* and *Methanomassiliicoccus*, which utilize dimethyl sulfide/methylated amines/methanol and H_2-CO_2/methanol respectively for methane production (Rosenberg 2014b). The dominance of these genera could possibly be coupled with the presence of *Desulfobacter*, which, as stated earlier, performs complete oxidation of acetate and thereby does not form substrate for methanogenic archaea that utilize formic acid as electron donor. In the two other lines where incomplete oxidation of VFA by the sulfate reducers took place, the growth of other methanogenic genera could be stimulated, e.g. *Methanospirillum* which is able to use, beside H_2-CO_2, formic acid for methanogenesis (Rosenberg 2014b).

An inhibition of OTUs related to methane production compared to the sulfide-producing bacteria was observed in both the iron treated lines. Even though the Fe(III) lines had the greatest counts of methanogenic archaea, the ratio between *methano-* OTUs (sulfide-related OTUs)$^{-1}$ was lower in both the iron treated lines compared to the untreated line. This was consistent with the findings of the activity measurement, which showed a reduction in both sulfate reduction and methane production in these lines.

CONCLUSION

Beside the main purpose of precipitating sulfide in force mains, treatment with Fe(II) and Fe(III) changed the microbial community significantly and also the activity of the biofilms. How much the presence of nitrate in the Fe(III) treated line affected this change compared to pure Fe(III) treatment cannot be deduced from this study. The microbial community of sulfate-reducing bacteria and methanogenic archaea in the lines showed significant differences and were each dominated by different genera (Figure 3). Treatment with Fe(II) and Fe(III) did also impose changes on the overall microbial community. This is concluded based on the number of significant different genera represented in the biofilms, and further reflected in an altered production of VFAs in the batch experiments. Another key finding was that both Fe(II) and Fe(III) seemed to have had an inhibitory effect on both sulfate reduction and methane production. As the inhibition observed was not specific for the high energetic electron acceptor (Fe(III)), it cannot be concluded whether this was due to a shift in the microbiome or due to the effect previously proposed by Zhang *et al.* (2009), where metal sulfide deposits or deactivation of enzymes caused this inhibition.

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AUTHOR CONTRIBUTIONS

B.K., A.H. and J.V. conceived and designed the experiments; B.K. and P.K. performed the experiments; B.K. and P.K. analyzed the data; B.K. wrote the paper; P.K., A.H. and J.V. revised the paper.

CONFLICTS OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; and in the decision to publish the results.

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