

# Effects of aerobic–anaerobic transient conditions on sulfur and metal cycles in sewer biofilms

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## ABSTRACT

Interactions between sulfur and metals were studied in aerobic and anaerobic biofilms grown on domestic waste water at 15 °C. The dominant metals in the waste water were iron, zinc and copper, which were present in average concentrations of 0.5 mg/l, 0.6 mg/l and 0.1 mg/l, respectively. Copper and zinc were found to accumulate in the anaerobic biofilm owing to precipitation of metal sulfides. Iron supplementation (approximately 5.5 mg Fe/l) to the anaerobic waste water reduced the zinc and copper precipitation due to sulfide precipitation with iron. However, even at these elevated iron concentrations in the waste water, sulfide precipitation in the biofilm was controlled largely by zinc and copper. In the aerobic biofilm, addition of iron resulted in accumulation of iron and phosphate, probably owing to precipitation of iron phosphates and iron (oxy)hydroxides. The potential importance of extracellular polymeric substances (EPS) on metal sorption in sewer biofilms was studied. EPS consisted mainly of proteins (13–260 mg/(g volatile solids)) and to a lesser extent of carbohydrates (8–26 mg/(g volatile solids)). The EPS composition remained relatively constant during experimental runs, but differed significantly between them. No relationships between the metal content of the biofilm and the amount of extracted EPS were found, which suggests that EPS did not play a major role in the metal accumulation.

## INTRODUCTION

Build-up of hydrogen sulfide (H<sub>2</sub>S) in the atmosphere of sewers is associated with various problems including odor, corrosion and health-related aspects (Boon, 1995). Sulfide in sewers is produced mainly when anaerobic conditions prevail and sulfate is used as the terminal electron acceptor for organic matter degradation (Postgate, 1984; Vincke *et al.*, 1999). The biofilms covering the submerged sewer walls and sewer sediments are the main sites for sulfate reduction in sewer networks (Boon, 1995; Vincke *et al.*, 1999). Several potential sinks for dissolved sulfide exist within the biofilms. The sulfide may react with metals to produce insoluble metal sulfides (Morton *et al.*, 1991). The sulfide may also be oxidized chemically or biologically if aerobic regions exist (Norsker *et al.*, 1995). When the bulk water contains dissolved oxygen (DO), the outer layers of the biofilm will generally be aerobic (Norsker *et al.*, 1995). An internal sulfur cycle has been observed in aerobic biofilms grown on domestic waste water (Kühl & Jørgensen, 1992; Lens *et al.*, 1995a; Okabe *et al.*, 1998). If the sulfide production exceeds its potential sinks, sulfide will enter the flowing waste water. Analogous to the reactions in the biofilms, the sulfide may be oxidized or react with metals within the bulk water (Nielsen *et al.*,

2003). In addition, hydrogen sulfide can be released from the waste water, potentially resulting in a hydrogen sulfide build-up in the sewer atmosphere.

Other exchange processes between the biofilm and the bulk water may also be of significance for the sulfur cycle in the biofilm. Metals may accumulate in the biofilm owing to entrapment of precipitates such as metal (oxy)hydroxides or metal phosphates (Lieher, 1995). The precipitated metals can subsequently be involved in reactions of the sulfur cycle. Metals may also accumulate in the biofilm owing to binding to negatively charged functional groups within the biofilm; i.e. cell walls, cell membranes, cell cytoplasm and extracellular polymeric substances (EPS) can serve as sorption sites in biofilms (Flemming, 1995; van Hullebusch *et al.*, 2003). EPS typically contain a large number of anionic functional groups and may have a significant metal-binding capacity. EPS have therefore been suggested to be important in determining the metal sorption properties of biofilms (Flemming, 1995).

Only a few studies of metals in sewer biofilms have been conducted (e.g. Gutekunst, 1988). The importance of metals in the sulfur cycle in sewer biofilms is therefore essentially unidentified. Domestic waste water typically contains a range of heavy metals in low concentrations, i.e. in the µg/l range (Henze *et al.*, 2002; Hvitved-Jacobsen,

2002). Iron and zinc are typically the most abundant metals, present in concentrations of 0.3–5 mg/l (Morton *et al.*, 1991; Flores-Rodríguez *et al.*, 1994; Henze *et al.*, 2002). Both metals react readily with sulfide to produce insoluble metal sulfides (Stumm & Morgan, 1996). Accordingly, iron and zinc are expected to be important metals in the sulfur cycle of sewer biofilms. Okabe *et al.* (1998) found metal sulfide precipitation to be an important sink for sulfide in aerobic biofilms grown on waste water from a municipal waste water treatment plant. The metal sulfide precipitation was ascribed entirely to precipitation with iron. However, no measurements of metals were reported and other metals may have been important as well.

The objective of the present work was to study interactions between sulfur and various metals in sewer biofilms, with particular emphasis on effects of the DO (aerobic versus anaerobic waste water), iron and sulfate in the waste water. Sulfur and metal interactions in the biofilm matrix were studied by characterizing the chemical composition of the biofilm matrix using a sequential extraction scheme and an EPS extraction scheme to characterize the inorganic and organic parts of the biofilm matrix, respectively. Interpretation of the element concentrations extracted in the sequential extraction scheme permits identification of different chemical forms of the elements present in the biofilm. Accordingly, a sequential extraction scheme was used to gain new insight into the significance of metals in the sulfur cycle in sewer biofilms.

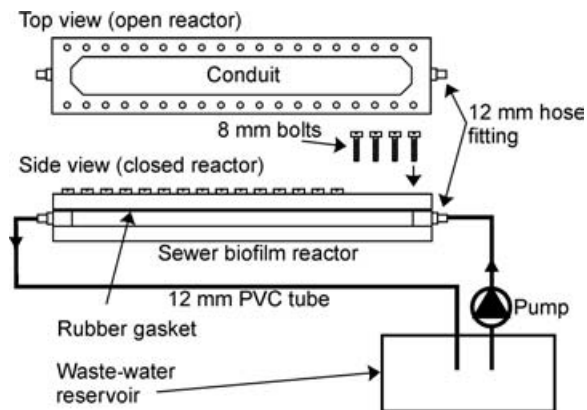
## MATERIALS AND METHODS

### Waste-water sampling

Waste-water samples (25 liters) were collected at the influent pumping station at Bennekom Municipal Waste-water Treatment Plant (The Netherlands). The treatment plant serves approximately 22 000 person-equivalents and the waste water is without industrial inputs. The sewer catchment of Bennekom is a combined sewer system and thus receives both domestic waste water and surface run-off. Samples were collected between 09:00 a.m. and 10:30 a.m. during dry weather flow conditions from 11 August to 16 December 2003.

### Biofilm flow cells

The sewer biofilms were cultivated in two identical flow cells as depicted in Fig. 1. Each flow cell was made of polyvinylchloride (PVC) and constructed as a conduit with a rectangular cross-section and tapered ends. The internal dimensions were 2 cm × 5 cm × 50 cm and the volume of each flow cell was 0.39 liter. The top of the flow cells could be removed, thereby providing access to the biofilm covering the interior walls. The biofilm flow cells were circulating waste water from a reservoir using Totton NEMP 50/7 centrifugal pumps (Totton Pumps, England) with polypropylene (PP) pump housing and ceramic spindles to avoid possible interference from metallic parts. The average liquid velocity in the flow



**Fig. 1:** Experimental set-up for studying interactions between sulfur and metals in sewer biofilms; for further explanation, see the text.

cells ( $\pm$  standard deviation) was 30 ( $\pm$  3) cm/s, which is considered sufficient to prevent deposition of the organic solids in the waste water (Tchobanoglous, 1981). The reservoirs were placed in a water bath thermostated at 15 ( $\pm$  0.5) °C and the waste water was changed at 48 h intervals. The waste water in the aerobic flow cell set-up was constantly aerated (DO = 4 ( $\pm$  1) mg/l) in the reservoir using compressed air (20 l/h). Anaerobic waste water was prepared by storing freshly collected waste water at room temperature for 1–2 h. Before replacing the waste-water feed, depletion of DO in the waste water was verified.

### Biofilm growth and experimental runs

Effects of perturbations of growth conditions on sulfur and metal interactions in sewer biofilms were studied in two experimental runs (I + II). Prior to runs I and II, the biofilms were grown aerobically in series, circulating waste water from a 25 liter reservoir for 65 and 16 days, respectively. This was done to ensure that, when experiments were started, the biofilms had reached an overall steady-state condition with respect to biofilm thickness and composition. Between the two experimental runs, the flow cells were cleaned using hot water and a brush.

In run I, effects of changing from aerobic to anaerobic growth conditions were investigated. Accordingly, one flow cell was fed aerobic waste water from a constantly aerated 10 liter reservoir (Ia), while the second flow cell was fed anaerobic waste water from a closed 10 liter reservoir with no aeration (Ib). Thus experiment Ia was not perturbed and served as a blank control in which fluctuations of element concentrations and EPS composition reflected the natural variation due to the variability of the waste-water composition. Run I was terminated after 8 days.

In run II, the two flow cells were operated in parallel in order to study the effects of increased iron and sulfate concentrations in the waste water on aerobic (IIa) and anaerobic (IIb) biofilms. Iron and sulfate was added to the waste water as ferric chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , Merck, Germany) and di-sodium sulfate ( $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ , Merck,

Germany) in concentrations of approximately 5 mg Fe/l and 50 mg S/l, respectively. Such concentrations are frequently found in high-strength waste water and in waste water with industrial inputs (e.g. Holder *et al.*, 1984; Morton *et al.*, 1991). Both flow cells were first fed iron-supplemented waste water for a period of 5 days, followed by a period of 5 days with both iron and sulfate supplemented to the waste water.

### Sampling and biofilm characterization

Biofilm samples were harvested using a plastic spatula. When samples were taken, it was attempted to obtain them intact over the entire biofilm depth in order to have an equal representation of inner and outer parts of the biofilm. Samples were obtained from areas near the center line of the flow cell where the biofilm was of uniform thickness.

The average biofilm thickness was measured regularly by determination of the weight of the flow cells after the biofilms had drained for 5 min (Lens *et al.*, 1995b). By subtracting the dry weight of the clean flow cell and assuming a density of the wet biofilm of 1.00 g/cm, the biofilm volume was calculated. The average biofilm thickness was calculated by dividing the biofilm volume by the biofilm area. The interior of the flow cells was not completely covered by biofilm; biofilm sampling, sloughing and turbulent flow conditions at the inlet resulted in uncovered spots. The biofilm area was therefore estimated visually from digital images of the biofilm.

### Sequential extraction procedure

Sequential extractions were performed with approximately 1 g of wet biofilm in four steps, according to the sequential extraction scheme developed by Tessier *et al.* (1979) with slight modifications (Osuna *et al.*, 2004). The efficiency of the scheme was tested on BCR (Commission of the European Communities Bureau of Reference) reference materials by van Hullebusch *et al.* (2005). Table 1 summarizes extraction reagents and conditions in the applied sequential extraction scheme. The extractions were performed on a shaker and were initiated immediately after sampling. After each extraction step, the samples were centrifuged for 10 min at 10 000 revolutions/min

**Table 1:** Sequential extraction scheme applied in the experiments (Osuna *et al.*, 2004)

Extraction step	Extraction reagent	Extraction time	Temperature
I	10 ml NH <sub>4</sub> CH <sub>3</sub> COO (1 M, pH = 7)	1 h	20 °C
II	10 ml CH <sub>3</sub> COOH (1 M, pH = 5.5)	1 h	20 °C
III	5 ml H <sub>2</sub> O <sub>2</sub> (30%, pH = 2)	3 h	35 °C
IV	10 ml Aqua Regia (HCl/HNO <sub>3</sub> , 3 : 1)	50 min	Microwave oven

(r.p.m.). The supernatant was filtered and stored for multi-element analysis. The pellet was subsequently resuspended in the extraction reagent for the next extraction step. In extraction step IV, the pellet was transferred to a Teflon vessel, resuspended in 10 ml Aqua Regia (37% HCl : 65% HNO<sub>3</sub>, 3 : 1) and digested at 160 °C for 40 min using a Milestone Ethos Microwave Labstation. Extracts from steps I to III were acidified (140 mM HNO<sub>3</sub>) to ensure that the extracted metals remained in solution.

### Multi-element analyses

Multi-element analyses of Ca, Co, Cu, Fe, Mn, Ni, P, S and Zn were performed on biofilm extracts from the sequential extractions and on waste-water samples digested in Aqua Regia by microwave-assisted digestion. The multi-element analyses were performed using a Varian Vista-MPX Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). Two sets of matrix-matched (140 mM HNO<sub>3</sub> and 20% Aqua Regia) standards and blanks for ICP-OES were prepared in 18.2 MΩ/cm Millipore water. Standards were prepared using CertiPUR element standards (Merck, Germany). Spike recovery of yttrium was used for quality control in all blanks, standards and samples. Glassware (Duran®, Schott, Germany) for multi-element analyses was acid washed in 4 M HNO<sub>3</sub> for 24 h and subsequently rinsed in Millipore water prior to use.

### EPS extraction

EPS were extracted using a cation-exchange resin (Dowex 50 × 8, Na<sup>+</sup> form, 20–50 mesh, Fluka 44445) according to the method of Jahn & Nielsen (1995) with slight modifications. Dewatered biofilm (1 g, 5 min) was mixed with 10 g Dowex 50 × 8 and 25 ml extraction buffer (2 mM Na<sub>3</sub>PO<sub>4</sub>, 4 mM NaH<sub>2</sub>PO<sub>4</sub>, 9 mM NaCl, 1 mM KCl, pH 7.0) and stirred for 2 h at 4 °C in a cylindrical beaker at 900 r.p.m. using a magnetic stir bar. After the extraction, the suspension was centrifuged at 12 000 g for 15 min and the supernatant analyzed for carbohydrate and protein.

Carbohydrate was determined in the EPS extracts using the phenol–sulfuric acid method of Dubois *et al.* (1956), with glucose (Merck, Germany) as standard. Protein was measured according to the Lowry method using bovine serum albumin (Fraction V) (Sigma, UK) as standard (Lowry *et al.*, 1951).

### Analytical techniques

DO was measured using a PreSens Microx TX 2 fiber optic oxygen meter in combination with a needle-type fiber optic oxygen sensor (PreSens GmbH, Germany). The pH was measured using a Hamilton Polylite Pro pH-electrode (Hamilton Company), the oxidation–reduction potential (ORP) was measured using a WTW Sentix platinum–silver/silver chloride ORP electrode (WTW Measurement Systems Inc.). ORP measurements are referred to with the standard hydrogen electrode as a reference ( $E_H$ ). Sulfate (SO<sub>4</sub><sup>2-</sup>) was analyzed by ion chromatography using a

Dionex AS 17 column at a temperature of 30 °C. The eluent was KOH: 1 mM for 1.5 min to 20 mM in 3.5 min and from 20 to 40 mM in 2 min at a flow rate of 1.5 ml/min. The background eluent conductivity was suppressed using a Dionex ASRS-ULTRA suppressor. Sulfide was measured according to the methylene blue method (APHA *et al.*, 1995). Total solids (TS) and volatile solids (VS) and chemical oxygen demand (COD) were analyzed according to standard methods (APHA *et al.*, 1995).

## RESULTS

### Waste-water composition

The pH and temperature of the waste water from Bennekom were 7.9 ( $\pm 0.2$ ) and 15.9 ( $\pm 3.0$ ) °C, respectively. Concentrations of  $\text{SO}_4^{2-}$ , COD, TS and VS of the waste water were measured 15 times on independent waste-water samples. Average ( $\pm$  standard deviation) concentrations of  $\text{SO}_4^{2-}$ , COD, TS and VS in the waste-water samples were 29.4 ( $\pm 2.1$ ) mg S/l, 562 ( $\pm 151$ ) mg/l, 726 ( $\pm 59$ ) mg/l and 352 ( $\pm 81$ ) mg/l, respectively. Total concentrations of Ca, Cu, Fe, P, S and Zn in the waste water are summarized in Table 2. Several other metals of potential importance in the sulfur cycle were also analyzed, including Co, Mn and Ni, but were present only in very low concentrations ( $< 50$   $\mu\text{g/l}$ ). They were therefore omitted in the further research of this study.

The sulfate analyses gave results that were on average 12% higher than the total sulfur concentration ( $n = 15$ ). Waste water from sewers typically contains organically bound sulfur at 3–6 mg S/l (Boon, 1995). In addition, the initial sulfide concentration of the waste water was generally less than 0.2 mg S/l. The total sulfur concentration was therefore expected to be 10–20% higher than the sulfate concentration. The reason for this discrepancy is not known.

### Biofilm thickness

During biofilm cultivation, the mass of biofilm in the flow cells grew linear. Within a period of 14 days, the biofilm thickness was approximately 600  $\mu\text{m}$  and reached an apparent steady-state condition. Visual inspections revealed that the biofilm was of uniform thickness near the center line on the top and the bottom of the flow cells and that a thick biofilm ( $> 1000$   $\mu\text{m}$ ) developed on the sidewalls

and in the corners. This was probably due to lower flow velocities and corresponding lower shear forces in these areas.

The average ( $n = 17$ ) concentrations of TS and VS of the biofilms were ( $\pm$  standard deviation) 60.7 ( $\pm 14.5$ ) mg/(g wet biofilm) and 35.3 ( $\pm 5.1$ ) mg/(g wet biofilm), respectively. The TS and VS of the biofilms were relatively constant and there was no significant difference between the two flow cells.

### Effect of DO concentration of waste water (experimental run I)

The ORP of the waste water was generally between  $-100$  and  $-220$  mV in the anaerobic flow cell and above 200 mV in the aerobic flow cell. Batch experiments showed that sulfide production in the waste water started when the ORP was reduced below approximately  $-50$  mV (data not shown). The DO concentration of the waste water in the aerobic flow cell was between 3 and 6 mg  $\text{O}_2/\text{l}$ , depending on the strength of the waste water. Thus the DO concentration was not limiting for the activity of the aerobic heterotrophic biomass (Vollertsen *et al.*, 1999).

Sequential extractions of biofilms fed aerobic and anaerobic waste water (experimental run I) are presented in Fig. 2. In both the aerobic and the anaerobic biofilm, zinc was extracted mainly in steps II and III ( $> 90\%$ ) and copper was extracted almost exclusively in steps III and IV ( $\approx 100\%$ ). In the aerobic biofilm, the zinc concentrations in the different extraction steps remained relatively constant, while the copper concentrations were subject to some variability in step III. The most significant effect of switching from aerobic to anaerobic waste water was an increase in concentration of copper in step III and of zinc in steps II and III. During 8 days of anaerobic conditions, the copper concentration in step III increased from 0.3 to 1.4 mg Cu/(g volatile solids) and the zinc concentrations in steps II and III increased from 0.9 to 3.9 mg Zn/(g volatile solids) and 2.0 to 4.0 mg Zn/(g volatile solids), respectively.

Iron was extracted primarily in the residual fraction (step IV) in both the aerobic and anaerobic biofilm (Fig. 2). In the aerobic biofilm, the iron concentrations in all extraction steps were relatively constant. In the anaerobic biofilm, the iron concentration in step IV was initially higher than that in the aerobic biofilm, but decreased during the experiment to a similar concentration.

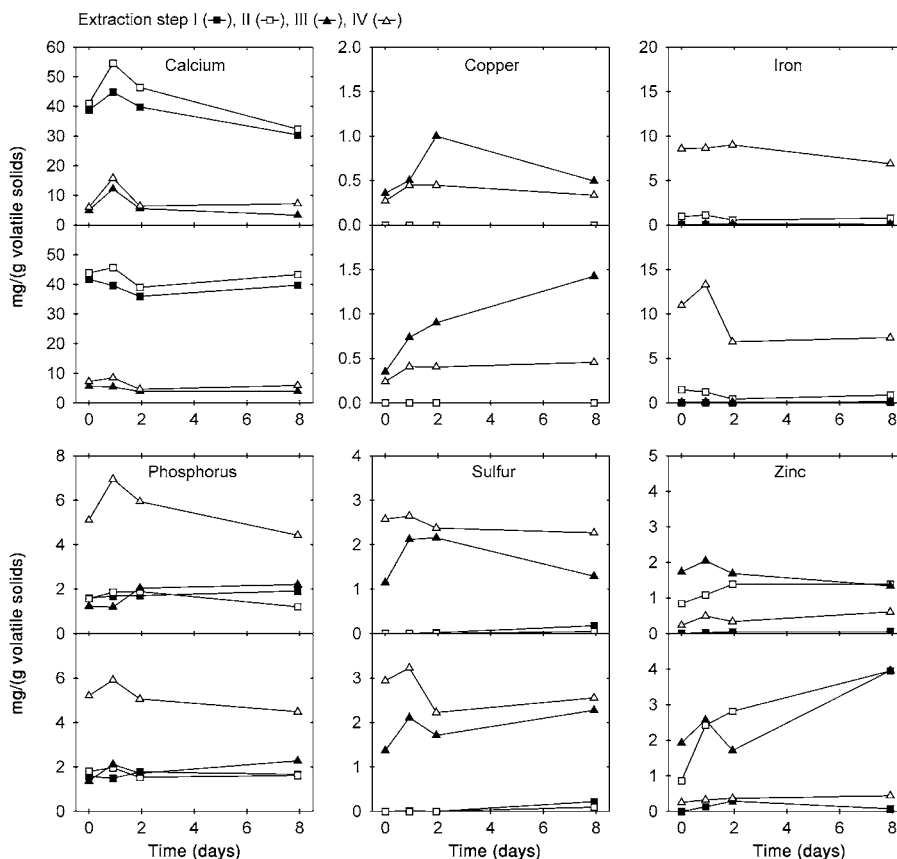
Sulfur was predominantly extracted in steps III and IV (Fig. 2). In the aerobic biofilm, the sulfur concentration in step III increased from 1.2 to 2.1 mg S/(g volatile solids) during the first day, but decreased again to the initial level when the experiment was terminated. In the anaerobic biofilm, the sulfur concentration in step III increased from 1.4 to 2.3 mg S/(g volatile solids) during the experiment, whereas the sulfur concentration in step IV decreased from 2.9 to 2.6 mg S/(g volatile solids).

In both biofilms, significant amounts of phosphorus were extracted in all extraction steps (Fig. 2). However, the major part ( $\approx 50\%$ ) of the phosphorus was extracted in step IV. The phosphorus concentrations in the different

**Table 2:** Concentrations of sulfur, phosphorus and selected metals in waste-water samples from Bennekom, the Netherlands

Element	Average <sup>a</sup> (mg/l)	Standard deviation <sup>a</sup> (mg/l)
Ca	47.4	6.9
Cu	0.1	0.0
Fe	0.5	0.2
P	4.9	0.4
S	26.1	0.8
Zn	0.6	0.4

<sup>a</sup> $n = 15$ .

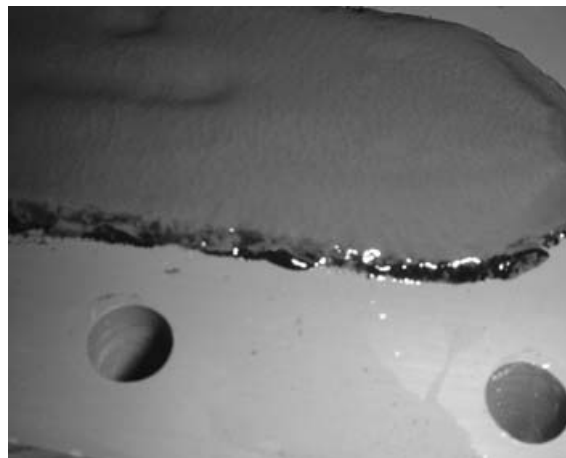


**Fig. 2:** Sequential extractions of two 65 day-old biofilms fed aerobic (top) and anaerobic (bottom) domestic waste water (experimental run I).

extraction steps were similar in both biofilms. Calcium (>80%) was extracted mainly in steps I and II. During the experiment, the total calcium concentration decreased from 90.6 to 73.1 mg Ca/(g volatile solids) in the aerobic biofilm and from 98.0 to 92.7 mg Ca/(g volatile solids) in the anaerobic biofilm.

Visual inspections revealed that both biofilms appeared similar upon the start of experimental run I. They were characterized by a pale brown filamentous surface and a distinct black coloring near the substratum (Fig. 3). After conditions were changed from aerobic to anaerobic, it was observed that black spots developed in the anaerobic biofilm matrix. In run Ib, the change from aerobic to anaerobic waste water resulted in a major sloughing of the biofilm approximately 8 days after the change in feeding. The experiment was therefore discontinued and a new biofilm was grown for run II.

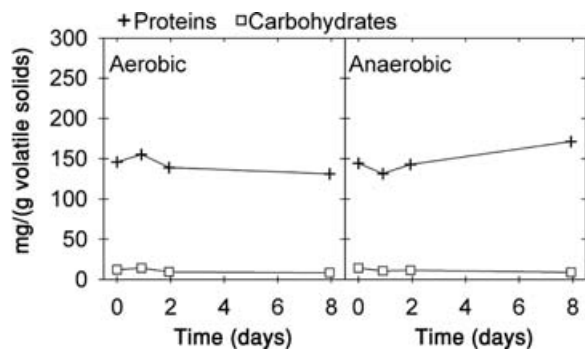
The EPS extractions (Fig. 4) showed that proteins were the main component extracted. The initial concentrations of proteins and carbohydrates in the two biofilms were similar at approximately 145 and 15 mg/(g volatile solids), respectively (Fig. 4). During the experiment, the concentrations remained relatively constant in both biofilms. The protein concentration increased slightly in the anaerobic biofilm, while it decreased slightly in the aerobic biofilm, but the changes were not statistically significant.



**Fig. 3:** A 65 day-old biofilm with an average thickness of approximately 600  $\mu\text{m}$ , grown on aerobic ( $\text{DO} = 4(\pm 1) \text{ mg O}_2/\text{l}$ ) waste water. A blackening of the deep biofilm layers is clearly visible on the edges of the biofilm.

### Effects of waste-water composition (experimental run II)

Fig. 5 summarizes the metal analyses of the biofilm extracts from experimental run II. Concentrations of sulfate and iron in the supplemented waste water were



**Fig. 4:** Protein and carbohydrate concentrations in EPS extracts from the aerobic and anaerobic biofilms of experimental run I.

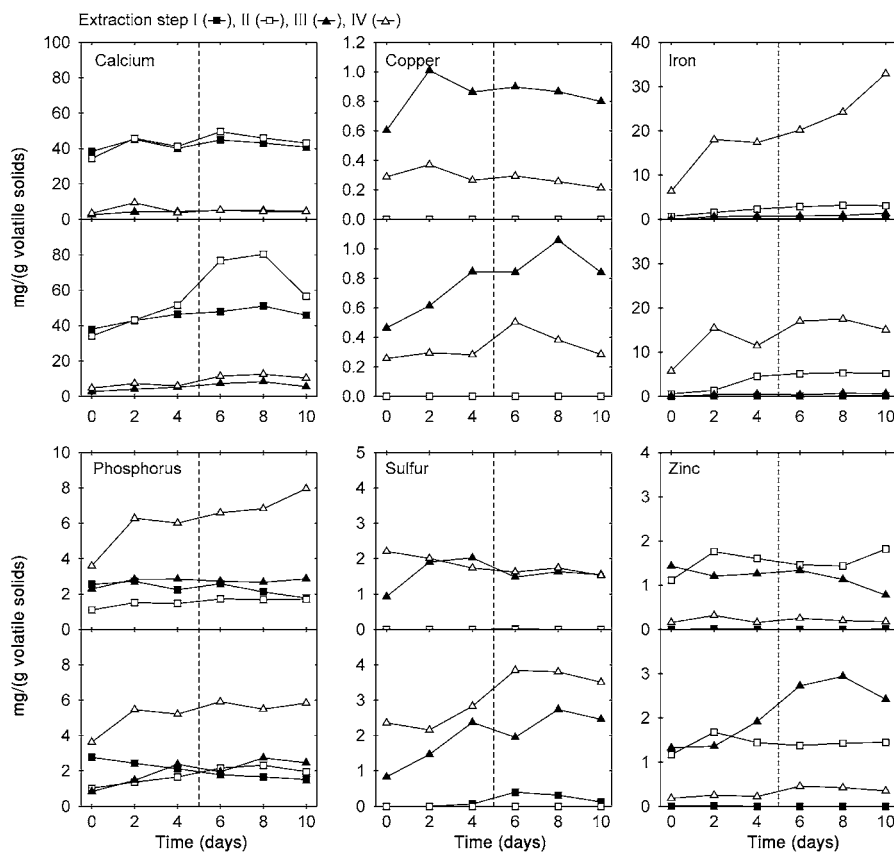
82.8 ( $\pm 4.5$ ) mg S/l and 5.35 ( $\pm 0.30$ ) mg Fe/l, respectively. These corresponded to 282% and 1054% increases in the *in situ* water-phase concentrations of sulfate and iron, respectively. The concentrations of extracted elements in both biofilms at the start of the experiment ( $t=0$ ) were comparable to the concentrations at start of experimental run I and within the range of concentrations of the blank control (experiment Ia).

The biofilm-fed aerobic waste water responded rapidly to the iron addition, which resulted in increased amounts of iron and phosphate in the residual fraction (extraction

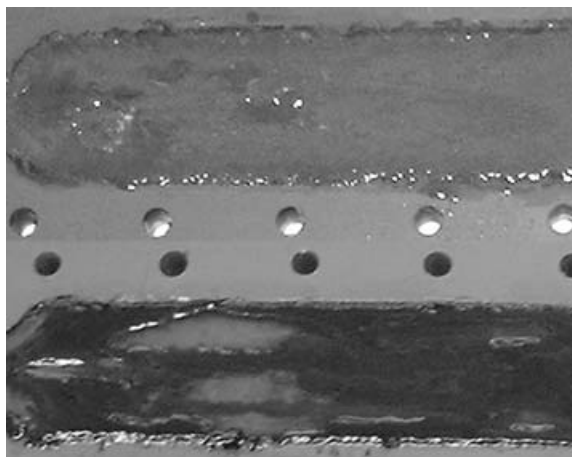
step IV). Over the 10 days of the experiment, the total iron content of the aerobic biofilm accumulated from 7.0 to 37.5 mg Fe/(g volatile solids) at an average rate of 3.0 mg Fe/(g volatile solids) per day.

In the aerobic biofilm, the zinc concentration increased in extraction step II and decreased in step III, but the total amount of zinc extracted remained stable at approximately 2.9 g Zn/(g volatile solids). The copper concentration in step III increased rapidly during the first 2 days, but stabilized thereafter at 0.8 mg Cu/(g volatile solids) (Fig. 5). A similar, but slower, increase in copper extracted in step III was observed in the anaerobic biofilm, which increased from 0.4 to 0.8 mg Cu/(g volatile solids) over 10 days. In the anaerobic biofilm, the zinc concentration in step III increased during the experiment from 1.3 to 2.4 mg Zn/(g volatile solids) (Fig. 5). This was similar to the anaerobic biofilm in experiment Ib (Fig. 2). However, the increased zinc concentration in extraction step II during experiment Ib was not observed when iron and sulfate were added to the waste water.

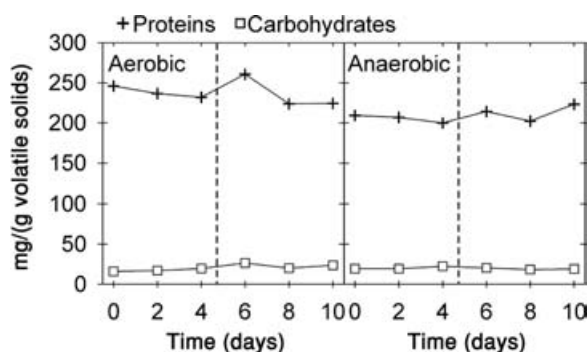
The extracted sulfur remained relatively constant in the aerobic flow cell also after sulfate was added to the waste water (Fig. 5). In the anaerobic biofilm, the sulfur concentration increased from 0.8 to 2.5 mg S/(g volatile solids) and 2.4 to 3.5 mg S/(g volatile solids) in extraction steps III and IV, respectively. This contrasts with experiment Ib, where the sulfur concentration in step IV did not increase.



**Fig. 5:** Sequential extractions of 16 day-old aerobic (top) and anaerobic (bottom) biofilms fed iron- and sulfate-supplemented waste water (experimental run II). Iron was added throughout the experiment whereas sulfate addition started 5 days into the experiment, as indicated by the dashed vertical line.



**Fig. 6:** Comparison between aerobic (top) and anaerobic (bottom) biofilm after being fed iron-supplemented waste water for 5 days; for further explanation, see the text.



**Fig. 7:** Protein and carbohydrate concentrations in EPS extracts from the aerobic and anaerobic biofilms fed iron- and sulfate-supplemented waste water (experimental run II). Iron-supplemented waste water was fed throughout the experiment and sulfate addition started after 5 days, as indicated by the vertical dashed line.

When sulfate-supplemented waste water was fed to the biofilm flow cells, the calcium concentration in extraction step II of the anaerobic biofilm increased considerably from 51.5 to 76.7 mg Ca/(g volatile solids), but the concentration decreased again to 56.6 mg Ca/(g volatile solids) when the experiment was terminated. Apart from this effect, no other significant effects attributable to the addition of sulfate to the waste water were observed in the sequential extractions.

Visually it was observed that the surface of the biofilm changed from pale brown to black when ferric chloride was added to the anaerobic waste water, while the biofilm fed aerobic waste water did not change color (Fig. 6).

The EPS extractions from run II are presented in Fig. 7. Both the extracted protein and carbohydrate fractions remained relatively constant in both biofilms during run II. Generally, more EPS was extracted compared with the EPS extractions from experimental run I. Initial concentrations of proteins and carbohydrate in the aerobic and the anaerobic biofilm were 246 and 210 mg protein/(g volatile solids) and 16 and 19 mg carbohydrate/(g volat-

ile solids), respectively (Fig. 7). Again, the protein concentration increased slightly in the anaerobic biofilm and decreased slightly in the aerobic biofilm.

## DISCUSSION

### Biofilm growth and characterization

The organic matter content of the biofilms was comparable with literature values of aerobic biofilms grown on domestic waste water (Raunkjær *et al.*, 1997). However, the inorganic matter content was relatively high. Raunkjær *et al.* (1997) found that the VS accounted for 77–83% of the TS in a biofilm grown in a rotating drum biofilm reactor operated with a peripheral flow velocity of 0.5–0.7 m/s. In this study, the VS concentration was on average 58% of the TS concentration of the biofilm. The higher inorganic content of the biofilm may have resulted from deposition of inorganic solids owing to the lower flow velocities applied in this study. In addition, this study used raw waste water while Raunkjær *et al.* (1997) used pre-settled waste water, which may have reduced the inorganic content significantly. The steady-state biofilm thickness of approximately 600  $\mu\text{m}$  is considered to be relatively low for an aerobic sewer biofilm, which typically exceeds 1000  $\mu\text{m}$  in thickness (Norsker *et al.*, 1995).

The presence and development of populations of sulfate-reducing bacteria (SRB) in biofilms grown on aerobic waste water has been well documented (Norsker *et al.*, 1995; Okabe *et al.*, 1998; Santegoeds *et al.*, 1998). Although SRB were not determined by microbial or molecular ecology tools in the present study, the presence of an active population of SRB in the biofilms is strongly indicated by the distinct black layer of the deep biofilm regions (Figs. 3 and 6) and the blackening of the anaerobic biofilms (experimental runs Ib and IIb). The black color probably resulted from the accumulation of metal sulfide precipitates, for example FeS and CuS, in the biofilm following sulfide production by SRB. This is a well-known colorization and is used in diagnostic tests for SRB (Postgate, 1984; Lens *et al.*, 1995a). The sulfide production in the waste water observed in batch experiments (data not shown) demonstrated that SRB were indeed present and active in the waste water and thus most probably served as the inoculum for the biofilm SRB population.

The sloughing that was observed in the anaerobic biofilm in run I was probably related either to anaerobic gas production within the biofilm or to the aerobic/anaerobic transient conditions causing mineralization of the deeper biofilm layers (Characklis *et al.*, 1990). A similar phenomenon has been reported in the literature for an undefined biofilm when growth conditions were changed from aerobic to anaerobic (Characklis *et al.*, 1990).

### Characterization of the extracted organic phase – EPS

The concentrations of proteins and carbohydrates in the EPS extractions are in reasonable agreement with previously reported values for sewer biofilms using the

same extraction method. Jahn & Nielsen (1995) extracted 154 mg protein/(g total organic carbon) and 12 mg carbohydrate/(g total organic carbon) from a gravity sewer biofilm. Assuming a VS/TOC ratio of 0.55 in accordance with Henze *et al.* (2002), this corresponds to 280 mg protein/(g volatile solids) and 22 mg carbohydrate/(g volatile solids).

EPS has been shown to effectively complex various heavy metals (Rudd *et al.*, 1984). In addition, metals may accumulate in the biofilm owing to entrapment of insoluble metal precipitates in the EPS. The quantity of EPS extracted remained virtually constant during the experimental runs, but were the highest in run II (Figs. 4 and 7). The differences in extracted EPS might be due to the age of the biofilms (Wuertz *et al.*, 2000), as the biofilms in runs I and II were 65 and 16 days old, respectively. The initial concentrations of metals were generally equal in the two experiments, i.e. there was no relationship between the amount of EPS extracted and the metal concentrations. Recent studies indicate the significance of EPS for sorption of metals to be smaller than was estimated in earlier studies. Wuertz *et al.* (2000) found that zinc accumulated on the cell surfaces rather than in the EPS of activated sludge as well as in aerobic biofilms when 100 ml of 0.1–4 mM  $\text{ZnSO}_4 \cdot 5\text{H}_2\text{O}$  were added to 10 g of sample. Correspondingly, Späth *et al.* (1998) found that more than 80% of cadmium and zinc were sorbed by the cellular fraction and the remaining part was bound to EPS in an undefined aerobic biofilm spiked with different cadmium and zinc concentrations ranging from 0.4 to 4.0 mM.

### Characterization of the inorganic solid phase – sequential extraction

In the sequential extraction scheme, elements were extracted according to the lability of the phase with which they were associated. It must be noted that the fractions of the sequential extraction scheme are operationally defined, and do not represent single chemical species. In the first extraction step (I), exchangeable metals adsorbed to the biofilm were extracted. This fraction includes water-soluble metals, weakly adsorbed metals retained on the solid surface by relatively weak electrostatic interactions, metals that can be released by ion-exchange processes and metals that can be co-precipitated with carbonates (Filgueiras *et al.*, 2002). In extraction step II, the pH was buffered at 5.5, which served to solubilize carbonates, thereby releasing the associated metals. In step III, the biofilm sample was treated with  $\text{H}_2\text{O}_2$  in acid medium in order to extract metals associated with organic matter and metal sulfides. The residual fraction (extraction step IV) includes mainly metal (oxy)hydroxides, metal phosphates and crystalline minerals.

In the waste water, copper, iron and zinc in particular varied significantly, while the concentrations of the remaining elements changed only slightly (Table 2). Experiment Ia reflected changes in concentrations of sequentially extracted elements owing to the variability of the waste-water composition. However, in the biofilm extracts,

mainly calcium, copper and phosphorus exhibited significant variability (Fig. 2). The variability of copper and phosphorus concentrations was, surprisingly, seen in the least labile phases (extraction steps III and IV).

Gutekunst (1988) investigated the chemical speciation of heavy metals in a biofilm from sewers both with and without industrial inputs. The concentrations of copper and zinc were determined at 0.2–2.6 and 1.1–6.0 mg/(g total solids), respectively. This is in good agreement with the total concentrations of copper and zinc found in this study, which were in the ranges 0.3–1.1 and 1.6–4.9 mg/(g total solids), respectively (Figs. 2 and 5). The sequential extraction scheme applied by Gutekunst (1988) was developed by Förstner (1983) and differed primarily from the modified Tessier scheme applied in this study by two extraction steps targeted at easily and moderately reducible compounds (primarily Fe and Mn oxides) performed after extraction step II. In the study of Gutekunst (1988), copper was released predominantly when the sample was extracted with  $\text{H}_2\text{O}_2$  in acid solution, which is in good agreement with findings of this study. Zinc was, however, associated predominantly with the reducible compounds. This extraction step was not included in our study, which precludes a comparison of the results. Recent work confirms that extraction steps targeted at reducible compounds also release zinc sulfide, thereby severely underestimating the amount zinc present as zinc sulfide (Peltier *et al.*, 2005).

The observed increase in concentrations of sulfur, zinc and copper in step III (Fig. 2) upon changing from aerobic to anaerobic waste water (experiment Ib) indicated that zinc and copper sulfides precipitated. The molar proportions of the increases in sulfur, zinc and copper in step III over 8 days were 1 : 1.09 : 0.68. Thus the metal accumulation was higher than that which would have been expected from precipitation of metal monosulfides alone. This suggests that copper precipitated as  $\text{Cu}_2\text{S}$  rather than  $\text{CuS}$  (McNeil & Little, 1992) or that increased amounts of copper and zinc were organically bound (Späth *et al.*, 1998).

When iron and sulfate were added to the anaerobic waste water (experiment Iib), sulfur, zinc and copper in extraction step III increased at molar proportions of 1 : 0.35 : 0.16 over 10 days. The reduced precipitation of zinc and copper relative to sulfur when iron was added to the waste water indicated that metal sulfides accumulated only partly as zinc and copper sulfides. Sulfur probably also accumulated, owing to precipitation of iron sulfide when the biofilms were fed iron-supplemented waste water. Quan *et al.* (2003) found that the sequence of metal sulfide precipitation in a laboratory-scale upflow anaerobic bioreactor filled with granular sludge and cow manure could be related directly to the solubility products ( $K_{\text{sp}}$ ); i.e. metal sulfides of the lowest solubility precipitated first. Zinc sulfide ( $K_{\text{sp}} = 1.2 \times 10^{-23}$ ) and copper sulfide ( $K_{\text{sp}} = 8.5 \times 10^{-45}$ ) are less soluble than ferrous sulfide ( $K_{\text{sp}} = 3.7 \times 10^{-19}$ ) (Weast & Astle, 1982). Accordingly, ferrous sulfide is expected to precipitate after zinc sulfide and copper sulfide, which may explain the importance of zinc and copper for sulfide precipitation even in the presence of high concentrations of iron.



The observation that iron addition resulted in both iron and phosphorus accumulation in extraction step IV of the aerobic biofilm (experiment IIa; Fig. 5) was probably due to precipitation of iron phosphates or adsorption of phosphorus on precipitated iron (oxy)hydroxides. More iron than phosphorus accumulated than would have been expected from precipitation of iron phosphates alone. Thus a major part of the iron probably precipitated as iron (oxy)hydroxides, which are the most stable forms of iron in the slightly alkaline, aerobic waste water (Stumm & Morgan, 1996).

The calcium in extraction step II was presumably calcium carbonate. SRB mediate calcium carbonate precipitation (Visscher *et al.*, 2000). Thus the increased calcium concentration in extraction step III when sulfate was added to the waste water (experiment IIb) was probably due to stimulation of the SRB, producing both sulfide (to precipitate metals) and carbonate (to form  $\text{CaCO}_3$ , for example). The observation that the addition of sulfate to the waste water did not significantly affect the metal speciation suggests that the sulfate present in the unsupplemented waste water was not limiting the active SRB population from producing enough sulfide to precipitate the metals present. The limiting sulfate concentration for SRB has been reported to be approximately 5 mg S/l for biofilms grown on domestic waste water (Nielsen *et al.*, 1988). The sulfate concentration in the waste water decreased on average 4.8 mg S/l over 48 h of anaerobic incubation ( $n=3$ ). Thus the sulfate concentration was significantly higher than the limiting sulfate concentration for SRB throughout the experiments, even when sulfate was not added to the waste water.

Generally, the results of the sequential extractions are characterized by a considerable degree of variability. The variability may be due to spatial heterogeneity of the biofilm composition, both in terms of element composition and water content. The handling of the samples prior to extraction is also recognized as a critical step that may introduce errors (Kersten & Förstner, 1986). Chemical fractionation of anoxic freshwater sediments has shown that copper and zinc extracted in step III and iron extracted in step II are particularly sensitive to sample aeration and are transformed to reducible associations (Kersten & Förstner, 1986). In addition to possible errors introduced by sampling and handling, limitations of sequential extraction schemes are well documented in the literature, including poor reproducibility and overlap of fractions (e.g. Rudd *et al.*, 1988). A recent study compared three sequential extraction schemes and found the modified Tessier sequential extraction scheme, which was applied in this study, to be the best in terms of reproducibility and repeatability for the examination of organic sulfides (van Hullebusch *et al.*, 2005). Several sulfide minerals are not solubilized in step III of the modified Tessier sequential extraction scheme (Dold, 2003). Metal sulfides were therefore probably extracted in both steps III and IV, thereby creating an overlap of sulfides in both fractions. Previous studies on metal fractionating in anaerobic granular sludges have indicated that copper sulfide is extracted largely in step IV rather than in step III

(van Hullebusch *et al.*, 2005). In addition, the  $\text{H}_2\text{O}_2$  treatment applied in extraction step III is not entirely selective to organic matter and sulfides and may also attack iron oxides (Filgueiras *et al.*, 2002). This lack of selectivity demonstrates the difficulties for accurate separation of metals bound to organic matter, sulfides and oxides by chemical sequential extraction schemes, and should be kept in mind when one is interpreting the results.

It is expected that most sulfides were present as metal sulfide precipitates, which are considered to be stable under reducing conditions (Stumm & Morgan, 1996). However, the modified Tessier sequential extraction scheme applied in this study does not differentiate between organic- and metal-bound sulfides. Further characterization of metal sulfide precipitation in the biofilm matrix therefore requires other analytical methods to be employed; for example, combining sequential extractions with acid-volatile sulfide analyses (Jong & Parry, 2004) and/or X-ray adsorption spectroscopy techniques (Prange & Modrow, 2002).

## CONCLUSIONS

This study investigated the nature of metal accumulation in aerobic and anaerobic sewer biofilms. Perturbations of the growth conditions resulted in a number of observable effects on the fractionation of elements extracted from the sewer biofilms:

Zinc, copper and sulfur accumulated when conditions were changed from aerobic to anaerobic waste water. The accumulation was seen mainly in extraction step III, which was expected to extract most metal sulfides.

When iron (5 mg/l) was added to the waste water, the extent of zinc and copper accumulation in the anaerobic biofilm was reduced. This was probably due to increased precipitation of the sulfide with iron.

When aerobic biofilms were grown on iron- and sulfate-supplemented waste water, significant amounts of iron and phosphorus accumulated in the least labile phase. This was interpreted as precipitation of iron phosphates or iron (oxy)hydroxides, which may effectively adsorb phosphorus.

Extracted biofilm EPS consisted mainly of proteins and the composition remained relatively constant during the experiments. No correlations between the extracted EPS and metal concentrations could be established. This might indicate that the metal retention in both aerobic and anaerobic sewer biofilms is governed by metal precipitation rather than sorption to the biofilm matrix.

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