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Fe(III) reduction and vivianite formation in activated sludge

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Abstract

Kinetics of iron reduction, formation of vivianite and the microbial community in activated sludge from two sewage treatment plants (STPs) with low (STP Leeuwarden, applying enhanced biological phosphate removal, EBPR) and high (STP Cologne, applying chemical phosphate removal, CPR) iron dosing were studied in anaerobic batch experiments. The iron reduction rate in CPR sludge ($2.99 \text{ mg-Fe g VS}^{-1} \text{ h}^{-1}$) was 3-times higher compared to EBPR sludge ($1.02 \text{ mg-Fe g VS}^{-1} \text{ h}^{-1}$) which is probably caused by its 3-times higher iron content. Accordingly, first order rate constants in both sludges are comparable ($0.06 \pm 0.001 \text{ h}^{-1}$ in EBPR vs $0.05 \pm 0.007 \text{ h}^{-1}$ in CPR sludge), thus potential rates in both sludges are comparable. The measured Fe(III) reduction rates suggest that all iron in STP Leeuwarden and STP Cologne can be turned over within 15 h and 44 h respectively. Mössbauer spectroscopy and X-ray diffraction (XRD) indicated vivianite formation within 24 h in both sludges. After 24 h, 53% and 34% of all iron were bound in vivianite in the EBPR and CPR sludge respectively. Next generation sequencing (NGS) showed that the microbial community in the CPR sludge comprised more genera with iron-oxidizing and iron-reducing bacteria. Iron reduction and vivianite formation commence once activated sludge is exposed to oxygen free conditions. Our study reveals that the biogeochemistry of iron in STPs is very dynamic. By understanding the interactions between iron and phosphate crucial processes in modern sewage treatment, such as chemical phosphate removal or phosphate recovery from sewage sludge, can be optimized.

Keywords: Vivianite, Iron reduction, Activated sludge, Sewage, Phosphate

1 Introduction

Phosphate has to be removed from sewage to prevent its emission to surface waters. Dosing iron (Fe) salts is an effective method to eliminate the phosphate (WEF, 2011). It is the most widespread method and allows, in contrast, to enhanced biological phosphorus removal (EBPR) the realisation of sewage treatment plants (STPs) with net energy production (Wilfert et al., 2015). For iron based chemical phosphate removal (CPR) ferrous (Fe(II)) or ferric iron (Fe(III)) salts are used. Adsorption via Fe(III) oxides, which are rapidly formed when Fe(III) comes in contact with water, is the main mechanisms for initial phosphate removal when Fe(III) are dosed (Lu et al., 2016; Mao et al., 2016; Wendt von, 1973). Several factors like aging/recrystallization of the precipitates, bacterial activity and pH variations can result in the transformation of these initial precipitates also affecting their phosphate retention capacity (Senn et al., 2017; Smith et al., 2008; Szabo et al., 2008).

Fe(III) reduction occurs in STPs and can be chemically, direct biologically or indirect biologically induced (Kappler et al., 2004; Nielsen et al., 2005, 2005; Nielsen, 1996; van den Brand et al., 2015). Fe(III) reduction is often considered to cause mobilization of phosphate, however, in environmental systems doubts have been risen about this hypothesis (Gächter and Müller, 2003; Golterman, 2001, 1995; Roden and Edmonds, 1997). Also observations in wastewater systems indicate that the mobilization of phosphate induced by Fe(III) reduction is not necessarily true. During anaerobic digestion, when strong reducing conditions prevail for a long period, all Fe(III) is likely reduced to Fe(II) (Ivanov et al., 2003). Still, phosphate was retained in the solid fraction of digested sludge at molar Fe:P ratios of around 2 (Ivanov et al., 2003; Thomas, 1965). In another study it was shown that phosphate was removed within 30 h from solutions containing complexed Fe(III), phosphate and Fe(III) reducing bacteria (Azam and Finneran, 2014). In the same study, bulk phosphate was removed within two days via Fe(III) reduction in real septic water. In both experiments complexed Fe(III) was used, in this form Fe(III) is not capable to bind phosphate. The mineral vivianite, $(\text{Fe}_3(\text{II})[\text{PO}_4]_2 \cdot 8\text{H}_2\text{O})$ was identified in these experiments using X-ray diffraction (XRD). Similarly,

in longer running experiments it was found that Fe(III) reduction caused phosphate removal and vivianite formation in septic tanks (Zhang, 2012).

For sewage, limited information on phosphate removal in response to Fe(III) reduction exist. It was reported that Fe(III) reduction resulted in effective removal of phosphate during anaerobic digestion of sewage sludge and that Fe(III) reducers can be used to eliminate phosphate from sludge liquor (Stabinov et al., 2005). In another study, activated sludge was kept under anaerobic conditions, it was claimed that Fe(III) reduction (with a parallel decrease in dissolved phosphate) resulted in the formation of vivianite without giving experimental evidence (Cheng et al., 2015). In a similar study (with a significant drop in pH), Fe(III) reduction did not result in substantial phosphate removal (Cheng et al., 2017). In purely chemical experiments, it was shown that within 1-5 h efficient phosphate removal could be achieved in secondary effluents when Fe(II) was dosed in the absence of oxygen and XRD indicated that vivianite was formed. The residual phosphate levels at the optimum pH=8 were lower as compared to experiments where Fe(III) was dosed (Ghassemi and Recht, 1971).

Vivianite was found in digested sludge and in sludge before the anaerobic digestion (Frossard et al., 1997; Singer, 1972; Wilfert et al., 2016). Before digestion, sludge passes anoxic, sometimes anaerobic but also aerated zones. In an earlier publication (Wilfert et al., 2016) it was hypothesized that the presence of vivianite in surplus sludge before the anaerobic digestion can be explained by fast Fe(III) reduction rates (Nielsen, 1996), fast vivianite formation (Azam and Finneran, 2014) and slow oxidation kinetics of vivianite (Miot et al., 2009; Roldan et al., 2002). Fe(III) reduction has been detected in STPs, however the products of Fe(III) reduction are unknown. In this study the products of iron reduction in sewage sludge were characterised and quantified for the first time.

Information regarding the iron reducing community in activated sludge are also lacking. It is currently not known how iron dosing affects the microbial population in the activated sludge. Identifying Fe(III) reducing bacteria is important because Fe(III) reduction can also be induced by

other bacteria via indirect mechanisms e.g. electron shuttling (Kappler et al., 2004) or sulphide (Pyzik and Sommer, 1981) or via other non-enzymatic pathways (Lovley, 2013).

We determined Fe(III) reducing rates using HCl extraction of the produced iron species, studied the iron reducing community using deep next generation sequencing (NGS) and analysed products of Fe(III) reduction in activated sludge systems using XRD and Mössbauer spectroscopy. Two STPs were sampled, one relies on Fe(III) dosing and the other plant combines Fe(II) and Fe(III) dosing with enhanced biological phosphate removal (EBPR). We hypothesized that (1) Fe(III) reducing rates are higher in the CPR sludge, (2) Fe(III) reducing genera show a higher relative abundance in the CPR sludge and that (3) vivianite is formed in both treatment plants as Fe(III) reduction commences. Currently, the time scale of iron reduction and vivianite formation in activated sludge systems are unknown despite the important role vivianite plays for phosphate recovery and removal in STPs.

Understanding the formation of vivianite in sludge is necessary to develop new phosphate recovery technologies, e.g. for stimulating the growth of large vivianite particles that can be separated from the sludge for subsequent phosphate recovery (Lin et al., 2017; Liu et al., 2018; Wang et al., 2018; Wilfert et al., 2018).

2 Methods and Material

2.1 Sample origin

Activated sludge samples were obtained from aerated tanks of the STPs Cologne-Rodenkirchen (Capacity: 70,000 p.e.; expressed in 150 g TOC/d) where Fe(III) is dosed for CPR and from the STP Leeuwarden (226,000 p.e.; expressed in 150 g TOC/d). In the STP Leeuwarden, EBPR, supported by dosing Fe(II) and Fe(III) salts, is applied. The sludge retention times (SRTs) in the activated sludge systems are 15 days in the STP Leeuwarden and 12 days in the STP Cologne. The elemental composition of the initial sludge (Table S1) was determined using a microwave assisted acid

digestion (MD, 9.0 mL 70% HNO_3 on 1 g wet sample and heating for 20 minutes at 200 °C) followed by inductively coupled plasma optical emission spectrometer (ICP-OES). Total and volatile solids (TS and VS respectively) were determined according to standard methods (APHA, AWWA, WEF, 1998). After sampling, the sludge was settled for 3-4 hours to increase the TS content by a factor of 7 for Leeuwarden and 5 for Cologne (Table S1 and Table S3). The supernatant was discarded and further experiments were performed with settled sludge (Table S3). Due to the transport/settling time we assume that anaerobic conditions occurred in the sludge.

2.2 Experimental design

To determine the total Fe(III) reduction rates in the activated sludge, $\text{Fe(III)Cl}_3 \cdot 7\text{H}_2\text{O}$ (1.0 g / kg settled sludge) was added to the settled sludge inside an anaerobic glovebox (95% N_2 and 5% H_2). In the following “all iron” refers to the initially present iron (Leeuwarden 100 and Cologne 1190 mg Fe/kg settled sludge) plus the Fe(III) that was added to the sludge (200 mg Fe / kg settled sludge). 100 mL of this mixture was added into butyl rubber stoppered serum bottles (n=3 per time interval) and equipped with magnetic stirring bars. Samples were incubated at 25 °C, for up to 24 h while stirring (400 rpm) and sampled at different time intervals. This was the experimental group, aiming to obtain total Fe(III) reduction rates. To determine chemical Fe(III) reduction rates in the sludge, a control group was treated analogue to the samples in the experimental group, however, this gravity settled sludge was autoclaved (30 min, 121 °C) before the experiments. Fe(III) reduction rates were calculated using the slope of the linear fitting of decreasing Fe(III) concentrations versus time. The biological Fe(III) reduction rates reported are the difference between total and chemical Fe(III) reduction rates.

2.3 Sampling and sample preparation

At each time interval, prior to (destructive) sampling, temperature, pH and ORP of the samples were determined potentiometric (Figure S1). Total Fe(III)/Fe(II) concentrations in the samples were

determined spectrophotometric after a 0.5 M HCl extraction (Nielsen et al., 2005; Rasmussen and Nielsen, 1996).

At the beginning and at the end of the experiment about 2 mL sample were taken for NGS analyses. These samples were centrifuged (2 minutes, 8000 G), the pellet was collected and stored at -20 °C for one day to extract the DNA using the Mo-Bio PowerSoil kit (Mo-Bio Laboratories, Inc, USA). The purity of the extracted genomic DNA met the requirements of NGS (Table S2). Then the extracted DNA was stored at -20 °C until sequencing commenced. Sequencing and basic data analysis were delegated to GATC Biotech (Germany). In case of paired end sequencing, amplicons were sequenced in both directions, the resulting read pairs were merged based on overlapping bases using fast length adjustment of short reads (FLASH) with maximum mismatch density of 0.25 (Magoč and Salzberg, 2011). The sequencing data was compressed by performing sequence clustering based on 99% similarity basis accounting for PCR and sequencing errors (< 1%) using cd-hit (Li and Godzik, 2006). Chimera check was performed with UCHIME (Edgar et al., 2011). Data that was not qualified as reliable was discarded. All qualified sequences were submitted to the NCBI database for a BLAST search (E-value cutoff $1e^{-06}$). All hits were utilized and the similarity was set above 97%.

The remaining sample was filled in plastic centrifuge tubes, the lids were sealed with parafilm and samples were centrifuged (10 min, 3500 G). Back in the glovebox the supernatant was filtered (0.45 μ m) to determine the dissolved Fe(II)/Fe(III) concentration using the ferrozine method (Viollier et al., 2000) and the dissolved elemental and ionic composition using ICP-OES and IC respectively.

For XRD and Mössbauer analyses, the centrifuge pellet from the sample centrifugation was two times washed with oxygen free acetone and centrifuged (10 min, 3500 G) and then spread on glass plates inside the glovebox. The samples were apparently dry within 30–60 minutes but drying was continued in the dark, overnight inside the glovebox. For XRD analyses, the dried and grinded sample from one of the triplicates was filled in 0.7 mm glass capillaries and sealed with modelling

clay and superglue inside the glovebox. Just before the measurements started, glass capillaries were burnt off to be able to mount them in the sample holder. The measurements were done on a PANalyticalX'Pert PRO diffractometer with Cu-K α radiation (5-80 °2 θ , step size 0.008°). For Mössbauer analyses, dried and grinded samples were filled in plastic rings, sealed with Kapton tape and super glue and then wrapped in parafilm. Still in the glovebox the samples were put in gas tight glass bottles and stored until Mössbauer analyses. Transmission ^{57}Fe Mössbauer spectra were collected at 100 K with conventional constant-acceleration and sinusoidal velocity spectrometers using a ^{57}Co (Rh) source. Velocity calibration was carried out using an α -Fe foil. The Mössbauer spectra were fitted using Mosswinn 4.0 (Klencsár, 1997).

3 Result

3.1 Fe(III) Reduction

Figure 1 shows the average Fe(II), Fe(III) and total iron concentrations over time during the experiments. All iron concentrations in this graph were measured using the ferrozine method after an extraction step with 0.5 M HCl. On average 88% of all iron was extracted from EBPR activated sludge, while 84% could be extracted from the CPR sludge. In both sludges, Fe(III) dominated at the start of the experiment. Initially, Fe(III) made up 93% and 92% of all iron in EBPR and CPR sludge respectively. In EBPR sludge, about 75% and in the CPR sludge 58% of all iron was reduced within 24 h respectively. The chemical Fe(III) reduction rate, determined in experiments with autoclaved sludge, was about 5% of the total iron reduction rate. The specific iron reduction rate, that was actually measured in the samples, in the CPR sludge (2.99 mg-Fe g VS $^{-1}$ h $^{-1}$, $R^2=0.95$) was almost 3 times higher than the one in EBPR sludge (1.02 mg-Fe g VS $^{-1}$ h $^{-1}$, $R^2=0.97$).

Fe(III) bio-reduction of Fe(III) oxyhydroxides follows first order kinetics (Bonneville et al., 2009; Hacherl et al., 2003). Hence, the first order rate constant (k) was determined by linear fitting of $\ln(\text{Fe(III)})$ versus time. By assuming first order kinetics for biological iron reduction, (i.e. the iron reduction rate depends on the concentration of iron only) the rate constant allows comparing iron

turnover rates between sludges with identical iron phases but different iron contents. The k value (95% Confidence Interval) for EBPR sludge was $0.06 \pm 0.001 \text{ h}^{-1}$ and for CPR sludge $0.05 \pm 0.007 \text{ h}^{-1}$. A t -test showed that there is a statistical difference between the constants ($p < 0.05$, $t = 0.03$). The k value of the EBPR sludge was only 1.14 times higher than the one obtained from measurements on CPR sludge. In practice, one can consider the potential iron reduction rate in STP Leeuwarden as being comparable to the one in STP Cologne. Thus, Fe(III) reduction rates should be very similar when both sludges contain the same quantities of iron. The positive correlation between the iron content and iron reduction rates in sludge samples is illustrated in Figure 4. Here, data from earlier studies on iron reduction rates in sewage sludge were plotted.

Figure 1: Iron concentrations in activated sludge during batch tests determined after HCl extraction. Fer refers to the ferrozine method. Tot refers to total iron. A & C refer to sludge from STP Leeuwarden and STP Cologne respectively, while B & D were the corresponding sludges after autoclaving.

3.2 Vivianite formation

3.2.1 XRD

The degree of crystallinity in both samples was low, probably due to a large organic fraction and due to the presence of inorganic amorphous phases (all spectra including peak assignments are included in the supporting information, Figure S2). Despite a low crystallinity, several crystalline phases were identified in the samples. Samples from the activated sludge tanks in the STP Leeuwarden contained only quartz as crystalline material at the beginning and after 24 h of anaerobic incubation (Table 1). The samples from the STP Cologne also contained quartz at the beginning and end of the experiment but the dominant crystal phase was calcium carbonate. After 24 h of anaerobic incubation characteristic peaks of vivianite appeared in the samples. Vivianite contributed 29% to the crystalline material in the CPR sludge after 24 h of incubation (Table 1). In our study, XRD was used as a qualitative analytical tool to identify the presence of minerals/vivianite (Figure S2). The amorphous share and thus the contribution of vivianite to the total sample were not determined. Mössbauer spectroscopy was used for quantifying vivianite and other iron minerals in the samples.

Table 1. Semi-quantitative results from XRD measurements on EBPR sludge (STP Leeuwarden) and CPR sludge (STP Cologne).

ID	Compound	Share (% of crystalline material)	
		t=0 h	t=24 h
Leeuwarden sludge	Quarz (SiO ₂)	100	100
Cologne sludge	Quarz (SiO ₂)	19	15
	Vivianite (Fe ₃ [PO ₄] ₂ ·8H ₂ O)	-	29
	Calcium Carbonate (CaCO ₃)	81	57

3.2.2 Mössbauer spectroscopy

Vivianite was identified and quantified in all samples by Mössbauer spectroscopy. Note, results from Mössbauer spectroscopy refer to total iron in the samples. The total iron content in both samples differed considerably (Table S3).

The spectra showed Mössbauer parameters for vivianite in agreement with data that was obtained before (Gonser and Grant, 1976). However, the ratio of the two iron sites in vivianite should be 2 (Mori and Ito, 1950; Rouzies and Millet, 1993) but deviated from 2 in our measurements (1.4 for EBPR sludge, 1.1 for CPR sludge, Table 2). We believe that impurities in the vivianite are the reason for this deviation. Substitution of iron by other cations in iron phases can effect Mössbauer measurements (Gil et al., 1992), also the site ratios (Amthauer and Rossman, 1984). For baricite (the analogue of vivianite with partial substitution of iron by magnesium) iron substitution takes preferably place at site 2 (Yakubovich et al., 2001). If iron substitution in vivianite also takes dominantly place at site 2 then an iron site ratio < 2 can be expected. Indeed, vivianite is commonly known to contain impurities (Rothe et al., 2016). In sewage, dissolved metals, such as calcium and magnesium, occur that could be incorporated in the vivianite structure. With this line of reasoning we hypothesize that the vivianite in our samples is rather a vivianite-like structure, i.e. that some of the iron was replaced (data indicate that the replacement takes preferably place at site 2) by other metals such as magnesium or calcium. When we refer to vivianite in the following we actually refer to the vivianite-like structures with unknown degrees of impurity. Oxidation of vivianite can also change the site ratios but it was observed that initial oxidation rather increases than decreases the site ratio i.e. that it leads rather to an iron site ratio > 2 (Rouzies and Millet, 1993).

Table 2. Mössbauer results obtained with the samples from STP Leeuwarden and STP Cologne at the beginning of the experiments and after 24 h of anaerobic incubation.

Sample	Temperature (K)	IS (mm·s ⁻¹)	QS (mm·s ⁻¹)	Γ (mm·s ⁻¹)	Phase	Spectral contribution (%)
EBPR sludge t=0	100	0.37	0.68	0.49	Fe ³⁺ /Fe ^{II}	95
		1.25	2.36	0.30	Fe ²⁺ (Vivianite I)	2
		1.25	2.97	0.30	Fe ²⁺ (Vivianite II)	3
EBPR sludge t=24 h	100	0.39	0.70	0.54	Fe ³⁺ /Fe ^{II}	47
		1.16	2.64	0.39	Fe ²⁺ (Vivianite I)	22
		1.22	3.17	0.39	Fe ²⁺ (Vivianite II)	31
CPR sludge t=0	100	0.36	0.74	0.48	Fe ³⁺ /Fe ^{II}	91
		1.19	2.45	0.32	Fe ²⁺ (Vivianite I)	3
		1.19	2.98	0.32	Fe ²⁺ (Vivianite II)	5
CPR sludge t=24 h	100	0.38	0.72	0.46	Fe ³⁺ /Fe ^{II}	66
		1.14	2.59	0.34	Fe ²⁺ (Vivianite I)	16
		1.19	3.03	0.34	Fe ²⁺ (Vivianite II)	18

Note: Experimental uncertainties: Isomer shift: I.S. ± 0.01 mm s⁻¹; Quadrupole splitting: Q.S. ± 0.01 mm s⁻¹; Line width: $\Gamma \pm 0.01$ mm s⁻¹; Spectral contribution: $\pm 3\%$.

Mössbauer spectroscopy showed that vivianite was initially present only in small fractions in the EBPR sludge (5% of all iron bound in vivianite) and CPR sludge (8% of all iron bound in vivianite) respectively (Table 2). After 24 h of anaerobic incubation the vivianite content in both samples increased substantially. At the end of the experiments vivianite was the dominating iron phase in the EBPR sludge, here the Fe(II) in vivianite summed up to 53% of all iron. In CPR sludge about 34% of all iron was in vivianite at the end of the experiments. The vivianite structures in EBPR sludge (after 24 h) have Mössbauer parameters that are closer to those of standard. Also the site ratio is

higher – it seems the vivianite in EBPR sludge is better defined i.e. more crystalline compared to the vivianite in the CPR sludge.

The Fe(II) content in the activated sludges determined by HCl extraction combined with photometric determination of Fe(II)/Fe(III) was different to the Fe(II) content determined by Mössbauer spectroscopy. After 24 h, Mössbauer spectroscopy suggested that in EBPR sludge 53% and in CPR sludge 34% of all iron is Fe(II). In contrast, HCl extraction showed that in the same samples 83% (EBPR sludge) and 66% for (CPR sludge) of all iron is Fe(II). For EBPR sludge, the extraction efficiency was relatively high (94%). From CPR sludge only 71% of all iron was extracted after 24 h by using HCl and hence a comparison between HCl extraction and Mössbauer spectroscopy is difficult. Using Mössbauer spectroscopy for obtaining the oxidation state of the iron is not straight forward when samples are not measured at 4 K. In Table 2, Fe³⁺ refers to high-spin Fe(III) (that could e.g. be an iron oxide); Fe²⁺ to high-spin Fe(II) (vivianite or disordered/impure vivianite) and Fe(II) to low-spin Fe(II) (probably pyrite). For spectra obtained at 100 K, the Fe³⁺ (iron oxide) can overlap with Fe(II) (pyrite) – it can be any of the two alone or a mixture, but not an intermediate species. Thus, assuming HCl extraction is accurate in accounting for all Fe(II), then the difference between Mössbauer and HCl extraction should be iron that is bound in pyrite (about 30%). The elemental composition of the sludge solids indeed suggests that up to 50% of all iron could be bound in pyrite in EBPR sludge since the molar Fe:S ratio in the sludge was about 1 (Table S1). Thus, we can assume 30% of all iron in EBPR sludge is bound in pyrite, 53% in vivianite and the remaining 17% in another, unknown iron phase which is probably a ferric compound. Differences between HCl extractions and Mössbauer could be related to differences in sample handling (wet vs. dried sample) or the presence of other low spin Fe(II) species with Mössbauer parameters close to the one of pyrite (but with different stoichiometry / elemental composition).

3.3 Microbial communities in the activated sludges

Next generation sequencing was carried out to identify microbial groups that have been related to iron reduction before. These analyses serve as a first step for generating a deeper understanding of iron reducing communities in sewage sludge (regarding e.g. dominating groups of iron reducers, share of iron reducers of the total microbial population etc.). Currently, this knowledge is not available. The dominant phylum in both sludges was *Proteobacteria* comprising between 68 and 89% of all operational taxonomic units (OTUs) (Figure S3). *Betaproteobacteria* formed the dominant class of all OTUs observed (38–63% of all OTUs, Figure S4). The predominant microbial genera in the sludges are shown in Figure 2 and in Table S4. As expected for activated sludge communities the diversity was high with many different OTUs each comprising only a small fraction in the total community (Saunders et al., 2016; Zhang et al., 2012). The genus diversity in the two sludges were high with 103 OTUs in CPR sludge and 107 OTUs in EBPR sludge. Both sludges showed a similar Simpson index (between 0.95 and 0.96) and Shannon index (between 3.62 and 3.74) indicating a comparable richness and evenness on the genus level. The top 3 genera in EBPR sludge were *Flavobacterium* (11.7%), *Novosphingobium* (9.4%) and *Rhodoferrax* (8.9%). While in CPR sludge, the top 3 genera were *Novosphingobium* (10.8%), *Dechloromonas* (9.3%) and *Rhodoferrax* (8.9%). Compared with the EBPR sludge, the CPR sludge showed a higher abundance of the genera *Dechloromonas* (9.3% vs 1.6%), *Aquabacterium* (4.5% vs 2.2%), *Rubrivivax* (3.4% vs 0.6%), *Thauera* (2.5% vs 0.9%), *Leptothrix* (3.2% vs 0.6%) and *Simplicispira* (3.0% vs 0.4%), but a lower abundance of the genera *Flavobacterium* (4.6% vs 11.7%), *Trichococcus* (0.6% vs 6.8%) and *Janibacter* (0.2% vs 3.1%).

After anaerobic incubation for 24 h, a slight difference in the abundance of several genera was observed. In EBPR sludge the abundance of *Novosphingobium* increased from 9.4 to 12.0%. In CPR sludge the abundance of *Dechloromonas* decreased from 9.3 to 3.6%.

Figure 2: Genus wise OTU distribution (Top 20 OTUs contain 68% of total OTU assigned reads). Sequencing of 16S ribosomal RNA gene aiming the V3-V5 region.

3.4 Dissolved elemental composition

From EBPR sludge significant amounts of phosphate were released within the first few hours of the experiment (Figure 3, Figure S5). This was accompanied by a release of potassium and magnesium. Phosphate accumulating organisms (PAOs) are probably responsible for this phenomenon (Jardin and Pöpel, 1994). Despite apparent Fe(III) reduction, dissolved iron levels remained constant at 0.16 ± 0.06 mmol Fe/kg sludge. In the control group of the EBPR sludge iron and potassium remained relatively constant over time, whereas phosphate, calcium and magnesium increased within 24 h of anaerobic storage. This increase could be due to cell lyses in response to the autoclaving.

In contrast to EBPR sludge, phosphate levels did not increase in CPR sludge, the dissolved phosphate levels remained at 0.07 ± 0.02 mmol P/kg sludge throughout the experiment. The dissolved iron concentration clearly increased from 0.3 mmol Fe/kg sludge at the beginning to about 1.24 mmol Fe/kg sludge after 24 h of anaerobic incubation. The aqueous iron is supposed to

be Fe(II) rather than Fe(III) because under anaerobic condition Fe(III) is reduced to Fe(II) and maintained in this oxidation state at the low ORP (Table S1).

Figure 3: Element concentrations in suspension determined by ICP during batch experiments. A & B: With EBPR sludge from STP Leeuwarden (activated & autoclaved respectively), C & D: With CPR sludge from STP Cologne (activated & autoclaved respectively).

4 Discussion

4.1 Fe(III) reduction in activated sludge

The iron reduction rates measured in the activated sludge are in the range of earlier measurements (Nielsen et al., 1997). By compiling all these data, the trend for increasing iron reduction rates in sludge with higher iron content is obvious (Figure 4), which is consistent with the view that iron reduction exhibits first order kinetics. However, some data significantly deviate from the general

trend (Figure 4). These differences can be caused by 1) differences in the type of iron phases that are present in the sludge; 2) the presence of different types of iron reducing bacteria (IRB); 3) different mechanisms of microbial Fe(III) reduction; 4) different reaction conditions and 5) presence and availability of organics as electron donors. We could not find any indications for a significant formation of crystalline iron oxide phases in activated / digested sewage sludge in literature. Accordingly, in our study, Mössbauer spectroscopy and XRD indicate that poorly crystalline Fe(III) phases were present in the sludge (the line width of the Mössbauer signal is considerably higher compared to very crystalline structures with values of about 0.25 mm/s). Such iron phases usually have a high surface area and the iron in these compounds is readily available to Fe(III) reducers compared to more crystalline ferric iron forms (Lovley, 2013). The apparent increase of iron reduction rates with the higher iron content in the sludge, presumably under similar experimental conditions, follows first order kinetics. This suggests that the iron reduction rates are rather physical (mass transport) or chemical (concentration/activity of dissolved Fe(III)) limited than biological rate limited. In this context, it could be a limitation of mass transport that is usually a function of the surface area of the iron phases in the sludge. In other words, by assuming that the type of iron compounds in the sludges are the same, then a higher iron content will result in higher availability of iron because of reduced transport limitations. Experimentally, this hypothesis can be tested by adding different quantities of iron phases with identical crystallinities to sludge under the same experimental conditions until the reduction rate is plateauing. The iron reduction rates should linearly increase with the increasing iron content in the sludge. Such experiments can show that iron reduction in the sludge is indeed physical driven, i.e. by the surface area of the iron phases. In the other studies, iron phases in the sludge were not characterized. We can therefore not conclude that the type of iron in the sludges could explain the observed deviations. However, we suggest that iron phases in activated sludge are in general amorphous and that the difference in iron compounds between sludges are not the main reason for observed deviation. The type of organic substrates that are present in the sludges can explain deviations too. Adding lactate to sludge increased iron

reduction rates by about 20-30% (Nielsen et al., 1997). In the context of environmental or sludge samples this increase of the reduction rates, compared to the in-situ rates, is rather low. It can, though, explain some of the deviations observed in figure 4.

The absolute Fe(III) reduction rates in CPR sludge were 3 times higher compared to EBPR sludge. However, the potential of Fe(III) reduction rates for these two sludges are similar assuming first order kinetics.

Figure 4: Iron reduction rates in activated sludge samples. Rates marked with dots and circles refer to rates that were determined after adding lactate to the sludge. The other rates (triangles and crosses) were determined without organics addition. The rates are plotted versus the iron content of activated sludge. Rates determined by Nielsen (1996) are average values.

Besides direct microbial Fe(III) reduction also indirect microbial induced chemical reduction via sulphide production or via organic electron shuttles could be responsible for the turnover of iron in the samples. Out of 1 mol of sulphate 1 mol of sulphide can be formed that can reduce 2 mol of Fe(III) to Fe(II). In CPR sludge, IC measurements showed that dissolved sulphate levels dropped from 132 to 117 mg SO_4^{2-} /kg sludge indicating that at most 0.31 mmol Fe(III)/kg sludge can be reduced. In EBPR sludge sulphate dropped from 33.5 to 14.3 mg SO_4^{2-} /kg sludge indicating that a maximum of 0.4 mmol Fe(III)/ kg sludge could be reduced. The indirect Fe(III) reduction by sulphide could therefore, at maximum, explain 14 and 2% of the observed iron reduction in EBPR and CPR sludge respectively. During the experiments neither a sulphide smell nor black colouration

of the samples was observed. To what degree other mechanisms contributed to the reduction of Fe(III) in our samples is unknown.

Control incubations with autoclaved sludge were performed to identify the chemical/non-biological Fe(III) reduction. In both control incubations, a slight but steady increase of Fe(II) over time was measured but the computed Fe(III) reduction rates are only about 5% of the total rates. This is in accordance to earlier studies with sediments (Lovley et al., 1991). Chemical iron reduction in our study could be induced by reduced compounds in the sludge, such as reduced sulphur species that can be produced in-situ or originate from the incoming raw sewage (Hvitved-Jacobsen et al., 2013; van den Brand et al., 2015). Additionally, organic compounds are able to transfer electrons to Fe(III). Organic molecules are e.g. used as electron shuttles by microorganisms (Kappler et al., 2004), are probably released as external electron donors to access insoluble Fe(III) phases (Richter et al., 2012) and organic ligands, like cysteine, are capable of reducing Fe(III) (Cornell et al., 1989). Since interactions between Fe(III) and dissolved sulphide are usually relatively quick (Poulton et al., 2004) we suggest that organic or reduced compounds such as FeS_x are responsible for the Fe(III) reduction in the control incubations. Autoclaving could increase the abiotic Fe(III) reduction potential due to cell lyses that causes a release of organics with reducing properties. Autoclaving at elevated temperatures can modify the iron pool of the sludge samples (Schwertmann and Cornel, 2007). More crystalline iron phases could form that are less easily reducible. Additionally, autoclaving can increase the sludge surface which enhances adsorption and affects net iron release. However, at least for EBPR sludge most of the iron was added after autoclaving. We suggest to consider further non-destructive control incubations in future, such as cooling of the samples to reduce the activity of microorganism, to be able to distinguish between chemical and biological Fe(III) reduction.

In the STP Leeuwarden it would take 15 h and in STP Cologne 46 h to reduce the originally present iron (i.e. iron from the sewage and iron that is dosed for CPR) in the sludge from Fe(III) to Fe(II).

Note that significant amounts of Fe(III) would still be present after this time when the iron turnover is calculated by using the first order rate constants. In Cologne 10 and in Leeuwarden 40% of all iron would not be reduced after 46 and 15 h respectively. Yet, it was decided not to use the rate constants because the reaction can change order when it progresses and then another step could become rate limiting.

The SRTs in the plants are about 14 days, half of this time in oxygen free tanks. In STP Leeuwarden besides anoxic also anaerobic tanks exist. Earlier Mössbauer measurements on surplus sludge (i.e. sludge after settling but before dewatering that is brought into the digester) from the STP Leeuwarden showed that indeed all iron in this sludge was Fe(II) (Wilfert et al., 2016). These findings match with the first part of our hypothesis: Fe(III) reduction and subsequent formation of vivianite is quick and once vivianite is formed its re-oxidation is slow. In the STP Cologne, however, earlier Mössbauer data obtained at 4 K, indicate that not all but only about 57% of all iron in the surplus sludge was Fe(II), this sludge was taken right before it was pumped into the anaerobic digester (Wilfert et al., 2018). The activated sludge that we used in our experiments was taken from an aerated tank and here Fe(III) was dominant (90%). Note, about 15% of all iron was added as Fe(III) and that the extraction efficiency was about 90% at $t=0$. Still, there is obviously a difference in the iron chemistry between surplus and activated sludge. Iron containing sludge can encounter oxygen free conditions for some time in STPs, e.g. in settlers, inside flocs with limited oxygen diffusion or in anaerobic/anoxic tanks that are required for successful nutrient elimination. Apparently, in the STP Cologne, with a much higher iron content compared to sludge from the STP Leeuwarden, the reduction is not quick enough to turn over all Fe(III). This could be due to the presence of aerated tanks or because the conditions in our batch experiments are different to the ones encountered in treatment lines. Else, Fe(III) reduction would theoretically be quick enough to reduce all Fe(III) and re-oxidation of Fe(II) to Fe(III) should be slow assuming the presence of pure vivianite (Roldan et al., 2002). Most but not all Fe(II) could be re-oxidized after intense aeration of sludge that was stored under anaerobic conditions (Nielsen et al., 2005). Vivianite oxidation is

supposedly a slow process (Miot et al., 2009; Roldan et al., 2002). The incomplete oxidation of iron in the study could be explained by the presence of vivianite. Activated sludge from the STP Cologne, contained little vivianite and little Fe(II), showing that our hypothesis is not entirely true. On the other hand, surplus sludge sampled in STP Cologne contained about 60% of all iron as Fe(II) (Wilfert et al., 2018). We have no coherent explanation for these differences in iron speciation between activated and surplus sludge.

4.2 Microbial analyses in activated sludge

The metagenomic analyses were performed for both sludges before and after anaerobic incubation for 24 h under endogenic conditions. Since marginal growth and decay will occur the communities would be expected to be highly similar or identical. For the EBPR samples this is the case, but for the CPR samples there seem to be differences. This might be related to the change in iron valency. DNA extraction methods are sensitive to many factors and the presence of different forms of metals might influence the DNA extraction (Matheson et al., 2009). Since the transformation of Fe(III) to Fe(II) in the sludge sampled in STP Cologne was quicker and to a larger extent it seems logically that the detrimental effects on the genetic analyses was larger for the Cologne samples. The discussion section will therefore focus on the microbial community in both of the sludges at the start of the anaerobic incubation (t=0 h).

Similar to earlier studies *Proteobacteria* were the dominant phylum in the activated sludges (Ferrera and Sánchez, 2016). Table S4 shows the top 20 genera in EBPR and CPR sludge with their abundances. Half of the top 20 genera in both sludges are common genera that are encountered in STPs (Lu et al., 2007; Thomsen et al., 2007). Their traits are given in Table S5. The top genera in EBPR sludge and CPR sludge were similar, however they occurred with different abundances (Table S4).

IRB can directly reduce Fe(III) to Fe(II) and can carry out other metabolic conversions as well (Lovley, 2013). Among the genera found in the activated sludge samples, direct iron reduction has been related to members of genera *Rhodospirillum*, *Dechloromonas*, *Ferribacterium* and *Aeromonas*

(Cummings et al., 1999; Finneran et al., 2003; Liu et al., 2014; Ventura et al., 2015). Genera *Dechloromonas*, *Aquabacterium*, *Leptothrix* and *Simplicispira* are related to Fe(II) oxidation / Fe(III) reduction (Chang, 2015; Ilbert and Bonnefoy, 2013).

NGS suggests that, compared with EBPR sludge, CPR sludge contained more bacteria from genera that are related to Fe(II) oxidation / Fe(III) reduction (33.1 vs 17.7%). The higher abundance of iron metabolising bacteria in CPR sludge could be caused by its higher iron content.

Enumerating genera that were related to iron reduction before (using NGS or qPCR) and assigning them with average single cell iron reduction rates to estimate the absolute iron reduction rate in the sludge that is solely carried out by iron reducing microorganisms is too simplistic. Iron reducers are commonly able to carry out multiple metabolic pathways. Furthermore, this approach does not account for inactive microorganisms. Other molecular methods (qPCR or FISH), that target on functional genes are currently not available for iron reducers. Iron reducing organisms are phylogenetic diverse and iron reduction follows several different pathways (Weber et al., 2006). A general molecular proxy for iron reduction has not been identified yet (Akob et al., 2012). A possible approach could be to look at a few specific groups or iron reducers for which molecular proxies were identified (e.g. *Geobacteraceae*) and compare their activity with the overall measured iron reduction rate. Members of the genera *Geobacter* are often dominating the iron reducing community but were not found in our samples.

Overall, the iron metabolising community in environmental samples requires more in depth analyses. Future investigations on different sludges from other STPs should be carried out (on the metagenomics and if available on functional genes for iron reduction). In which way the iron reducing microorganisms between the sludge in Leeuwarden and Cologne differ, if the iron reducing community changes with changing iron content in the sludge and in which way iron

reducing organisms affect the absolute and specific iron reduction rates in the sludge remains currently unknown.

4.3 Vivianite is formed in both treatment plants as Fe(III) reduction commences

Phosphate removal is efficient in STP Cologne with effluent levels of 0.33 mg P / L (average in June 2017) although significant amounts of iron are present as Fe(II) in the surplus/digested sludge of this plant (Wilfert et al., 2018). It is commonly assumed that Fe(III) is more efficient in retaining phosphate. This would only be correct if iron phosphate species with a molar Fe:P lower than the one of vivianite (Fe:P=1.5) form. We could not find indications for strengite formation on a time scale of STPs (Wilfert et al., 2015). Also iron phosphate adsorption complexes show usually higher molar Fe:P ratios than vivianite (Gerke, 2010). Accordingly, we observed no substantial phosphate release despite more than 50% of all Fe(III) was reduced. Phosphate could be retained by Fe(III) that was still present but at a significant part was retained as vivianite. XRD and Mössbauer spectroscopy confirmed the presence of vivianite at the end of the incubation in activated sludge from STP Cologne. Taking the molar Fe:P ratio of this sludge (1.24, Table S3), the molar P:Fe ratio of vivianite (0.66) and the percentage of iron bound in vivianite (34%, Table 2) into account then about 30% of all phosphate is bound in vivianite (7% at the start of the experiment). In STP Leeuwarden, about 8% of all phosphate is bound in vivianite at the beginning of the experiment. Our data shows that substantial amounts of vivianite can form as a response to Fe(III) reduction and that this Fe(III) reduction does not necessarily result in net phosphate release. Vivianite has been identified in wastewater treatment systems with oxygen free stages as an important phosphate phase. Our data shows that its formation can take already place before the anaerobic digestion process when oxygen free conditions occur in treatment lines. This explains why considerable amounts of vivianite have been found in surplus sludge before digestion. Mössbauer measurements showed that sludge sampled in the aerated tanks of the STPs contained small amounts of vivianite, indicating that its formation already takes place in the activated sludge systems. This vivianite

fraction was not detectable by XRD, either because it is highly dispersed vivianite with small particle size or because its amount was below the detection limit of XRD. How fast oxidation of the vivianite-like structures in the sludge is has yet to be determined.

Our experiments furthermore show what will happen when sewage sludge is brought to an anaerobic digester. Here sludge is usually retained for 2-4 weeks under methanogenic conditions and all Fe(III) should be transformed to Fe(II). Since dissolved phosphate is present vivianite can form. Thermodynamic calculations indicate that, under conditions that occur in digesters, vivianite is more stable than most other phosphate minerals (Nriagu, 1972). If enough iron is present in the sludge, as in the case of the STP Cologne, formation of vivianite during anaerobic digestion is not hampered by the formation of insoluble iron sulphide compounds FeS_x (Wilfert et al., 2015). This line of reasoning underlines the important role vivianite plays for STPs using iron for CPR. When vivianite forms, the net phosphate release is non-existent during the anaerobic digestion as long as sufficient quantities of iron are present to buffer sulfide. In the STP Cologne phosphate concentrations in digested sludge liquor were only 0.8 mg P/L.

Figure 5: Conceptual model of vivianite formation in sewage sludge after Fe(III) addition and subsequent Fe(III) reduction in sludge from a EBPR plant (with low initial Fe(III) content) and from a CPR plant where Fe(III) is already present.

In both sludges vivianite was formed although different treatment designs are applied. We suggest that vivianite formation in these STPs differs (Figure 5). In the STP Cologne, most iron bound phosphate is initially adsorbed to amorphous ferric iron oxides. During the reduction of ferric iron (phosphate) phases, phosphate is released and re-precipitates with dissolved Fe(II) as vivianite. Not only dissolved but also adsorbed phosphate contributes to vivianite formation. Because of the very low solubility of vivianite, adsorbed phosphate binds with Fe(II) to form vivianite. Kinetics and importance of these two processes have to be established. In EBPR sludge, initially most phosphate is bound inside PAOs with only little amounts of phosphate adsorbed to ferric iron oxides. While storing samples under oxygen free conditions, PAOs release phosphate. At the same time Fe(II) is brought in solution via iron reduction, and vivianite is formed. Besides phosphate, PAOs release potassium and magnesium. Ammonium levels were increasing in solution from 0.65 to 3.69 mmol/L in the first 20 h and then sharp decreasing to 0.16 mmol/L at 24 h, theoretically struvite could form in the samples, but XRD could not detect it. Either it was below the detection limit of XRD or present with small particle size or vivianite formed on expenses of struvite due to a lower solubility of vivianite (Nriagu, 1972). Since vivianite has a lower solubility than struvite, phosphate recovery routes that rely on vivianite are more efficient than recovery routes that rely on struvite. Vivianite based phosphate recovery routes vivianite are under development (Wilfert et al., 2018).

5 Conclusion

Microbial driven iron reduction commences directly after exposing sludge to oxygen free conditions. The product of iron reduction is vivianite. Vivianite is an important mineral in STPs as it can contain most of the insoluble phosphate. The formation of vivianite has to be understood to be able to improve phosphate removal (i.e. using Fe(III) dosing) and to allow phosphate recovery via

vivianite (e.g. to understand growth mechanisms of vivianite and the influence of bacteria on vivianite crystallization to grow bigger vivianite particles that can easily be separated from the sludge). This study shows that biological driven iron reduction plays certainly an important role. In subsequent studies the role of microorganisms on mechanisms of vivianite formation in STPs and the re-oxidation kinetics of vivianite in sludge need to be investigated. Phosphate removal can be improved by understanding the differences in Fe(II)/Fe(III) dosing for phosphate binding. Our study further indicates that iron dosing changes the microbial population in sewage sludge by proliferating genera with iron metabolism (iron oxidation /reduction bacteria). Iron plays a crucial role for modern sewage treatment and is, contrary to common impressions, subject of manifold chemical changes during the treatment process.

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Supporting Information. Brief descriptions in nonsentence format listing the contents of the files supplied as Supporting Information.

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Highlights

1. Microbial iron reduction commences as soon as activated sludge becomes anaerobic.
2. The product of iron reduction is the ferrous iron phosphate mineral vivianite.
3. More genera with a metabolism related to iron were found in sludge with higher iron content.
4. Understanding vivianite formation will improve phosphate removal and recovery.