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Abstract

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Abstract: The oxic-settling anoxic (OSA) process interchanges activated sludge between alternating redox conditions (e.g. aerobic and anoxic) to reduce biosolids production. Iron

salts addition to wastewater is performed to remove phosphorous, but this study demonstrated that it may impair OSA performance. Batch test results showed that the addition of iron salt (as Fe^{2+}) decreased the volatile solids reduction of an intermittently aerated batch reactor (i.e., aerobic/anoxic), probably because iron reduced the destruction of extracellular polymeric substances (EPS) that serve as the structural framework of sludge flocs. The effect of different FeCl_2 dosages (0, 15, and 30 mg l^{-1}) on the sludge yield and EPS profile of an OSA system consisting of a sequencing batch reactor (SBR) attached to external aerobic/anoxic and anoxic reactors was compared to those of a control system consisting of an SBR attached to a single-pass aerobic digester. The two SBRs were fed with real wastewater and operated continuously in parallel for 230 d. Without FeCl_2 addition, the sludge yield (g MLVSS g^{-1} COD) of the SBR in the OSA system was 24.8% less than that of the control SBR. Moreover, the waste sludge of the OSA system had lower volatile solids content than that of the control system. When 15 or 30 mg l^{-1} FeCl_2 was added to the influent, OSA was unable to decrease the sludge yield of the SBR and the volatile solids content of the waste sludge. FeCl_2 dosing increased the EPS of sludge in the external aerobic/anoxic reactor, confirming that floc destruction in that reactor was reduced by the presence of iron.

Keywords: extracellular polymeric substances; ferrous chloride; oxic-settling-anoxic (OSA); sludge reduction; soluble microbial products.

1. Introduction

Sludge treatment and disposal constitutes a significant portion of wastewater treatment plant (WWTP) operation cost. The decreasing availability of disposal options (e.g., landfilling and incineration) has triggered a renewed research interest to minimise biosolids production during wastewater treatment. The oxic-settling-anoxic (OSA) process exposes biomass in alternating redox conditions by treating a portion of activated sludge in an external anoxic reactor and then returning it to the main aerobic reactor (Semblante et al., 2014; Semblante et al., 2015). Despite several full-scale OSA installations (Coma et al., 2013; Foladori et al., 2015), there remains some contention regarding the mechanism/s responsible for sludge reduction in OSA. Chon et al. (2011a) hypothesized that oxygen- and substrate-deficient conditions in the external reactor enhance the disintegration of the extracellular polymer substances (EPS), which are proteins, carbohydrates, and other macromolecules that serve as the structural framework of sludge flocs. Other researchers proposed that OSA enables ‘metabolic uncoupling’ and forces microorganisms to select energy replenishment over cellular propagation (Chudoba et al., 1992), or that it transforms the ecology of activated sludge such that slow-growing bacteria or bacteriovores are enriched (Chudoba et al., 1992; Ye et al., 2008; Navaratna et al., 2014). However, none of these mechanisms have been validated through investigations conducted with real wastewater. The maximum sludge reduction achieved by full-scale OSA (e.g. 18%) (Troiani et al., 2011; Coma et al., 2013) is significantly lower than those of laboratory-scale implementations (e.g. 58%) fed with synthetic wastewater (Chudoba et al., 1992; Saby et al., 2003; Chon et al., 2011a), which warrants further investigation using real wastewater.

The role of iron in the flocculation (Higgins and Novak, 1997) and floc destruction under anaerobic conditions (Novak et al., 2003) has been reported, but its impact on OSA performance has not been systematically studied. Iron salts are commonly added to wastewater in full-scale plants for phosphorous removal by chemical process (Paul et al., 2001; An et al., 2014). When Fe(II) salt is added to an aerobic reactor, iron is spontaneously oxidised to Fe(III) given the availability of oxygen in the system (i.e., $2\text{Fe}^{2+} + 2\text{H}^+ + \frac{1}{2}\text{O}_2 \rightarrow 2\text{Fe}^{3+} + 2\text{H}_2\text{O}$, $E^\circ_{\text{cell}} = +2.0$ V). Fe(III) forms hydroxyl complexes that serve as ‘ion

bridges' between negatively-charged sites of EPS and causes flocculation (Higgins and Novak, 1997). The binding of Fe(III) to EPS appears to make sludge flocs less easily dispersed or destroyed. For example, Niu et al. (2013) observed that the addition of FeCl_3 (5-10 g g^{-1} dry solids) prevented the destruction of flocs by shear stress. Mishima and Nakajima (2009) also observed that the addition of FeCl_3 (2,000-5,000 mg l^{-1}) decreased the release of EPS into the supernatant of a membrane bioreactor.

The oxidation reduction potential (ORP) of the external reactor of OSA is a key parameter that impacts sludge reduction. Saby et al. (2003) observed that decreasing the ORP of the external reactor from +100 mV to less than -250 mV increased sludge reduction in a laboratory-scale OSA from 23 to 58%. However, very low ORP levels are difficult to achieve under specific operational constraints, *e.g.*, at low hydraulic retention time (HRT) (Saby et al., 2003; Troiani et al., 2011). Troiani et al. (2011) showed that treating sludge in alternating anaerobic (ORP = -400 to -200 mV) and anoxic ranges (ORP = -200 to +50 mV) in a full-scale plant resulted in sludge reduction of 13-17%. In a full-scale plant, maintaining an ORP range is more practical than trying to maintain a specific ORP value. In this context, an OSA containing both aerobic and anoxic stages in the external reactor rather than a strictly anaerobic reactor may be additionally beneficial in terms of minimising the influx of soluble chemical oxygen demand (COD) and nutrients in the main bioreactor upon recirculation of treated sludge. Thus it is worthwhile to investigate the performance of an OSA containing both aerobic and anoxic stages in the external reactor, which has not been reported in literature.

This study investigates sludge reduction of an OSA system consisting of external aerobic/anoxic and anoxic reactors attached with an SBR receiving real wastewater. In addition, it determines the impact of FeCl_2 addition on OSA performance. Preliminary batch tests were performed to investigate the effect of FeCl_2 addition on volatile solids reduction under alternating redox conditions. Then, the effect of FeCl_2 dosing on sludge reduction by the OSA system was assessed relative to a control system consisting of an SBR attached to a single-pass aerobic digester. The use of real wastewater in this study is critical in that although real wastewater can undergo significant temporal variations, it produces more realistic biomass growth rates and sludge properties. The sludge yield, volatile solids reduction, and EPS concentrations of the reactors were monitored.

2. Materials and methods

2.1 Batch experiments

A series of preliminary batch experiments were conducted to assess sludge reduction under two redox regimes, *i.e.* aerobic/anoxic and anoxic. Batch aerobic/anoxic reactors with and without a single addition of 30 mg l^{-1} FeCl_2 were aerated in intermittent mode (*e.g.*, 8/16 hours aeration on/off) using an air diffuser placed at the bottom of the tank. Batch anoxic reactors with and without a single addition of 30 mg l^{-1} FeCl_2 were completely sealed with a silicone-lined cap equipped with a sampling port and a gas outlet port with an air trap to prevent air leakage. All reactors were kept in a 25 °C water bath, and continuously mixed by a magnetic stirrer.

The batch reactors were inoculated with activated sludge from the aerobic reactor of Wollongong WWTP. The sludge was centrifuged for 10 min at 3,267 \times g, and then re-constituted in synthetic wastewater to make up a total volume of 2 l. Synthetic wastewater representing medium-strength wastewater was used only in the batch experiments to study the impact of FeCl_2 on volatile solids reduction. It was composed of glucose (400 mg l^{-1}),

peptone (100 mg l^{-1}), urea (35 mg l^{-1}), KH_2PO_4 (17.5 mg l^{-1}), MgSO_4 (17.5 mg l^{-1}), FeSO_4 (10 mg l^{-1}), and sodium acetate (225 mg l^{-1}).

The batch reactors were incubated for 30 days. Samples for MLVSS, soluble microbial products (SMP) and EPS analysis (Section 2.3) were obtained after two weeks of incubation, with three and five sampling events for the batch aerobic/anoxic and anoxic reactors, respectively. Duplicate measurements were performed at each sampling event.

2.2 Continuous reactors

To scrutinize the observations in batch tests, further investigations were conducted via two laboratory-scale (5 l each) SBRs (denoted as $\text{SBR}_{\text{control}}$ and SBR_{OSA}) continuously fed with real wastewater. $\text{SBR}_{\text{control}}$ served as control and was attached to a single-pass aerobic digester, whereas SBR_{OSA} was attached to external aerobic/anoxic and anoxic reactors (Section 2.2.2).

2.2.1 Real wastewater

Primary effluent (e.g. effluent after primary sedimentation) from Wollongong WWTP was collected weekly and stored at $4 \text{ }^\circ\text{C}$ in the dark until used. The wastewater was allowed to reach room temperature prior to feeding to SBRs to avoid temperature shocks.

2.2.2 Reactor configuration and operation

The two SBRs (Figure 1) were inoculated with aerobic activated sludge from the Wollongong wastewater treatment plant and operated at 4 cycles/day and an HRT of 12 hours. Each cycle comprised of 15 min of filling, 5.5 hours of aeration, 1 hour settling, and 15 min of decanting. The two SBRs were fed from the same influent tank containing real wastewater that was continuously mixed with an impeller. The SRT of $\text{SBR}_{\text{control}}$ and SBR_{OSA} was maintained at 10 days by regular sludge wastage (W).

The two SBRs were operated for 87 days with addition of 15 mg l^{-1} of FeCl_2 in the influent tank starting from the 53rd day of operation. Both SBRs had a mixed liquor pH of 6-8 and dissolved oxygen (DO) concentration of 4-5 mg l^{-1} . At the 88th day of operation, $\text{SBR}_{\text{control}}$ was attached to a 2-l aerobic digester to form the ‘‘control system’’ (Figure 1a), and SBR_{OSA} was integrated with an external aerobic/anoxic reactor (2 l) and an anoxic reactor (2 l) to form the ‘‘OSA system’’ (Figure 1b). The aerobic digester and aerobic/anoxic and anoxic reactors were kept at $25 \text{ }^\circ\text{C}$ water bath, and continuously mixed by a magnetic stirrer.

[**Figure 1** Schematic diagram of the (a) ‘control system’ consisting of $\text{SBR}_{\text{control}}$ attached to a single-pass aerobic digester and (b) ‘OSA system’ consisting of SBR_{OSA} attached to external aerobic/anoxic (AE/ANX) and anoxic (ANX) reactors.]

The aerobic digester was continuously aerated using an air diffuser placed at the bottom of the reactor (Figure 1a). It was fed with sludge from $\text{SBR}_{\text{control}}$ thickened to $5\text{-}10 \text{ g l}^{-1}$ by centrifugation for 10 min at $3,267\times g$ (Q_{in}). The supernatant produced by the thickening step was discarded. Sludge was regularly discharged (Q_{out}) to achieve an SRT of 20 days. At steady-state, the aerobic digester had a pH of 5-7, DO concentration of 4-5 mg l^{-1} , and ORP of +180 to +340 mV.

The external aerobic/anoxic reactor was intermittently aerated (e.g., 8/16 hours aeration on/off), and the anoxic reactor was sealed using a silicone-lined cap (shaded area Figure 1b). The combined SRT of the aerobic/anoxic and anoxic reactors was 20 days. Sludge from

SBR_{OSA} was thickened to 5-10 g l⁻¹ by centrifugation for 10 min at 3,267xg, and then fed to aerobic/anoxic reactor (q_1). The same volume of sludge was removed from the aerobic/anoxic reactor, of which 33% was transferred from aerobic/anoxic to anoxic (q_2) and the remaining 67% was discarded (q_3). Accordingly, 16.5% of sludge was transferred from anoxic to the aerobic/anoxic reactor (q_4) and a further 16.5% was transferred from anoxic to SBR_{OSA} (q_5).

The wasted sludge (q_5) was thickened to 16-24 g l⁻¹ by centrifugation for 10 min at 3,267xg to obtain the supernatant, which was returned to SBR_{OSA}. The pellet was discarded. At steady-state, the aerobic/anoxic reactor had a pH of 5-7, DO concentration of less than 1 mg l⁻¹, and ORP of +50 to +100 mV (measurements obtained when aeration was off), and the anoxic reactor had an ORP range of -400 to -300 mV.

To study the effect of FeCl₂ on OSA, FeCl₂ dosing was halted on the 152nd day of operation and then resumed at 30 mg l⁻¹ on the 196th day of operation. It is noteworthy that the background total iron concentration in the wastewater during the period of this study was 1.52±0.68 mg l⁻¹ (n=12). Thus even during the period of no FeCl₂ dosing, the influent to the SBRs was not completely devoid of iron.

Wastewater and sludge samples were collected twice weekly to monitor reactor performance. There were eight sampling events for SMP and EPS analysis, with duplicate measurements performed at each sampling event.

2.2.3 Calculations of sludge yield and sludge reduction by OSA

To account only for the production of biomass, mixed liquor volatile suspended solids (MLVSS) concentration was used to quantify sludge production instead of mixed liquor suspended solids (MLSS).

The observed sludge yield (Y_{obs}) was defined as

$$Y_{obs} = \frac{P}{C} = \frac{g \text{ MLVSS}}{g \text{ COD}} \quad \text{Equation 1}$$

where P is the sludge produced and C is the substrate consumed. The experimental Y_{obs} was derived from the slope of the linear regression of the plot of cumulative sludge produced versus the cumulative substrate consumed. Cumulative values were obtained by incrementing the variations in sludge production and substrate consumption in previous sampling intervals (Chon et al., 2011b).

In this study, sludge yield was presented in two ways. First, the Y_{obs} of the SBR_{control} and SBR_{OSA} were compared to understand the difference in MLVSS production by an aerobic reactor receiving anoxic sludge from external reactors relative to a control aerobic reactor (Supplementary Table S1). Second, the sludge yield of the control system (*i.e.*, the SBR_{control} and aerobic digester) and OSA system (*i.e.*, SBR_{OSA} and external aerobic/anoxic and anoxic reactors) were determined to account for potential MLVSS production in the external reactors as active biomass may consume products of cell lysis (Hao et al., 2010) (Supplementary Table S1).

The sludge reduction achieved by the external aerobic/anoxic and anoxic reactors was calculated using Equation 2:

$$\text{Sludge reduction by external reactors (\%)} = \frac{MLVSS_{in}/MLSS_{in} - MLVSS_{out}/MLSS_{out}}{MLVSS_{in}/MLSS_{in}} \times 100 \quad \text{Equation 2}$$

where $MLVSS_{in}/MLSS_{in}$ and $MLVSS_{out}/MLSS_{out}$ is the concentration of thickened sludge fed to and wasted from the aerobic/anoxic reactor, respectively.

2.3 Analytical techniques

Total suspended solids (TSS) and VSS of influent and effluent and MLSS and MLVSS of sludge were measured according to APHA Standard Method 2540 (Eaton et al., 2005). Sludge volume index (SVI) was measured using 1000 mL of sludge according to APHA Standard Method 2710-D (Eaton et al., 2005). COD was measured using a Hach DBR200 COD Reactor and a Hach DR/2000 spectrophotometer (program number 430 COD LR) according to US-EPA Standard Method 5220. Total organic carbon (TOC) and total nitrogen (TN) were quantified using a TOC/TN-VCSH analyzer (Shimadzu, Japan). Ammonia (NH_3) and orthophosphate (PO_4^{3-}) were measured using flow injection analysis (Lachat instruments, Milwaukee, USA) following APHA Standard Method 4500 (Eaton et al., 2005). Ammonia analysis involved the reaction of ammonia with phenol and hypochlorite to form a blue complex whose colour was intensified by nitroferricyanide, followed by measurement of the absorbance at 630 nm (APHA 4500-N) (Eaton et al., 2005). Orthophosphate analysis involved the reaction of orthophosphate with ammonium molybdate and antimony potassium tartrate under acidic conditions to form a complex that is reduced by ascorbic acid, followed by measurement of the absorbance at 880 nm (APHA 4500-P) (Eaton et al., 2005).

Soluble microbial products (SMP) was extracted by centrifuging sludge at 3,267xg at 4 °C followed by filtration of supernatant with 0.45 µm membrane to ensure removal of suspended solids. EPS was extracted from the same sample by resuspending the pellet in 10 mM NaCl adjusted to pH 10.5 using 1 M NaOH. The solution was purged with N_2 gas, immediately sealed off, and then shaken at 100 rpm at 25 °C. The solution was centrifuged at 3,267xg at 4 °C, and then filtered using 0.45 µm membrane to remove suspended solids and obtain EPS extract (Chon et al., 2011a). Proteins and carbohydrates were analysed using the modified Lowry method and phenol-sulphuric method, respectively (Hai et al., 2011; Wijekoon et al. 2013).

To determine the concentration of total iron in sludge, samples were digested according to US EPA Method 3050b that involved digestion using nitric acid and hydrogen peroxide followed by addition of hydrochloric acid (Peña-Icart et al., 2011). The iron concentration of digested samples were measured using an inductively-coupled plasma mass spectrophotometry (Agilent 7500CS, Agilent Technologies, USA).

Two-sample *t*-test was performed using Analysis Toolpak in Microsoft Excel to determine if there was significant difference in the EPS concentrations in the external aerobic/anoxic and anoxic reactors due to FeCl_2 dosing. $p < 0.05$ was considered to indicate statistical significance.

3. Results and discussion

The main part of this study *i.e.*, the continuous operation of the OSA process was carried out with wastewater collected from a municipal wastewater treatment plant. However, the continuous flow experimental program was based on a series of tests conducted with batch reactors fed with a synthetic wastewater. The batch tests were conducted to study the impact

of FeCl₂ addition on MLVSS reduction of the individual reactors of the OSA system (*i.e.*, anoxic/aerobic and anoxic reactors).

3.1 Impact of FeCl₂ addition on batch reactors

FeCl₂ addition to the batch aerobic/anoxic reactor resulted in an increased production of EPS_{protein} and lower extent of MLVSS reduction (Table 1). Because the ORP of this reactor varied from +30 to +80 mV, iron prevailed as Fe(III), which decreased the disintegration of the EPS. Changes in the EPS and SMP were primarily observed in the protein fraction due to the preferential binding of Fe(III) to proteins (Novak et al., 2003). An opposite trend was observed in the batch anoxic reactors (ORP = -400 to -300 mV), that is, the addition of FeCl₂ slightly increased MLVSS reduction and decreased EPS in the form of proteins (Table 1).

[**Table 1** MLVSS/MLSS reduction and average EPS and SMP of the batch reactors (average ± standard deviation; n = number of measurements).]

Fe(III) can lead to sludge flocculation due to ion bridging and surface charge neutralisation (Higgins and Novak, 1997). In the flocculation process, the outer EPS layer called the “loosely-bound EPS” and the inner EPS layer called the “tightly-bound EPS” are both compressed as flocs aggregate (Niu et al., 2013). Studies report that Fe(III) strongly retains biopolymers within flocs (Murthy and Novak, 2001), and decreases the extractability of the loosely-bound EPS (Niu et al., 2013). However, during anaerobic respiration, Fe(III) can be converted to Fe(II). This results in the release of EPS into solution, especially those in the form of proteins, and eventually to deflocculation (Novak et al., 2003). Park et al. (2006) further suggests that the reduction of Fe(III) is a prerequisite to the destruction of volatile solids under anaerobic digestion. Thus, in the current study, MLVSS reduction was facilitated in the batch anoxic reactor (ORP < -250 mV), and not in the batch aerobic/anoxic reactor (ORP = +30 to +80 mV) where bacterial population capable of Fe(III) reduction may not have been enriched. Indeed, in the presence of FeCl₂, the anoxic reactor showed nearly twice as much EPS_{protein} in solution (*i.e.*, SMP_{protein}) than the aerobic/anoxic reactor (Table 1), confirming that Fe(III) reduction (and hence volatile solids reduction) was impaired in intermittently aerated (*i.e.*, aerobic/anoxic) conditions.

The batch reactor investigations systematically demonstrate that aerobic/anoxic treatment of sludge could achieve similar MLVSS reduction as anoxic treatment in the absence of Fe(III). However, if significant (e.g. at least 30 mg l⁻¹) Fe(III) is present in the sludge, aerobic/anoxic treatment does not effectively reduce volatile solids. These observations form an important baseline for an explanation of the results from OSA operation with real wastewater.

3.2 Impact of FeCl₂ dosing on a continuous OSA system fed with real wastewater

3.2.1 Basic reactor performance and sludge properties

The COD removal efficiencies of SBR_{control} and SBR_{OSA} were stable during the start-up phase of the study (Figure 2). After the attachment of the external aerobic/anoxic and anoxic reactors, the COD removal efficiency of SBR_{OSA} temporarily decreased probably because it received additional COD from the returned sludge. Nonetheless, SBR_{OSA} quickly acclimatised and from then on, the COD (Figure 2) and TOC (Supplementary Figure S1) removal efficiency of SBR_{control} and SBR_{OSA} were similar, indicating that OSA had no impact on these parameters.

[**Figure 2** COD concentrations and removal by SBR_{control} and SBR_{OSA} at different FeCl₂ dosage.]

Similar $\text{NH}_3\text{-N}$ (Figure 3) and negligible TN (Supplementary Figure S2) removal was observed in the SBRs throughout the operational period. A negligible difference in the $\text{PO}_4^{3-}\text{-P}$ removal performance of the SBRs was also observed: $\text{SBR}_{\text{control}}$ and SBR_{OSA} had no $\text{PO}_4^{3-}\text{-P}$ removal up to a FeCl_2 dose of 15 mg l^{-1} , and achieved only up to 30% $\text{PO}_4^{3-}\text{-P}$ removal when FeCl_2 was added at a concentration of 30 mg l^{-1} FeCl_2 (Figure 3). This was because the Fe/P molar ratios (*i.e.*, 1.27 and 0.85 for FeCl_2 dosage of 15 mg l^{-1} and 30 mg l^{-1} , respectively) were lower than the theoretical ratio required for chemical precipitation (*e.g.*, 1.5). Notably, the SVI was below 100 ml g^{-1} for both SBRs irrespective of iron dosing (Supplementary Figure S3). This indicates that the SBRs possessed rapidly settling flocs (Tchobanoglus et al., 2003), and that OSA did not improve sludge settleability.

[Figure 3 $\text{NH}_3\text{-N}$ and $\text{PO}_4^{3-}\text{-P}$ concentrations in $\text{SBR}_{\text{control}}$ and SBR_{OSA} effluent at different FeCl_2 dosage.]

The observed negligible impact of OSA on COD (Chudoba et al., 1992; Saby et al., 2003; Goel and Noguera, 2006) and TN (Ye et al., 2008) removal is consistent with the literature. However, an additional aspect revealed in the current study was that fluctuations in influent wastewater strength (COD= 9 to 133 mg l^{-1} , $n=41$) similarly affected the COD (Figure 2) removal performance of the control SBR_1 and SBR_2 . This influent COD fluctuation was also observed to somewhat affect the volatile solids reduction capacity of the OSA system (data not shown) although the trend of volatile solids reduction discussed in Section 3.2.2 was consistent.

3.2.2 Impact of FeCl_2 dosing on OSA performance

The impact of FeCl_2 addition on OSA performance in terms of sludge reduction was analysed using Y_{obs} (Table 2) derived from the corresponding plots of cumulative sludge produced versus cumulative substrate consumed (Figure 4 and Supplementary Figure S4). Because of the significant variation in the wastewater strength between different runs, comparing the Y_{obs} values of a reactor at different runs did not give meaningful trends. Therefore, to eliminate interference from the varying influent, the effect of FeCl_2 dosage was observed by contrasting the Y_{obs} of SBR_1 and SBR_2 at each experimental phase only (Table 2).

[Figure 4 Cumulative sludge produced (g MLVSS) versus cumulative substrate consumed (g COD) of SBR_1 and SBR_2 at different FeCl_2 dosage.]

[Table 2 Y_{obs} of control and OSA system with different dose of FeCl_2 to the influent (real wastewater)]

SBR_1 and SBR_2 had similar Y_{obs} during the start-up phase (Table 1). In other words, the SBRs equally acclimatised to wastewater characteristics and operation conditions and the experiments had similar initial conditions.

When 15 and 30 mg l^{-1} of FeCl_2 was added to the influent, the Y_{obs} of SBR_{OSA} was higher than that of the control SBR_1 (Table 1), meaning that the OSA process was unable to reduce the MLVSS production. The Y_{obs} of the OSA system (*i.e.*, SBR_{OSA} +external reactors) was also greater than that of SBR_{OSA} , which could indicate that the external reactors had a net MLVSS production. This is supported by the fact that the MLVSS/MLSS reduction of the external reactors was mostly in negative values (Figure 5). On the contrary, without FeCl_2

addition, the Y_{obs} of SBR_{OSA} was lower than that of the control $\text{SBR}_{\text{control}}$ by 24.8% (Table 1), evidencing that OSA reduced the MLVSS production. Furthermore, without FeCl_2 dosing, (i) the Y_{obs} of the entire OSA system was lower than that of SBR_{OSA} , and (ii) the MLVSS/MLSS ratio of sludge fed to the aerobic/anoxic reactor was reduced (Figure 5).

[**Figure 5** Reduction (%) of MLVSS/MLSS ratio achieved by the external reactors superimposed with the MLVSS/MLSS ratio of the thickened sludge fed to the aerobic/anoxic reactor at different FeCl_2 dosage.]

To understand the impact of FeCl_2 addition on OSA performance, SMP and EPS profiles of the reactors were investigated (Figure 6a). The difference in the EPS profiles of $\text{SBR}_{\text{control}}$ and SBR_{OSA} was not ascertained due to the significant variability of data points in each experimental run (Supplementary Figure S5). Nonetheless, a significant increase in the $\text{EPS}_{\text{protein}}$ of the external aerobic/anoxic reactor (Figure 6) occurred when FeCl_2 concentration was changed from zero ($33.2 \pm 9.8 \text{ mg g}^{-1}$, $n=4$) to 30 mg l^{-1} ($55.7 \pm 10.8 \text{ mg g}^{-1}$, $n=5$) (Two sample t -test; $t(7)=3.57$, $p=0.014$). Correspondingly, $\text{SMP}_{\text{protein}}$ (Figure 6a) and $\text{SMP}_{\text{carbohydrate}}$ (Supplementary Figure S6) of the aerobic/anoxic reactor decreased. These findings suggest that FeCl_2 dosing reduced the disintegration of EPS especially in the aerobic/anoxic reactor, and consequently decreased the efficiency of OSA to degrade MLVSS. The deleterious effect of FeCl_2 dosing on EPS disintegration and sludge reduction was also observed in the batch aerobic/anoxic reactors (Section 3.1), and was possibly due to the inefficiency of aerobic/anoxic conditions to biologically reduce Fe(III) that bound EPS.

[**Figure 6** SMP and iron-associated EPS in the form of proteins of the (a) aerobic/anoxic and (b) anoxic reactors of OSA at zero and 30 mg l^{-1} FeCl_2 dosage.]

Notably, the $\text{EPS}_{\text{protein}}$ of the anoxic reactor (Figure 6b) slightly increased when FeCl_2 concentration was changed from zero ($23.7 \pm 10.0 \text{ mg l}^{-1}$, $n=4$) to 30 mg l^{-1} ($34.7 \pm 11.4 \text{ mg l}^{-1}$, $n=5$), but the change was not statistically significant (Two sample t -test; $t(7)=1.55$, $p=0.17$). This indicates that EPS degradation in the anoxic reactor was not as impacted by FeCl_2 dosing as the aerobic/anoxic reactor. Nonetheless unlike the batch anoxic reactor (Section 3.1), the external anoxic reactor of the OSA system did not exhibit enhancement of EPS disintegration with FeCl_2 dosing. This was probably because the anoxic reactor received less destructible flocs from the aerobic/anoxic reactor, whereas the batch anaerobic reactor stood alone. Moreover, it had a much lower SRT (10 days) than that of the batch anoxic reactors, which was 490 days (calculated from the sludge spent for analysis).

3.2.3 Mechanisms of sludge reduction in OSA with dual-redox external reactors

The vulnerability of OSA to FeCl_2 dosing belies the critical role that the aerobic/anoxic reactor plays in this particular OSA configuration. The dual-redox external reactor that was utilised in this study is distinct from the OSA configurations reported in literature, which commonly involves a single anoxic or anaerobic external tank (Saby et al., 2003; Goel and Noguera, 2006; Chon et al., 2011b; Coma et al., 2013). Anaerobic condition in OSA (e.g., $\text{ORP} = -250$ to -100 mV) has been found to improve sludge reduction (Saby et al., 2003). Nonetheless, this study demonstrates that sludge reduction can also occur in intermittently aerated (*i.e.*, aerobic/anoxic) and anoxic conditions that may be easier to implement in full-scale operation (Troiani et al., 2011). However, the volatile solids reduction capacity of this configuration, particularly that of the aerobic/anoxic reactor, is susceptible to iron dosing.

In the particular OSA configuration investigated in this study, it is possible that the aerobic/anoxic reactor facilitated the hydrolysis of proteins, carbohydrates, and other macromolecules, thereby enhancing subsequent degradation in the anoxic reactor. The aerobic/anoxic reactor could have also helped ensure that O_2 , NO_3^- , and COD are depleted as much as possible so that the anoxic reactor was deficient of oxygen and substrate. Furthermore, the intermittent aeration in the aerobic/anoxic reactor possibly created alternating redox conditions that could trigger faster biodegradation.

This study confirms that without $FeCl_2$ dosing, the OSA process reduces sludge in two ways: (i) it decreases the MLVSS/MLSS of sludge fed to the external reactors (Figure 5), and (ii) it decreases the sludge yield of the main bioreactor (Table 1). The reduction in volatile solids content of waste sludge may have implications on its treatability and odour reduction during post-processing and transport. The influence exerted by OSA on the biomass growth in the main bioreactor has been reported in earlier studies (Chudoba et al., 1992; Chen et al., 2001; Saby et al., 2003). For example, Chudoba et al. (1992) reported that alternating sludge between favourable and non-favourable growth conditions result in metabolic uncoupling in microorganisms, which forces the biomass that is returned to the main bioreactor to prioritise energy replenishment instead of cellular propagation. The current study provides compelling evidence of lower sludge production in the main bioreactor as a result of the OSA process.

OSA potentially affects biomass growth not only at the cellular level, but also at a microbial community level. Due to sludge interchange, bacteria that are not commonly selected in aerobic conditions may have opportunity to thrive in OSA. For instance, Goel and Noguera (2006) noted an enrichment of slow-growing bacteria such as polyphosphate-accumulating organisms and fermenters in the enhanced biological phosphate removal (EPBR)-SBR due to attachment to an external anaerobic reactor. Aside from having long incubation, fermenters are able to decompose flocs and enhance biomass decay. In a recent study, Ning et al. (2014) found through pyro-sequencing that an anoxic-oxic reactor in an OSA system had more fermentative *Sphingobacteria* than an anoxic-oxic reactor only. Further investigation on the possible enrichment of slow-growing bacteria and its impact on the Y_{obs} will help elucidate the impact of sludge interchange in sludge minimisation.

3.2.3 Verification of the effect of $FeCl_2$ dosing on solids concentration analysis

A previous study showed that iron can precipitate as hydrated vivianite ($Fe_3(PO_4)_2 \cdot 8H_2O$) that may cause over-estimation of MLVSS as it loses 17% of its weight upon incineration at 550 °C (Tien and Waugh, 1969). Vivianite has been observed in iron-amended anoxic reactors (Frossard et al., 1997). Nonetheless, in the current study, the formation of vivianite in the continuous reactors was unlikely due to the insufficiency of iron in the influent. The average molar Fe/P ratio in the influent tank was only 1.27 and 0.85 at the period when $FeCl_2$ concentration was 15 and 30 $mg\ l^{-1}$, respectively (Table 3). An et al. (2014) investigated the formation of vivianite in synthetic wastewater with $FeCl_2$ dosing and found that the Fe/P molar ratio should be more than 3 to enable significant vivianite formation. The authors attributed this to the partial formation of ferrous hydroxides, which hindered the formation of vivianite.

[**Table 3** PO_4^{3-} -P concentration and Fe/P molar ratio in the influent at different phases of the experiment.]

[**Figure 7** Total Fe concentration of the sludge superimposed with MLVSS/MLSS ratio of $SBR_{control}$ and SBR_{OSA} at zero and 30 $mg\ l^{-1}$ $FeCl_2$ dosage.]

It was also observed that the measured Fe concentration, i.e. combined Fe(II) and Fe(III), of the sludge of SBR_{OSA} steadily increased when FeCl₂ dosage was increased from zero to 30 mg l⁻¹ due to the accumulation of metal precipitates, whereas that of SBR_{control} increased and then decreased (Figure 7). The fluctuation in Fe concentration in SBR_{control} was probably due to the wash out of solids. If vivianite had formed and caused over-estimation of MLVSS, the MLVSS/MLSS ratio should have increased when FeCl₂ dosage was increased from zero to 30 mg l⁻¹. However, it was observed that the MLVSS/MLSS ratio of SBR_{OSA} decreased and that of SBR_{control} remained the same (Figure 7). This was because the accumulation of metal precipitates in the reactor increased MLSS as observed in other studies (Paul et al., 2001; Li, 2005), but not MLVSS.

4. Conclusions

Based on investigations conducted with a continuous flow OSA system receiving real wastewater, this study demonstrated for the first time that the addition of FeCl₂ is counterproductive to sludge reduction in the external intermittently aerated (*i.e.*, aerobic/anoxic) reactor. Batch tests showed that FeCl₂ dosing decreased the volatile solids reduction of a batch aerobic/anoxic reactor probably due to a decline in the destructibility of EPS. This parallels the findings in continuous OSA operation, wherein it was found that the external aerobic/anoxic reactor had greater EPS and lower SMP when there was FeCl₂ dosing. In contrast, FeCl₂ did not have any negative effect on sludge reduction of the batch anoxic reactor, and had less severe impact on EPS destruction in the external anoxic reactor during continuous operation. This was probably because anoxic conditions facilitated the biological reduction of Fe(III) causing deflocculation and eventual sludge degradation. Without FeCl₂ addition, the sludge yield of the SBR_{OSA} was 24.8% lower than that of the SBR_{control}. Results reported here validate two mechanisms of sludge reduction (in absence of iron dosing) by the OSA process: first, the external reactors reduce the volatile solids of waste activated sludge and second, the interchange of sludge decreases volatile solids production in the main bioreactor.

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LIST OF FIGURES

Figure 1 Schematic diagram of the (a) control system consisting of $SBR_{control}$ attached to a single-pass aerobic digester and (b) OSA system consisting of SBR_{OSA} attached to external aerobic/anoxic (AE/ANX) and anoxic (ANX) reactors.

Figure 2 COD concentrations of influent and effluent and COD removal by $SBR_{control}$ and SBR_{OSA} at different $FeCl_2$ dosage.

Figure 3 NH_3-N and $PO_4^{3-}-P$ concentrations of influent and $SBR_{control}$ and SBR_{OSA} effluent at different $FeCl_2$ dosage.

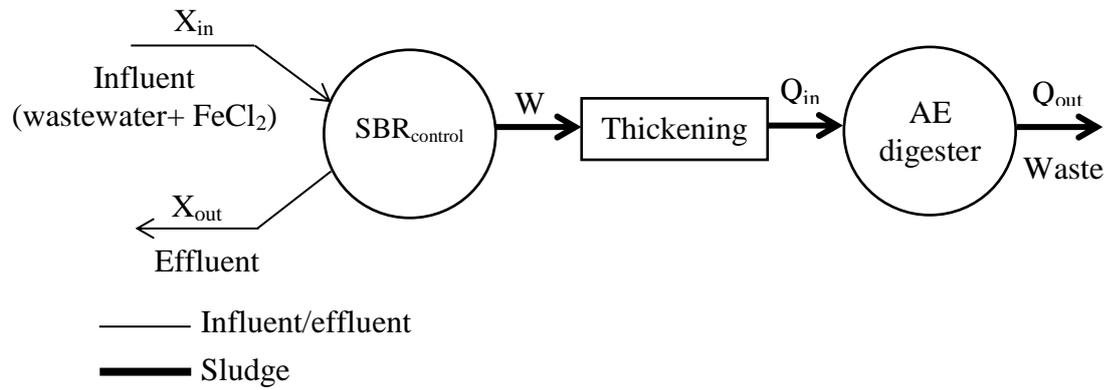
Figure 4 Cumulative sludge produced (g MLVSS) versus cumulative substrate consumed (g COD) of $SBR_{control}$ and SBR_{OSA} at different $FeCl_2$ dosage.

Figure 5 Reduction (%) of MLVSS/MLSS ratio achieved by the external reactors superimposed with the MLVSS/MLSS ratio of the thickened sludge fed to the aerobic/anoxic reactor at different $FeCl_2$ dosage.

Figure 6 SMP and iron-associated EPS in the form of proteins of the (a) aerobic/anoxic and (b) anoxic reactors of OSA at zero and $30\text{ mg l}^{-1} FeCl_2$

Figure 7 Total Fe concentration of the sludge superimposed with MLVSS/MLSS ratio of $SBR_{control}$ and SBR_{OSA} at zero and $30\text{ mg l}^{-1} FeCl_2$

(a) Control system



(b) OSA system

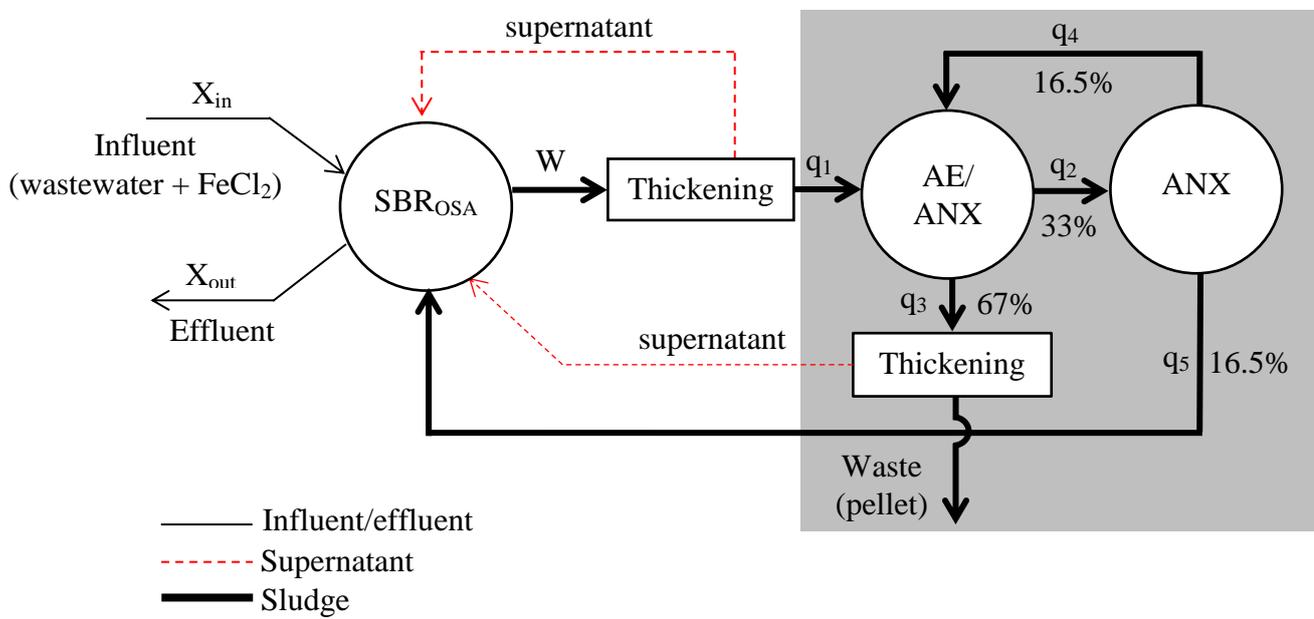


Figure 1

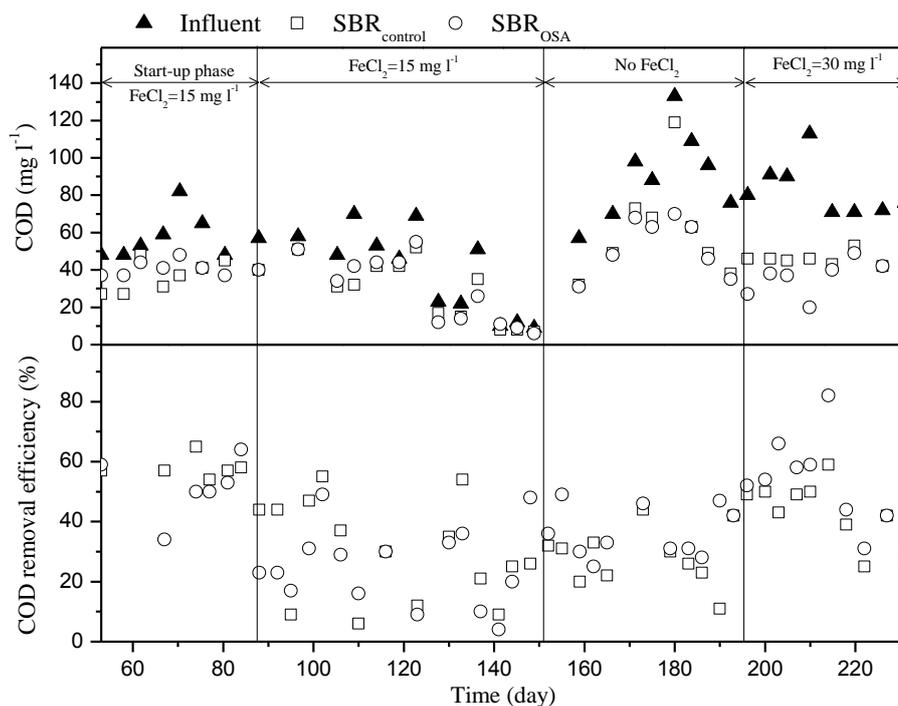


Figure 2

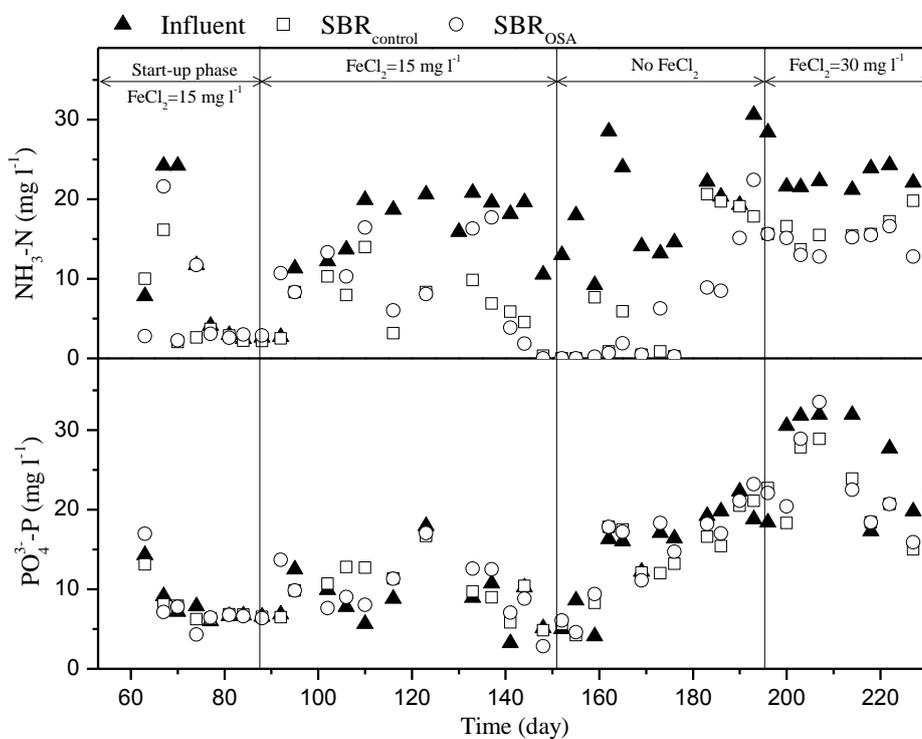


Figure 3

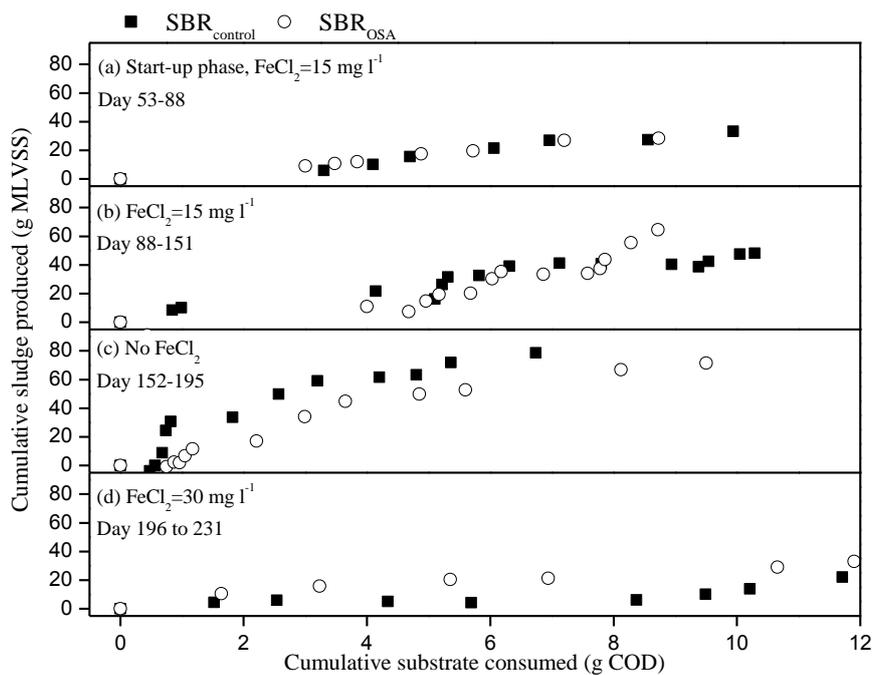


Figure 4

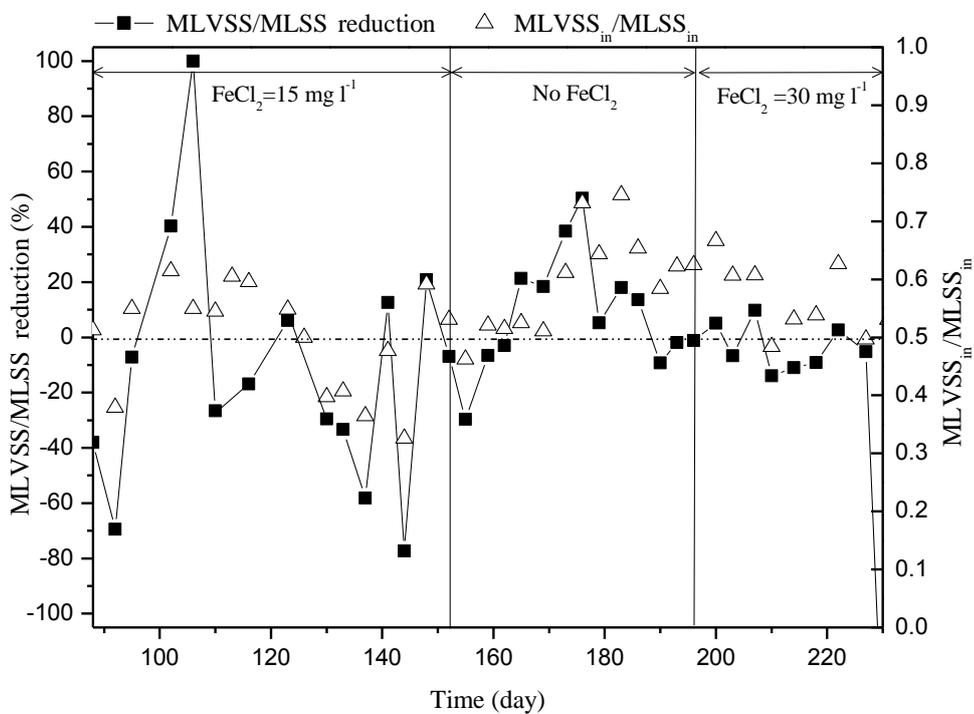


Figure 5

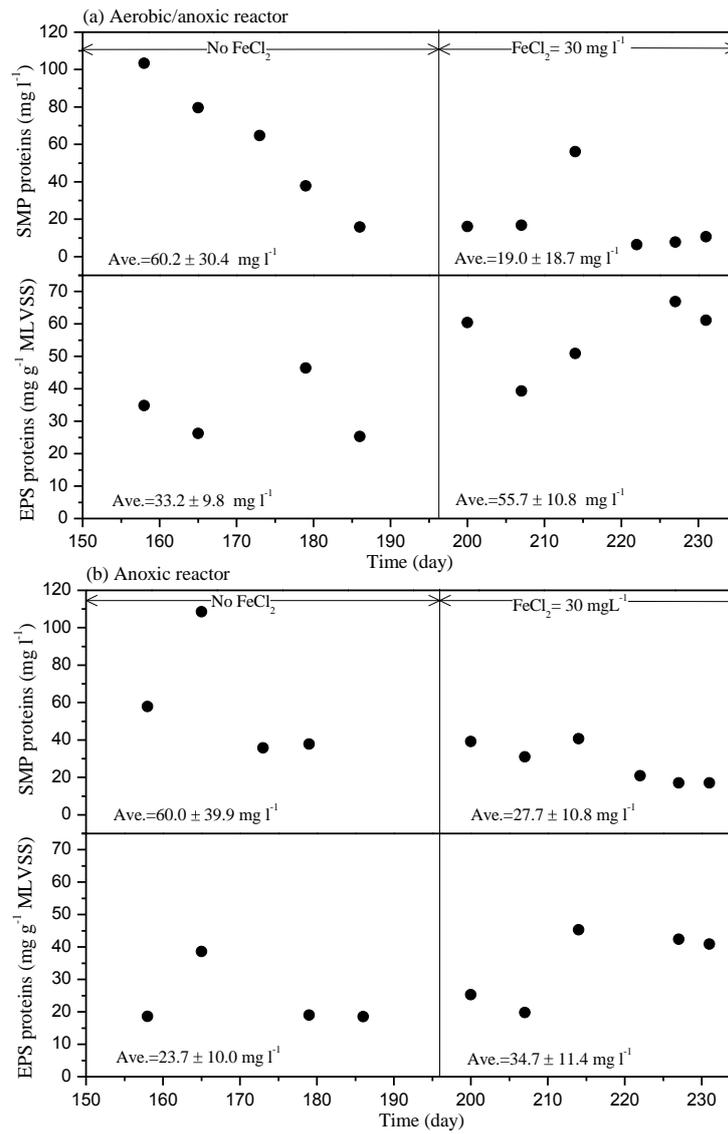


Figure 6

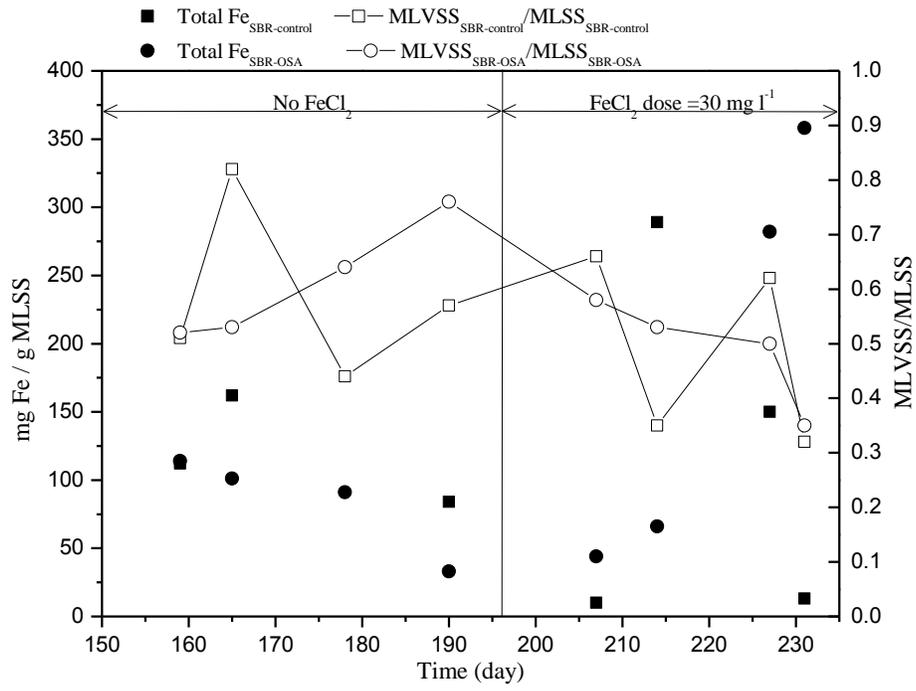


Figure 7

LIST OF TABLES

Table 1 MLVSS/MLSS reduction and average EPS and SMP of the batch reactors (average \pm standard deviation; n = number of measurements).

Table 2 Y_{obs} of control and OSA system with different dose of FeCl_2 to the influent (real wastewater).

Table 3 $\text{PO}_4^{3-}\text{-P}$ concentration and Fe/P molar ratio in the influent (real wastewater) at different phases of the experiment.

Table 1 MLVSS/MLSS reduction and average EPS and SMP of the batch reactors (average \pm standard deviation; n = number of measurements).

Batch reactor	MLVSS/MLSS reduction ^a (%)	EPS ^b				SMP ^b			
		Protein (mg g ⁻¹ MLVSS)	n	Carbohydrate (mg g ⁻¹ MLVSS)	n	Protein (mg l ⁻¹)	n	Carbohydrate (mg l ⁻¹)	n
aerobic/anoxic	25	5.4 \pm 3.6	5	1.0 \pm 0.6	5	9.8 \pm 1.6	5	6.3 \pm 2.8	5
aerobic/anoxic +FeCl ₂	18	10.1 \pm 2.9	5	1.3 \pm 0.4	5	7.7 \pm 0.7	5	14.0 \pm 6.3	5
anoxic	24	21.1 \pm 15.9	3	7.3 \pm 4.9	3	16.7 \pm 5.8	3	18.6 \pm 12.2	3
anoxic +FeCl ₂	29	12.8 \pm 8.7	3	6.1 \pm 5.1	3	30 \pm 11.3	3	21.5 \pm 17.4	3

^aMLVSS/MLSS reduction calculated at Day 30^bAverage of measurements obtained from Day 14 to 30**Table 2** Y_{obs} of control and OSA system with different dose of FeCl₂ to the influent (real wastewater).

Experimental phase	Y _{obs} (g MLVSS g ⁻¹ COD)							
	SBR _{control}	R ²	Control system ^b	R ²	SBR _{OSA}	R ²	OSA system ^c	R ²
Start-up ^a , FeCl ₂ = 15 mg l ⁻¹	3.61	0.94	-	-	3.51	0.98	-	-
FeCl ₂ = 15 mg l ⁻¹	4.29	0.91	3.90	0.73	7.62	0.87	9.72	0.85
No FeCl ₂	10.54	0.85	8.75	0.87	7.87	0.93	6.69	0.96
FeCl ₂ = 30 mg l ⁻¹	1.47	0.77	1.14	0.66	2.67	0.97	2.74	0.95

^a Before attaching the external reactors to the SBRs^b Control system consisted of SBR_{control} and aerobic digester^c OSA system consisted of SBR_{OSA} and external aerobic/anoxic and anoxic reactors

Table 3 PO₄³⁻-P concentration and Fe/P molar ratio in the influent (real wastewater) at different phases of the experiment.

Experimental phase	n	Minimum influent PO ₄ ³⁻ -P (mg l ⁻¹)	Maximum influent PO ₄ ³⁻ -P (mg l ⁻¹)	Average influent PO ₄ ³⁻ -P concentration (mg l ⁻¹)	PO ₄ ³⁻ -P standard deviation (mg l ⁻¹)	Minimum influent molar Fe/P	Maximum influent molar Fe/P	Average influent molar Fe/P
FeCl ₂ =15 mg l ⁻¹	12	3.22	17.96	8.79	3.75	0.62	3.47	1.27
No FeCl ₂	12	5.01	22.3	14.65	5.90	NA	NA	NA
FeCl ₂ =30 mg l ⁻¹	10	17.30	31.90	26.23	1.71	0.69	1.28	0.85

Effects of iron salt addition on biosolids reduction by oxic-settling-anoxic (OSA) process

Supplementary Data
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Table S1. Calculation of sludge yield

The MLVSS produced by SBR_{control} ($P_{SBR-control}$) and SBR_{OSA} ($P_{SBR-OSA}$) at a given time interval were quantified using a mass balance of biomass and shown in Equation S.1 and S.2, respectively:

$$P_{SBR-control} = \Delta MLVSS_{SBR-control} \times V_{SBR-control} \times (VSS_{e1} \times X_{out} + MLVSS_{SBR-control} \times W - VSS_i \times X_{in}) \times \Delta t \quad S.1$$

$$P_{SBR-OSA} = \Delta MLVSS_{SBR-OSA} \times V_{SBR-OSA} + (VSS_{e2} \times X_{out} + MLVSS_{SBR-OSA} \times W - VSS_i \times X_{in} - MLVSS_{ANX} \times q_5) \times \Delta t \quad S.2$$

where $MLVSS_{SBR-control, SBR-OSA}$ or ANX is the biomass concentration of the SBRs and the anoxic reactor, $V_{SBR-control}$ or $SBR-OSA$ is the effective reactor volume, VSS_i is the volatile suspended solids concentration of the influent, VSS_{e1} or $e2$ is volatile suspended solids concentration of the effluent, X_{in} or out is flow rate of the influent or effluent, W is the flow rate of sludge wasted from the SBRs, q_5 is the flow rate of sludge returned from the anoxic reactor to SBR_{OSA} (Section 2.2), and t is time. Notably, VSS_i is deducted from the calculation of $P_{SBR-control}$ and $P_{SBR-OSA}$ to discount the significant amount of volatile solids carried by real wastewater (e.g. 0.1-0.5 g/L), and $MLVSS_{ANX}$ is deducted from the calculation of $P_{SBR-OSA}$ to discount the biomass that was only recycled back to SBR_{OSA} from the anoxic reactor.

Second, the Y_{obs} of the control (e.g., combined SBR_{control} and aerobic digester) and OSA (e.g., combined SBR_{OSA} and external aerobic/anoxic and anoxic reactors) systems were determined. The synthesis of cells in the aerobic digester and external reactors may occur even under limited substrate conditions when microorganisms consume products of cell lysis (Hao et al., 2010), so those reactors may also contribute to MLVSS production of the whole system. The MLVSS production of control ($P_{control}$) and OSA (P_{OSA}) systems were calculated using Equations S.3 and S.4, respectively:

$$P_{control} = \Delta MLVSS_{SBR-control} \times V_{SBR-control} + \Delta MLVSS_{AE} \times V_{AE} + (VSS_{e1} \times X_{out} + MLVSS_{AE} \times Q_{out} - VSS_i \times X_{in}) \times \Delta t \quad S.3$$

$$P_{OSA} = \Delta MLVSS_{SBR-OSA} \times V_{SBR-OSA} + \Delta MLVSS_{AE/ANX} \times V_{AE/ANX} + \Delta MLVSS_{ANX} \times V_{ANX} + (VSS_{e2} \times X_{out} + MLVSS_{AE/ANX} \times q_3 - VSS_i \times X_{in}) \times \Delta t \quad S.4$$

where $MLVSS_{AE/ANX}$ or ANX is the biomass concentration of the aerobic/anoxic and anoxic reactors, $V_{AE, AE/ANX}$ or ANX is the effective digester or reactor volume, Q_{out} is the flow rate of sludge wasted from the aerobic digester, and q_3 is flow rate of sludge wasted from the aerobic/anoxic reactor. Notably, the sludge interchanged within the external reactors and between SBR₂ and the external anoxic reactor were retained in the OSA system hence it is not necessary to deduct those sludge flows from the calculation of P_{OSA} .

The amount of substrate consumed C by individual SBRs and the control and OSA systems was calculated according to Equation S.5:

$$C = (COD_i - COD_{e1/e2}) \times X_{in} \quad S.5$$

where COD_i and $COD_{e1/e2}$ are the soluble COD of the influent and effluent, respectively.

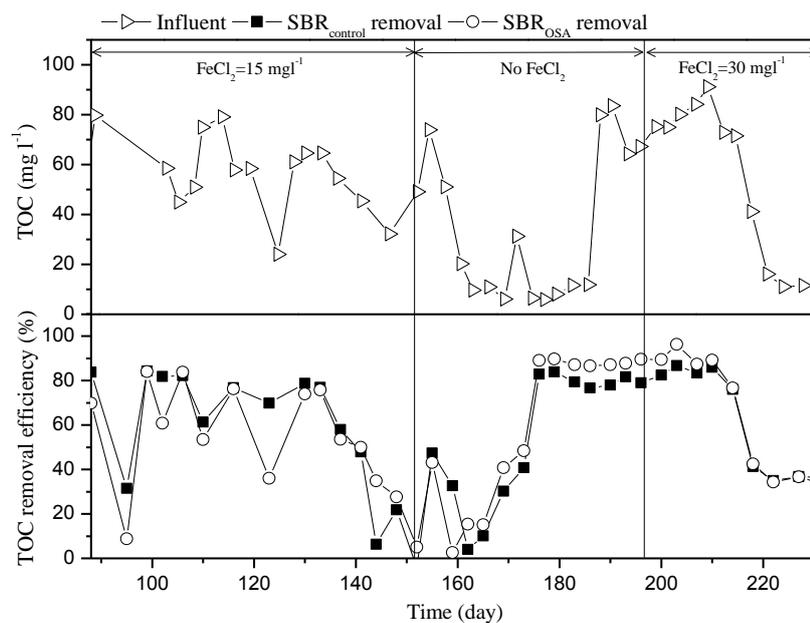


Figure S2. Influent TOC concentration and TOC removal efficiencies of SBR_{control} and SBR_{OSA} at different FeCl₂ dosage.

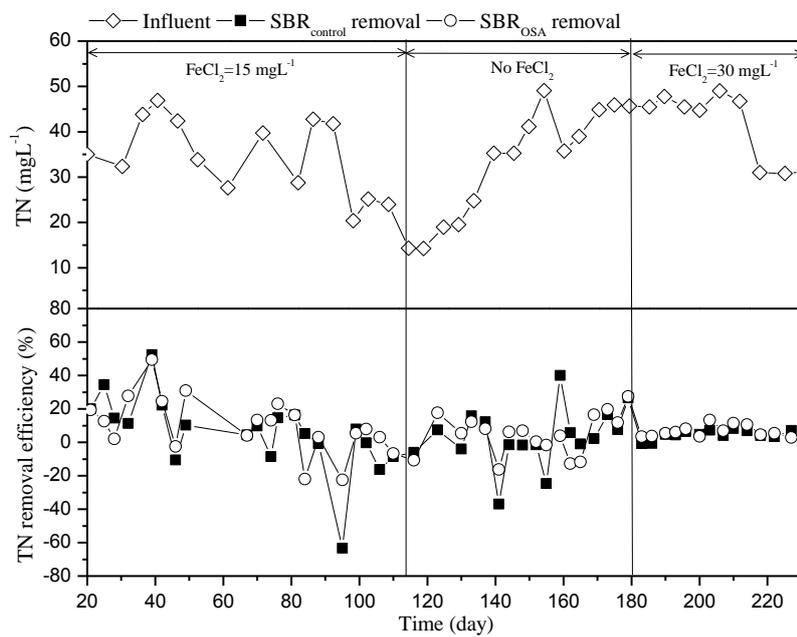


Figure S3. Influent TN concentration and TN removal efficiencies of SBR_{control} and SBR_{OSA} at different FeCl₂ dosage.

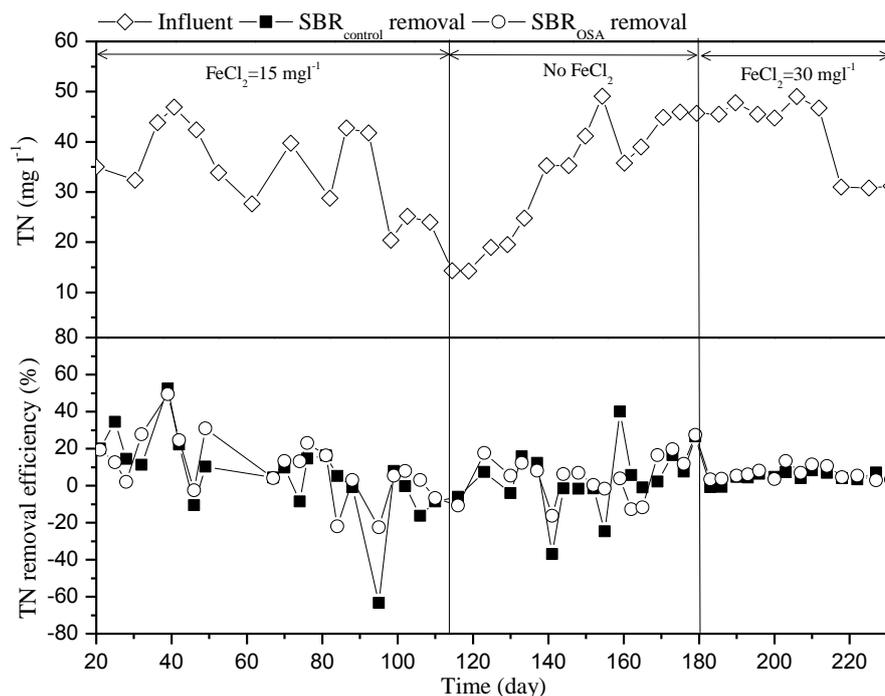


Figure S4. SVI of SBR_{control} and SBR_{OSA} at different FeCl₂ dosage.

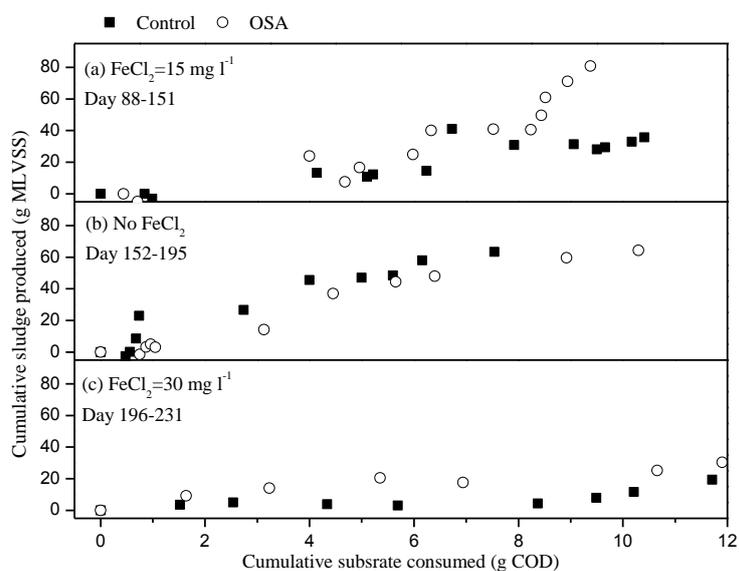


Figure S5. Cumulative sludge produced (g MLVSS) versus cumulative substrate consumed (g COD) of the control (*e.g.*, combined SBR_{control} and aerobic digester) and OSA (*e.g.*, combined SBR_{OSA} and external aerobic/anoxic and anoxic reactors) systems at different FeCl₂ dosage.

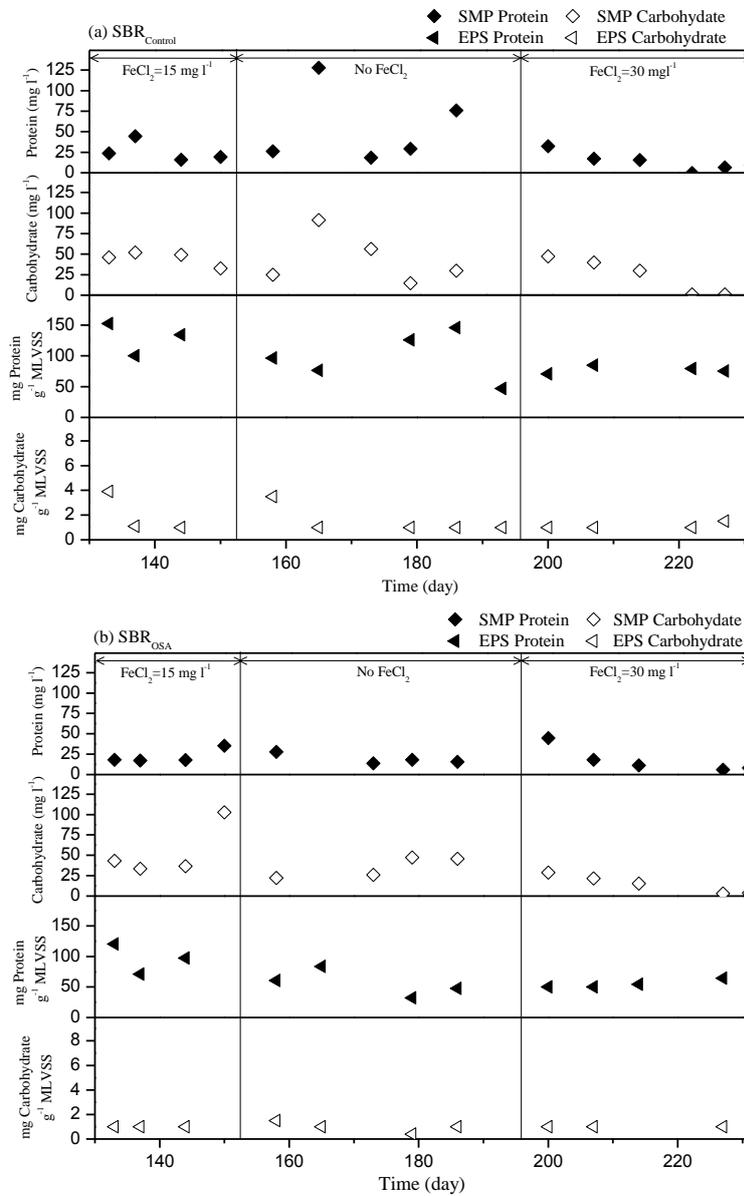


Figure S6. Iron-associated EPS and SMP profiles of $SBR_{control}$ (a) and SBR_{OSA} (b) at different $FeCl_2$ dosage.

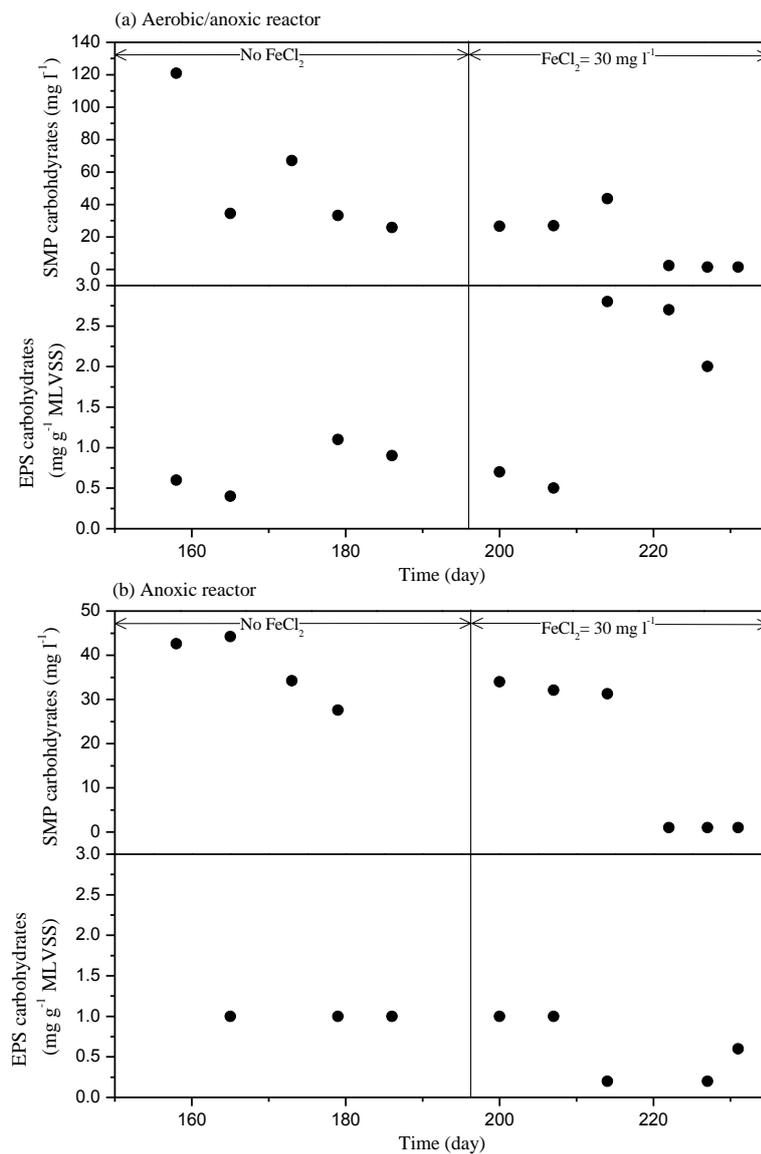


Figure S7. Iron-associated EPS and SMP in the form of carbohydrates of aerobic/anoxic (a) and anoxic (b) reactors at FeCl₂ dosage of zero and 30 mg l⁻¹.