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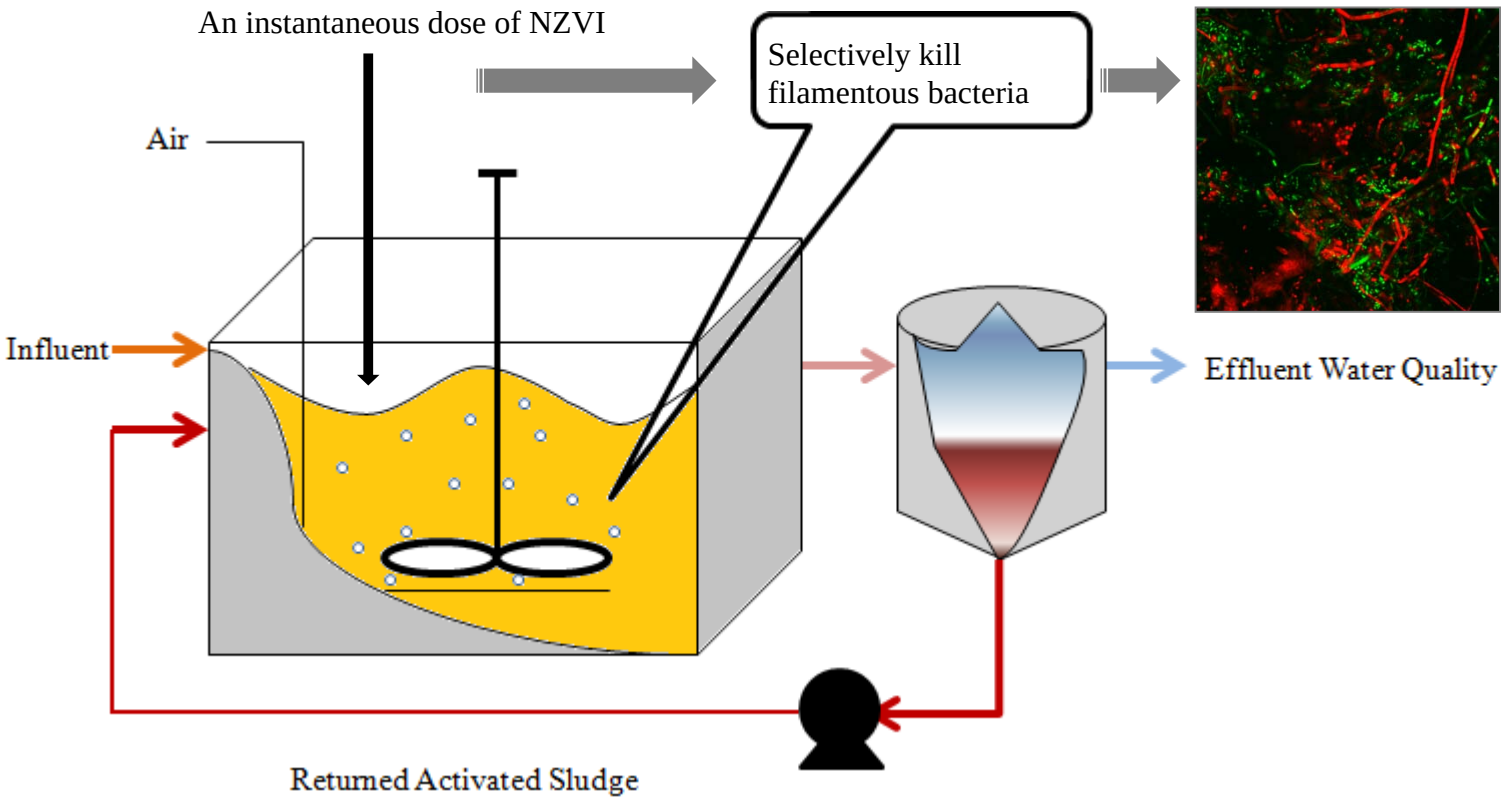

Title:

Filamentous sludge bulking control by nano zero-valent iron in activated sludge treatment systems

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Abstract

Sludge bulking causes loss of biomass in the effluent and deterioration of effluent water quality. This study explored the use of nano zero-valent iron (NZVI with an average particle size of 55 ± 11 nm) for sludge bulking control. In two Modified Ludzack-Ettinger (MLE) activated sludge treatment systems, a single dose of NZVI at the final concentration of 100 mg Fe/L in the mixed liquor reduced the number of filamentous bacteria Type 021N by 2-3 log units (a reduction of 99.9 and 96.7% in MLE tank #1 and #2, respectively). The side effect of the use of NZVI depended on sludge bulking conditions and biomass concentration. In the system with sludge bulking and significant sludge loss already (average biomass concentration of 1,022 ± 159 COD mg/L or at the ratio of 0.098 g Fe/g biomass COD), the use of NZVI increased effluent COD, NH_4^+-N and NO_2^--N concentrations, as also evident with loss of nitrifying populations and nitrifying activities resulting in more than 40 days to have the full recovery of the activated sludge system. In contrast, in the system with the early stages of bulking and the biomass concentration of 1,799 ± 113 COD mg/L (at the ratio of 0.056 g Fe/g biomass COD), the effluent water quality and overall bioreactor performance were only slightly affected for a few days.

Keywords: filamentous bacteria; nano zero-valent iron; sludge bulking control; qPCR; sludge volume index

Introduction

Sludge bulking, which is often caused by excessive growth of filamentous organisms in activated sludge, [1] results in poor sludge settling, sludge loss from secondary clarifiers and deterioration of effluent water quality. [2-5] The kinetic selection theory is commonly used to explain sludge bulking and to explore engineering solutions to control bulking. [4, 6] Most filamentous bacteria are slow growing organisms with lower maximum specific growth rate (μ_{max}) but higher affinity constant (Ks) than floc-forming bacteria [4] at low substrate concentrations. When the substrate concentration (S) is low (i.e., S << Ks), filamentous bacteria may have higher growth rates, thus outcompeting floc-forming bacteria (e.g., *Zoogloea*) in activated sludge. [7] Nowadays, selectors are therefore commonly used by creating a substrate concentration gradient to improve sludge settling. [6, 8] Metabolic selection is another approach for filamentous sludge bulking control as most filamentous bacteria cannot grow under anoxic or anaerobic conditions. [4] Other theories and factors such as substrate diffusion limitation, [9] intracellular polymer storage, [10, 11] and the difference in decay rates between filaments and floc-forming bacteria [12] have also been proposed. While an integrated framework combining kinetic selection and substrate diffusion limitation has been suggested for sludge bulking, [13] there is still no single mechanism that can fully explain the sludge bulking problems. In practice, the causes for filament growth in activated sludge treatment are complex and include factors such as low food-to-microorganisms (F/M), long solids retention time (SRT), low nutrients, low dissolved oxygen (DO), low pH or high sulfide levels. [1, 4, 14] Eikelboom type 021N (Type 021N), Type 1701, Type 0041, *Microthrix parvicella*, *Thiothrix* spp, *Gordonia* spp., among others, have been found to be reponsible for

most of the bulking and foaming events. [4, 15, 16]

Practical control methods for filamentous sludge bulking include specific and non-specific methods. [17, 18] Specific methods like the use of selectors are preferred as they eliminate the causes favorable for filamentous growth. In order to apply the principles of kinetic selection and metabolic selection, approaches such as modifying process operating conditions and process configurations are necessary but can be costly. [17] Non-specific methods can be a quick fix by adding toxicants (biocides) such as chlorine and hydrogen peroxide to improve sludge settleability. [1, 3] This approach is based on the fact that filaments protrude from the flocs are more susceptible to toxicant exposure, while most of floc-forming bacteria are embedded inside the flocs therefore protected from exposure to toxicants. Chlorination is the most widely applied method to control sludge bulking due to its low cost. Chlorine dose can be properly managed to control filamentous bulking without impairing nitrification performance. [1] However, chlorination often causes side effects on wastewater treatment performance by deflocculating activated sludge resulting in poor effluent water quality. [19, 20] There is also a concern about the development of chlorine-resistant filamentous bacteria in sludge. [3, 21] Other types of toxicants such as cetyl trimethylammonium bromide (CTAB), ozone and hydrogen peroxide are generally too costly to use, not mention the generation of harmful disinfection byproducts (DPBs), odor and chemical scum. [1, 3] Synthetic polymers and coagulants (e.g. lime, iron salts) may also be used to improve sludge sedimentation through bridging between flocs, [22] but coagulation/flocculation did not kill filamentous bacteria.

Nanomaterials having antiseptic (antimicrobial) properties may have beneficial uses in wastewater treatment. Nano zero-valent iron (NZVI) is one of the most commonly used and studied engineered nanoparticles due to its broad applications. [23, 24] NZVI has been evaluated in wastewater treatment for nitrogen removal through chemical reduction of nitrate [24, 25] and phosphate removal through chemical precipitation. [26] The associated release of Fe^{2+} due to oxidative dissolution of NZVI helps sludge flocculation and settling. [27] More importantly, NZVI is an effective biocide that can kill a broad range of microorganisms [28-30] with its mode of action through reductive decomposition of cell membrane due to strong reducing conditions ($E_H^{\circ}(Fe^{2+}/Fe)$ = -0.447 V) at the NZVI surface. [23, 29] The antibacterial effect of NZVI may also involve the generation of intracellular reactive oxygen species (ROS) by dissociative recombination of H_3O^+ ($H_3O^+ + e^- \rightarrow {}^{\bullet}HO + H_2$) catalyzed by Fe^0/Fe (II). [31] Remarkably, NZVI was also reported to be highly selective, [32] with its EC_{50} on cyanobacteria 20-100 times lower than that on algae, daphnids, water plants and fishes. It is well established that the filaments have higher cell loss from biocide because a larger fraction of their population compared to floc formers is exposed to the bulk liquid. [1] Similarly, because both filaments and NZVI have high surface/volume ratios, it is hypothesized that filamentous bacteria are more susceptible to NZVI exposure than floc-forming bacteria while agglomeration of nanoparticles in sludge may reduce the negative effect of NZVI on floc formers, thus resulting in selective removal of filamentous species. The main objective of this research was to explore the use of NZVI for sludge bulking control and to determine the side effect of the use of NZVI, which is likely related to sludge bulking conditions and biomass concentration (or NZVI/biomass ratio) in activated sludge wastewater treatment systems.

Materials and Methods

Nano zero-valent iron synthesis

NZVI particles were synthesized by the sodium borohydride reduction method ($2Fe^{2+} + BH_4^- + 3H_2O \rightarrow 2Fe^0 + H_2BO_3^- + 4H^+ + 2H_2$). A diluted carboxymethyl cellulose (CMC, Sigma-Aldrich, St. Louis, MO) solution (0.2%, w/w) served as a capping agent. [33] Briefly, 160 mL of the CMC solution was sparged with nitrogen for at least 20 min before use. Then 40 mL of freshly prepared $FeCl_2 \cdot 4H_2O$ (0.25 M) was gradually added to the CMC solution under nitrogen gas protection. Finally, a total of 50 mL freshly prepared $NaBH_4$ (0.4 M, Sigma-Aldrich) solution was added dropwise to the CMC solution that was magnetically stirred at 1,100 rpm at room temperature. Nitrogen sparging was continued for another 10 min to remove hydrogen gas. The final concentrations of NZVI in the solution were 0.04 M. The NZVI stock suspension was purged with nitrogen gas throughout the synthesis process to ensure that only nano-Fe^0 was formed. [23] The NZVI particles had an average size of 55 ± 11 nm as reported in our recent study. [34]

Bioreactor set-up and operation

Replicate lab-scale activated sludge systems (Tanks #1 and #2) employing Modified Ludzack-Ettinger (MLE) process were used in this study. The MLE process was chosen so that the approach of metabolic selection was applied in sludge bulking control while filamentous species such as Type 021N would not be affected. [4] Each system had a working volume of 7.4 L and consisted of anoxic and aerobic chambers separated by a glass baffle.

The effective volumes of the anoxic, aerobic, and internal settling chambers were 1.9, 3.8 and 1.7 L, respectively. The feed flow rate was set at 7.2 L/d resulting in an average hydraulic retention time (HRT) of 0.8 d. There was a recirculation at a flow rate equal to the influent flow rate from the aerobic chamber to the anoxic chamber in each MLE tank. For each bioreactor, a fine bubble diffuser in conjunction with the use of a magnetic stirrer provided mixing and aeration in the aeration chamber to maintain DO concentrations of 2-4 mg/L and only a magnetic stirrer was used to provide mixing in the anoxic chamber. Both bioreactors were inoculated with activated sludge obtained from the Columbia WWTP (Columbia, MO) and fed with synthetic wastewater. The synthetic wastewater (pH = 6.9 $\pm$ 0.1) prepared with tap water mainly contained nonfat dry milk powder with a target chemical oxygen demand (COD) concentration of 400 mg/L, 40 mg/L total nitrogen, 25 mg/L NH_4^+-N and 8 mg/L PO_4^{3-}-P. It also contained the following macro- and micronutrients per liter: 44 mg $MgSO_4$, 14 mg $CaCl_2{\cdot}2H_2O$, 2 mg $FeCl_2{\cdot}4H_2O$, 3.4 mg $MnSO_4{\cdot}H_2O$, 1.2 mg $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.8 mg $CuSO_4$, 0.3 mg $NiSO_4{\cdot}6\ H_2O$, and 1.8 mg $Zn(NO_3)_2{\cdot}6H_2O$. [35] The wastewater was prepared every 3 days and stored at room temperature (23 ± 1 ºC) in a covered 130 L (volume) plastic storage bin.

The bioreactors were operated and monitored for 150 days after the start-up period, and divided into two phases. Phase I lasted for the first 60 days at the target SRT of 10 days by wasting the mixed liquor directly from the aerobic chamber. Phase II started from day 61 onwards at a long SRT (20 days) associated with high bulking potential. To determine bulking conditions, the sludge volume index (SVI) was regularly monitored following the

145 standard methods [36] with modification (by taking 100-mL of the waste sludge). Through SVI
146 measurements and microscopic observations, an instantaneous dose of NZVI in the anoxic
147 chamber at the final concentration of 100 mg Fe/L in the entire mixed liquor was applied for
148 sludge bulking control on day 89 and day 104 for Tank #1 and #2, respectively. The NZVI
149 concentration was selected based on the results from a microtiter assay (details in Supporting
150 Information or SI).
151
152 **Filamentous bacterial DNA and polymerase chain reaction analysis**
153 Bacterial DNA samples were collected from each bioreactor before and after NZVI dosing at
154 a predetermined time. Total genomic DNA was extracted from the mixed liquor taken from
155 the aeration chamber using a MoBio UltracleanTM Soil DNA Isolation Kit (MioBio
156 Laboratories, Inc., Carlsbad, CA). An average of 0.5 g biomass was collected in DNA
157 extraction. The DNA was quantified by Nanodrop ND 1000 (NanoDrop Technologies,
158 Wilmington, NC, USA) and its purity was analyzed by measuring the 260/280 nm absorbance
159 ratio. The extracted DNA samples were stored at -20˚C before use.
160
161 A broad range of filamentous bacteria including *Microthrix parvicella*, Eikelboom type 021N,
162 *Gordonia* spp., *Thiothrix eikelboomii* were detected in the MLE systems by conventional
163 polymerase chain reaction (PCR) methods as described elsewhere. [37-39] All primers were
164 synthesized by Integrated DNA Technologies (Coralville, IA) and their detailed sequence
165 information is available in SI Table S1.
166
167 For quantitative microbial analysis, Type 021N was selected as a representative filamentous

168 species through quantitative real-time PCR (qPCR) analysis. Type 021N stands for a large
169 group of filamentous bacteria and their growth is strongly related to an unbalanced influent
170 composition, low molecular weight organic substrates and low oxygen concentrations in
171 aeration tanks. [1, 17, 40] The bacteria have also been shown to be present at moderate to high
172 SRT. [17] The qPCR assays were performed with the ABI 7500 Real time PCR System and the
173 7500 SDS system software (version 1.4, Applied Biosystems, CA), according to the protocols
174 described previously with modification. [38] To avoid PCR inhibition, NZVI/iron ions in the
175 DNA samples were removed with EDTA following a method described previously. [41]
176 Detailed information of qPCR procedure is available in SI Methods.
177
178 The copy numbers of 16S rRNA genes of Type 021N and total bacteria in all the samples
179 were determined at least in triplicate. Standard curves (mean cycle threshold (Ct) value of
180 triplicate assays versus log of cell number/PCR reaction) were constructed through serial
181 dilutions of plasmid DNA carrying a cloned 16S rRNA gene of Type 021N or total bacteria
182 using a TOPO® TA Cloning® kit (Invitrogen, CA). The PCR amplification efficiencies for
183 Type 021N and total bacteria were 92.4% and 94.8%, respectively (SI Figure S1). The
184 standard curves of the PCR assay without EDTA and $MgCl_2$ served as control to determine
185 the effect of EDTA and $MgCl_2$ on PCR amplification efficiencies (SI Figure S1). The
186 quantified 16S rRNA copy numbers were converted to cell numbers, according to the
187 genomic information (available at http://www.microbesonline.org), with the assumption that
188 Type 021N cell contains one 16S rRNA gene copy and total bacterial cell contains an average
189 of 3.6 16S rRNA gene copies. [42]

Effect of NZVI dosing on nitrifying bacterial population and nitrifying activity

The side effect of NZVI dosing in activated sludge treatment systems was inferred from its effect on the growth of sensitive nitrifying bacteria, which include ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). To analyze the impact of NZVI on the nitrifying population, the collected DNA samples were analyzed by Terminal Restriction Fragment Length Polymorphism (T-RFLP) targeting the 16S rRNA genes of AOB [43, 44] and NOB, [45] during which a fluorescent dye, 6-FAM, was incorporated at the 5' end of the labeled oligonucleotides. PCRs were conducted in a PCR DNA thermocycler (Eppendorf, Westbury, NY). The thermal profiles used for each PCR amplification have been described elsewhere. [46] The PCR amplification products were purified and digested with *MspI* restriction endonuclease (Promega, Madison, WI) at 37 °C for 3 hrs. The DNA products were then diluted 10 times and run through an ABI 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA) at the University of Missouri DNA Core Facility. An internal lane standard ranging from 20-600 bases (Genescan 600 LIZ) was added to each sample for precise sizing of each fragment by adjusting for lane to lane loading variation.

To determine the change in nitrifying bacterial activity, aliquots of mixed liquor were periodically taken from the aeration chamber to determine the specific oxygen uptake rates (SOUR), with detailed procedures described in Supporting Information.

211 **Microscopic, chemical and statistical analysis**

212 Activated sludge in the aeration chamber was periodically subjected to light microscopic
213 examination (Axioskop Zeiss microscope). One day after NZVI dosing into each MLE
214 bioreactor, the activated sludge samples were subjected to live/dead analysis after fluorescent
215 staining with the LIVE/DEAD® BacLight™ bacterial viability kit (Invitrogen Co., Carlsbad,
216 CA), according to the work reported elsewhere. [47] A laser-scanning confocal microscope
217 (Zeiss LSM 510 META) was used for fluorescence imaging of bacterial cells.

218

219 The influent and effluent water quality parameters such as COD, NH_4^+-N, NO_2^--N, NO_3^--N,
220 and orthophosphorus in the MLEs were measured in duplicate following the standard
221 methods. [36] Biomass concentration was measured in COD units. [48, 49] One-way ANOVA
222 analysis was conducted to assess the significance of the differences among groups, with $p <$
223 0.05 indicating statistical significance.

224

225 **Results and Discussion**

226 **Sludge bulking associated with long SRT operation and bioreactor performance**

227 Both MLE bioreactors were initially operated at the SRT of 10 days for about two months.
228 The SRT increased to 20 days from day 61 onwards. As long SRT operation often favors
229 filamentous bacterial growth [4] while short SRTs (< 5.7 d) suppress the growth of filamentous
230 bacteria (*Microthrix parvicella*), [50] an increase in SRT from 10 to 20 days encouraged sludge
231 bulking as indicated from the SVI measurements and confirmed by light microscopy (Figure
232 1 and SI Figure S2). In Tank #1, the SVI value increased from < 100 mL/g at the SRT of 10

233 days to 333 mL/g after about 20 days of operation at the SRT of 20 days. For comparison, the
234 SVI increase was slower in Tank #2, where the SVI increased from < 100 mL/g at the SRT of
235 10 days to 210 mL/g after about 40 days of operation at the SRT of 20 days (Figure 1).
236 Though SVI values above 150 mL/g indicate sludge bulking, [51] the different trends in SVI
237 change suggest the uncertainty and complex sludge bulking mechanisms involved in each
238 bioreactor, even though the two tanks were identical and operated at the same HRT and SRT.
239
240 Correspondingly, the degree of loss of sludge differed between the two bioreactors during
241 sludge bulking. At the SRT of 10 days with no evidence of sludge bulking, the average
242 biomass COD concentrations in Tank #1 and #2 were 2,332 ± 255 mg/L and 2,269 ± 235
243 mg/L, respectively (Figure 2). There was no significant difference in the biomass
244 concentration between the two bioreactors ($p = 0.67$). At the SRT of 20 days, due to sludge
245 loss in the effluent associated with bulking, the biomass COD concentration in Tank #1
246 gradually reduced to 1,022 ± 159 mg/L in on day 89. For comparison, in Tank #2, the
247 biomass COD concentration was only reduced to 1,799 ± 113 mg/L on day 104 (after about
248 40 days of operation at an average SRT of 20 days). The SVI data (Figure 1) and microscopic
249 observation (SI Figure S2) also confirmed that sludge in Tank #1 was already bulking
250 resulting in sludge loss while sludge in Tank #2 was in the early stages of bulking.
251
252 Figures 3 and 4 demonstrate that sludge bulking affected effluent water quality. At the SRT of
253 10 days (with no sludge bulking) and influent COD concentration of 403 ± 47 mg/L, the
254 effluent COD concentrations from Tank #1 and #2 were 21 ± 5 mg/L and 20 ± 5 mg/L,

255 respectively, resulting in a similar average removal efficiency of 95% (Figure 3). There were
256 also no significant differences in effluent NH_4^+-N ($p = 0.75$), NO_2^--N ($p = 0.73$) or NO_3^--N (p
257 $= 0.66$) concentrations between the two MLE bioreactors. The effluent NH_4^+-N
258 concentrations from Tank #1 and #2 were 0.4 ± 0.1 mg/L and 0.4 ± 0.2 mg/L, respectively,
259 with removal efficiencies of 98% and 99% respectively, indicating almost complete
260 nitrification (Figure 4). Correspondingly, the effluent NO_2^--N concentrations from Tank #1
261 and #2 were 0.4 ± 0.6 mg/L and 0.5 ± 0.7 mg/L, respectively, and the effluent NO_3^--N
262 concentrations were 20.2 ± 1.9 mg/L and 20.4 ± 1.9 mg/L, respectively.
263
264 As the SRT was increased to 20 days from day 61 onwards, the average effluent COD
265 concentrations before NZVI dosing in Tank #1 and #2 increased to 40 ± 13 mg/L and 37 ± 7
266 mg/L, respectively (Figure 3), which was mainly attributed to the loss of sludge in the
267 effluent due to sludge bulking. Meanwhile, the average effluent NH_4^+-N and NO_2^--N
268 concentrations in Tank #1 increased significantly to 1.2 ± 1.4 mg/L and 1.9 ± 1.6 mg/L,
269 respectively, while the effluent NO_3^--N concentration decreased to 15.9 ± 6.3 mg/L. The
270 much higher effluent NH_4^+-N and NO_2^--N concentrations in Tank #1 were linked to its more
271 serious sludge bulking and biomass loss in the effluent, suggesting that nitrifying bacteria are
272 more easily washed out and susceptible to perturbation associated with filamentous sludge
273 bulking. For comparison, with the sludge in the early stages of bulking in Tank #2, the
274 average effluent NH_4^+-N and NO_2^--N concentrations before NZVI dosing remained relatively
275 low at 0.5 ± 0.2 mg/L and 0.4 ± 0.2 mg/L, respectively.
276

Effectiveness of NZVI in killing filamentous bacteria

Among the filamentous bacteria studied, Type 021N and *Gordonia* spp. were detected most often (Figures S4) while *Thiothrix eikelboomii* was only detected in Tank #1 (data not shown). Type 021N bacteria were found to be excessive at the SRT of 20 days in Tank #1 on days 88 and 89 and Tank #2 on day 104 (Figure 5). The bloom of Type 021N species in Tank # 2 was delayed for about 15 days with smaller population for unknown reasons, which was in agreement with the sludge bulking conditions observed through SVI measurements and light microscopy.

In Tank #1, before NZVI dosing, the average concentration of Type 021N were increased from $(2.00 \pm 0.69) \times 10^6$ cells/g biomass on day 40 to $(2.67 \pm 0.09) \times 10^9$ cells/g biomass on day 88 and $(2.43 \pm 0.07) \times 10^9$ cells/g biomass on day 89 (Figure 5a). One day after NZVI dosing, however, Type 021N was reduced to $(1.37 \pm 0.54) \times 10^6$ cells/g biomass, a significant decrease (in 3 log units). In the following 20 and 40 days, the average concentrations of Type 021N were $(1.35 \pm 0.80) \times 10^6$ cells/g biomass and $(9.56 \pm 0.26) \times 10^6$ cells/g biomass, respectively, indicating a slight and slow recovery of the bacteria under long SRT operation. In Tank #2, Type 021N species increased from $(2.70 \pm 0.69) \times 10^6$ cells/g biomass on day 40 to $(1.09 \pm 0.01) \times 10^9$ cells/g biomass on day 104 (before NZVI dosing) (Figure 5a). One day after NZVI treatment, the number of Type 021N was reduced to $(3.58 \pm 0.20) \times 10^7$ cells/g biomass, a significant decrease (in about 2 log units or a 96.7% reduction). After 20 more days of operation at the SRT of 20 days, the number of Type 021N remained low at $(3.40 \pm 0.23) \times 10^7$ cells/g biomass. For comparison, the average concentration of total bacteria

ranged from (1.38 ± 0) × 10^{11} cells/g biomass to (1.68 ± 0.15) × 10^{11} cells/g biomass in Tank #1 and from (1.30 ± 0.05) × 10^{11} cells/g biomass to (1.49 ± 0) × 10^{11} cells/g biomass in #2 throughout the study period (Figure 5b), which were generally consistent with the range of biomass concentrations in municipal WWTPs. [42] Type 021N was characterized by having extremely high SVI values. [52] The results demonstrated the successful use of NZVI in sludge bulking control by significantly reducing the number of Type 021N bacteria.

Due to its high specific surface area, it is likely that NZVI was capable of directly attaching to the cell surface thus more likely killing filamentous bacteria, which also have high surface/volume ratio (SI Figure S3). Interestingly, the use of NZVI did not cause significant deflocculation one hour after the dosing while the nanoparticles appeared to be agglomerated. Live/dead staining results showed that unlike the control group (SI Figure S4), NZVI effectively killed the filamentous bacteria in both bioreactors, while a large fraction of floc-forming species were still alive with a larger amount of dead cells for the higher NZVI to biomass ratio in Tank #1 (Figure 6), because the filamentous bacteria are not protected by the floc. NZVI was also effective in killing *Gordonia* spp. through regular PCR analysis, as the disappeared bands right after NZVI dosing indicated the significant bactericidal effect of NZVI against *Gordonia* (SI Figure S5).

Impact of sludge bulking and NZVI dosing on nitrifying bacterial population and activity

T-RFLP analysis (SI Figure S6) shows the change in nitrifying bacterial community structure

in Tank #1 before and after NZVI dosing. On day 40, *Nitrosomonas* was the dominant genus of AOB while *Nitrospira* was dominant among NOB. With significant sludge bulking on day 89, there was a large decrease in the AOB population as indicated from the change in peak intensity (at 155 bp and 161 bp) of *Nitrosomonas*; in contrast, only one of the *Nitrospira* peaks (272 bp) was reduced considerably. Consistent with the higher effluent NH_4^+-N and NO_2^--N concentrations, the decrease in *Nitrosomonas* and *Nitrospira* population was attributed to overgrowth of filamentous bacteria and associated biomass loss in the effluent. Additional decrease in the numbers of *Nitrosomonas* and *Nitrospira* species were observed one day after NZVI dosing (day 90), showing the bactericidal effect of NZVI on nitrifying bacteria. Thereafter, the peak sizes of *Nitrosomonas* and *Nitrospira* species increased gradually as indicated from the data on days 110 and 130, correlating well with the effluent water quality data (Figures 3 and 4).

Furthermore, consistent with the change in nitrifying population and the effluent water quality, the autotrophic SOUR values in Tank #1 were decreased by 46 ± 4% due to sludge bulking on day 89 (SI Figure S7). One day after NZVI dosing, the nitrifying bacteria activity decreased further, with the full recovery of the activated sludge system taking more than 40 days. There was also a slight decrease in nitrifying activity in Tank # 2 on day 104 followed by a significant decrease (34 ± 1%) one day after NZVI dosing. Thereafter, the nitrifying bacteria activity was fully recovered within a few days.

Bioreactor performance recovery and other benefits associated with NZVI dosing

In Tank #1 with sludge bulking and sludge loss already (average biomass concentration of 1,022 ± 159 COD mg/L or at the ratio of 0.098 g Fe/g biomass COD), the use of NZVI caused a significant increase in effluent COD, NH_4^+-N and NO_2^--N concentrations with the full recovery of the activated sludge system taking more than 40 days (Figures 3 and 4). For comparison, in Tank #2 with the early stages of bulking and the biomass concentration of 1,799 ± 113 COD mg/L (at the ratio of 0.056 g Fe/g biomass COD), except for NH_4^+-N accumulation during the first week after NZVI dosing, the effluent COD concentration was stabilized at 22 ± 1 mg/L and effluent NH_4^+-N concentration was quickly reduced to 0.02 ± 0.03 mg/L (Figures 3 and 4). Hence, the effluent water quality and overall activated sludge bioreactor performance were only affected for a few days in Tank #2.

Additional benefits of the use of NZVI included an improved phosphorus removal and sludge settling. A single dose of NZVI resulted in fast and enhanced reduction of effluent PO_4^{3-}-P concentration (SI Figure S8), possibly due to iron phosphate precipitates (e.g., $Fe_3(PO_4)_2$, $FePO_4$, and $Fe_x(OH)_y(PO_4)_3$) [53] and the formation of iron oxides/hydroxides for P adsorption. [54] However, the effluent PO_4^{3-}-P concentrations resumed to the previous level three HRTs after NZVI treatment, indicating rapid loss of NZVI reactivity as nanoparticles were mainly associated with sludge. Nevertheless, due to the dissolution of NZVI, the oxidized forms (Fe^{2+}, Fe^{3+}) of iron could improve the sludge flocculation and settleability, as was also confirmed in this study where the SVI was generally below 100 mg/L in both bioreactors after the one-time NZVI treatment (Figure 1). Although more questions remain as to whether

or how the NZVI treated sludge would affect sludge digestion, it is expected that NZVI could be converted to iron ions and their complexes through fast NZVI dissolution (within an hour, data not shown) and therefore would not pose problems.

In chlorination-based bulking control, filamentous and floc-forming bacteria do not appear to largely differ in their chlorine susceptibility. Unlike chlorine, NZVI may serve as a new bulking control agent that can selectively kill filamentous organisms due to the unique fate and transport characteristics associated with NZVI dissolution and agglomeration (SI Figure S3). Nevertheless, the findings of this paper are more of an exploratory nature. A NZVI final concentration of 100 mg/L is very high, which shows side effects in a way that excess chlorine treatment does. More research is needed to show if there is a NZVI specific dose that can control filaments without causing nitrification inhibition by adjusting the particle size and dose of NZVI. Also there could be more complicated instances of filamentous bulking that are much more challenging to resolve in full-scale wastewater treatment plants. Further research is needed to design and test NZVI related nanomaterials for better sludge bulking control.

Conclusions

A new approach of filamentous sludge bulking control with NZVI was proposed. NZVI is an effective biocide. A single dose of NZVI at the final concentration of 100 mg Fe/L in the mixed liquor reduced filamentous bacteria such as Type 021N by 2-3 log units. Meanwhile, the nitrification efficiency was also reduced while the side effect of the use of NZVI

386 depended on sludge bulking conditions and sludge concentration. Because the filamentous
387 bacteria are not protected by the floc while agglomeration of nanoparticles in sludge may
388 reduce the negative effect of NZVI on floc formers, the research opens up the potential to use
389 NZVI as a more selective sludge bulking control agent.

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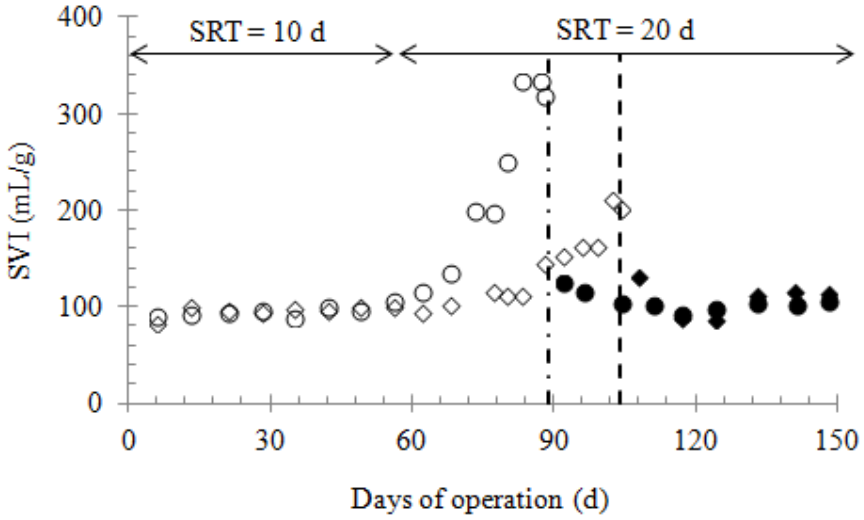

Figure 1. SVI values in Tank #1 before (○) and after (●) NZVI dosing on day 89 and SVI values in Tank #2 before (◇) and after (◆) after NZVI dosing on day 104. Two vertical hashed lines show the days of NZVI addition in Tanks #1 and #2, respectively.

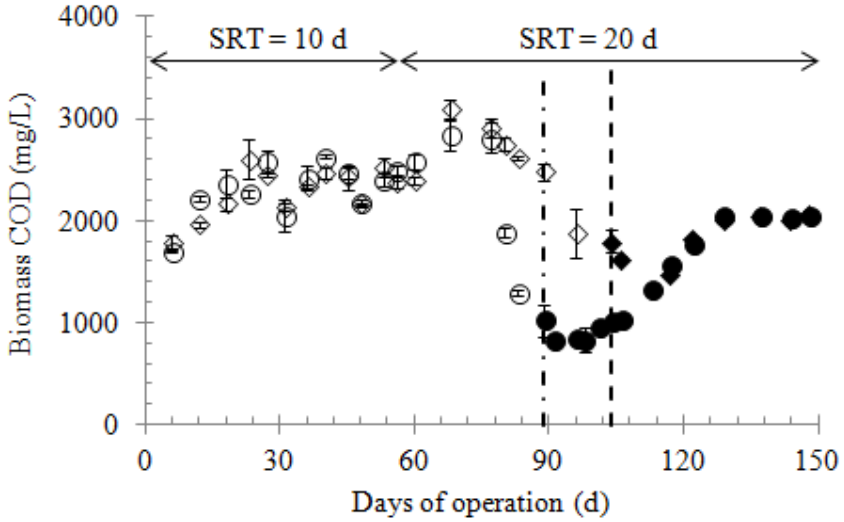

Figure 2. Biomass concentrations in Tank #1 (○) and Tank #2 (◇) before NZVI dosing and in Tank #1 (●) and Tank #2 (◆) after NZVI dosing on day 89 and day 104, respectively. Two vertical hashed lines show the days of NZVI addition in Tanks #1 and #2, respectively. The SRT was increased from 10 to 20 day from day 61 onwards. Error bars represent the range of duplicate samples.

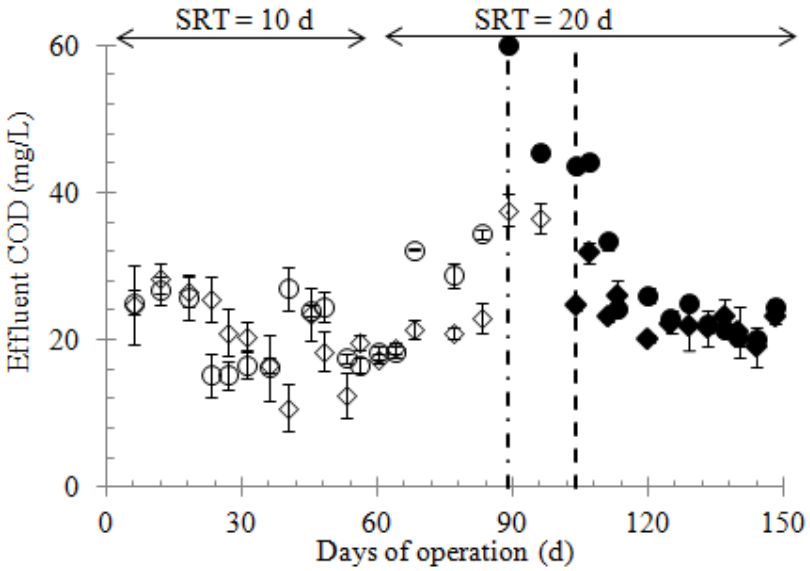

Figure 3. Effluent COD concentrations in Tank #1 (○) and Tank #2 (◇) before NZVI dosing and in Tank #1 (●) and Tank #2 (◆) after NZVI dosing on day 89 and day 104, respectively. Two vertical hashed lines show the days of NZVI addition in Tanks #1 and #2, respectively. Error bars represent the range of duplicate samples.

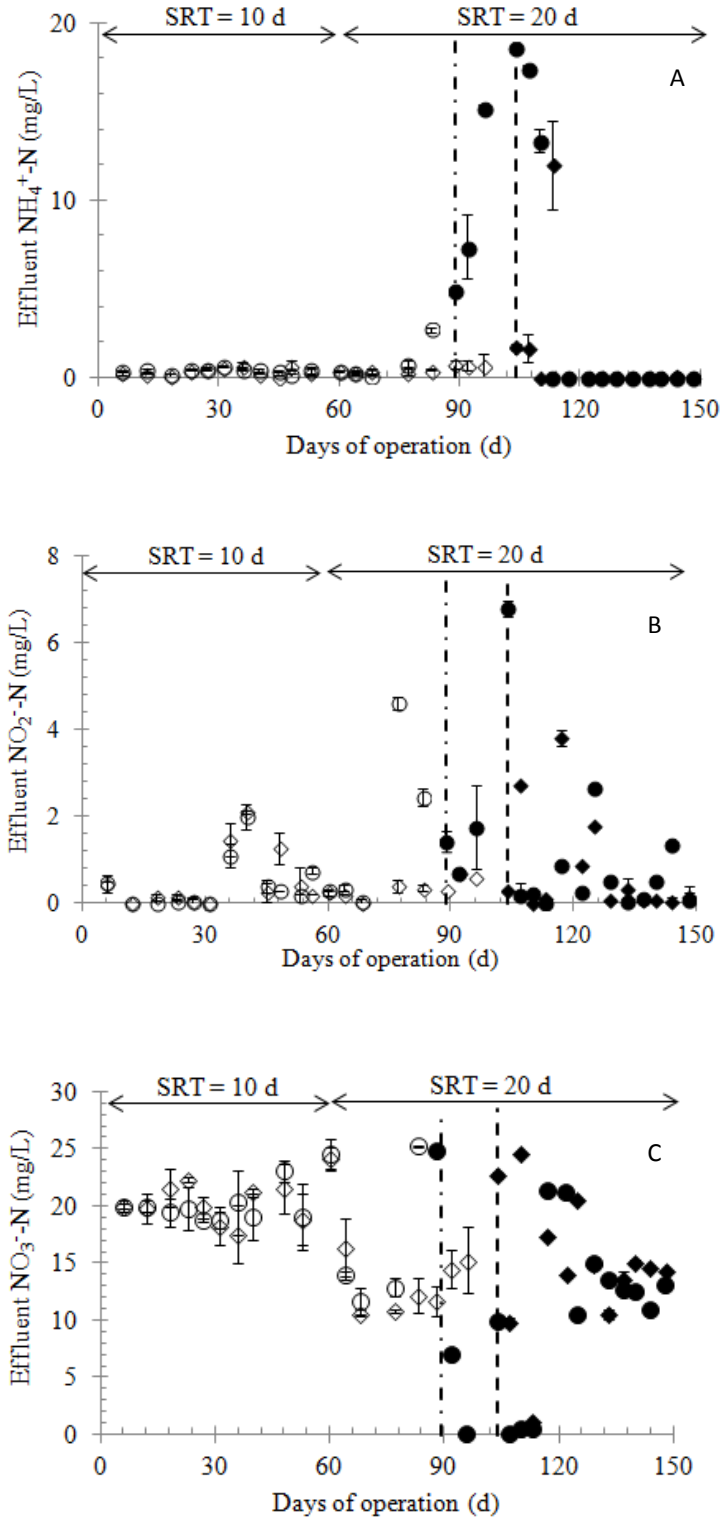

Figure 4. Effluent NH_4^+-N (A), NO_2^--N (B) and NO_3^--N (C) concentrations in Tank #1 (○) and Tank #2 (◇) before NZVI dosing and in Tank #1 (●) and Tank #2 (◆) after NZVI dosing on day 89 and day 104, respectively. Error bars represent the range of duplicate samples.

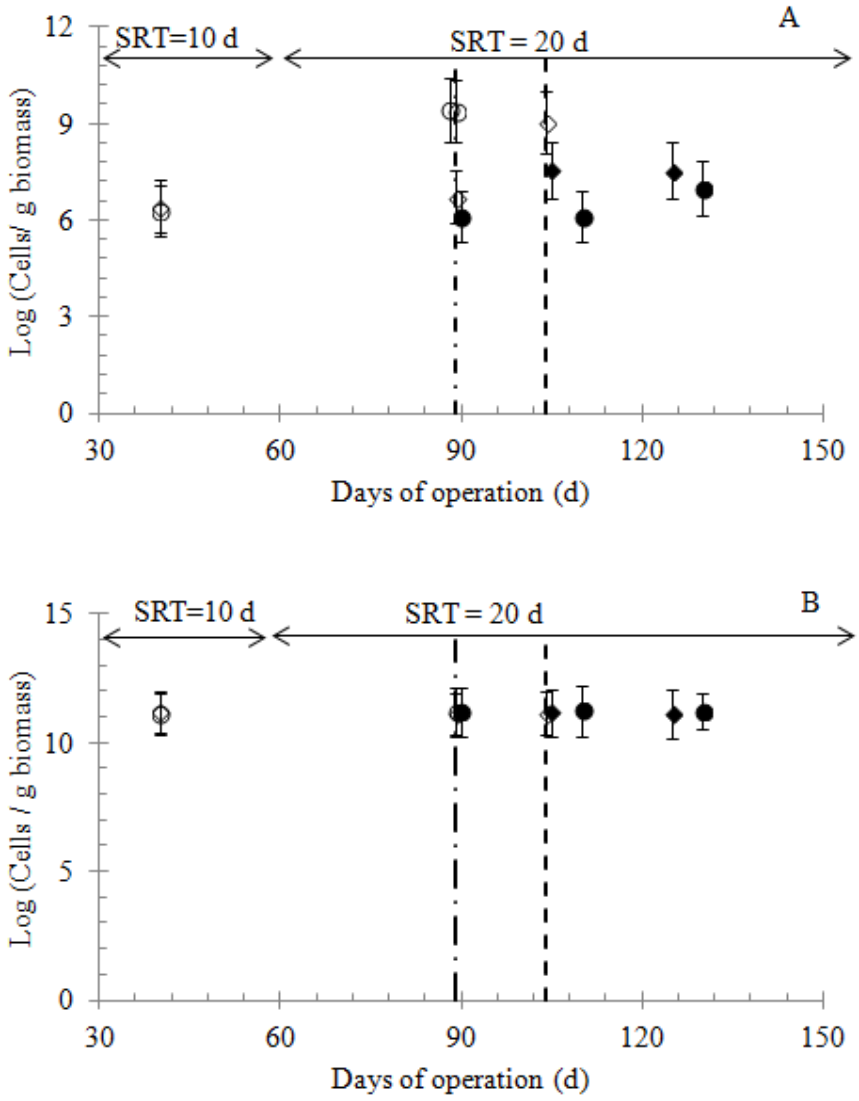

Figure 5. Type 021N (A) and total bacterial population (B) dynamics in Tank #1 and Tank #2 . A single dose of NZVI at the final concentration of 100 mg Fe/L in the mixed liquor was applied on day 89 and day 104 in Tank #1 and Tank #2, respectively. Two vertical hashed lines show the days of NZVI addition in Tanks #1 and #2, respectively. Error bars represent one standard deviation from the mean of at least triplicate samples.

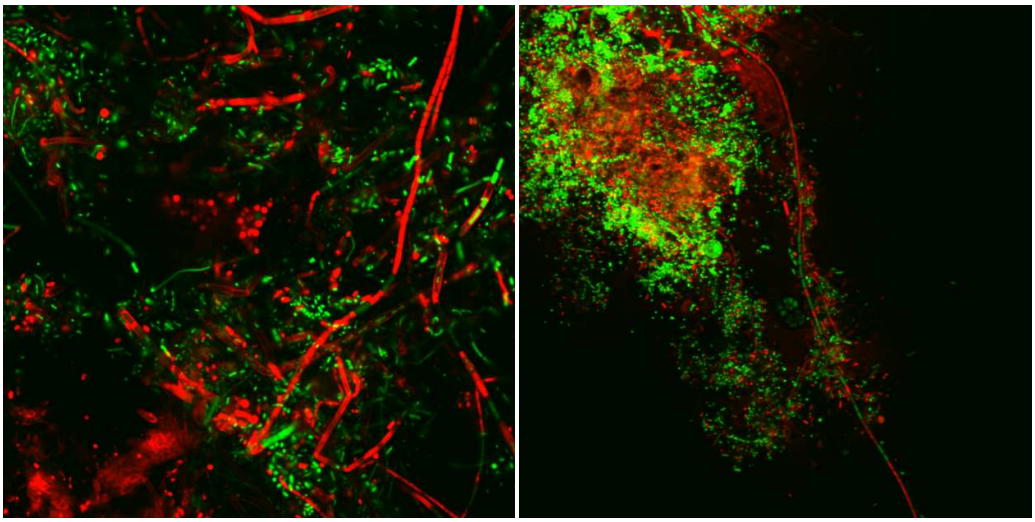

Figure 6. The viability of activated sludge from Tank #1 (with sludge bulking and significant sludge loss already, left) and Tank #2 (with the early stages of bulking, right) after the NZVI treatment on day 90 and 105, respectively. Under florescence microscopy, living cells were stained green and dead cells were stained red.