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Free nitrous acid breaks down extracellular polymeric substances in waste activated sludge

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ABSTRACT: Free nitrous acid (FNA) has been demonstrated to be effective in enhancing degradability of waste activated sludge (WAS). Considering that extracellular polymeric substances (EPS) are a major component in sludge flocs, the chemical breakdown of EPS components by FNA has been hypothesized to account for the improvement of sludge biodegradability in addition to enhanced cell lysis. EPS extracted from WAS was treated with FNA at 2.0 mg HNO_2-N/L (260 mgNO_2^--N/L and pH 5.5). The molecular weight distribution of EPS showed the breakdown of macromolecules into smaller molecules. The chemical structure analysis of EPS using Fourier transform infrared ascribed the breakdown to FNA-induced deamination of proteins, amino sugars and nuleic acids, implying that the main targets of FNA in EPS are protein-like substances. Particle size distribution analysis on the original WAS with the same FNA treatment revealed that FNA treatment of sludge significantly reduces the flocs sizes, which supported that FNA breaks down EPS in activated sludge flocs.

1. Introduction

Activated sludge processes produce substantial amounts of waste activated sludge (WAS), the treatment and disposal of which incur large costs.[1-3] Aerobic and anaerobic sludge digestion is the mainstream technology for sludge reduction and stabilization prior to disposal, but their effectiveness is limited by the poor degradability of WAS. Various methods including mechanical, thermal and chemical treatment have been proposed to enhance the WAS biodegradability prior to sludge digestion.[3-7] However, the above approaches require either intensive energy input (e.g. high pressure or high temperature) or large chemical consumption (e.g. chlorine, ozone or alkali), incurring substantial economic costs.

Free nitrous acid (FNA, i.e. HNO_2), a renewable and low cost chemical that can be produced on site by nitritation of the anaerobic digestion liquor,[8] has been demonstrated to be effective in causing cell lysis[9, 10] and improving sludge biodegradability.[11] For example, aerobic digestion tests showed that the degradation of WAS with FNA pre-treatment at 2.0 mg N/L for 48 h was more than two times higher than that of WAS without FNA pre-treatment.[10] Wang et al.[12] reported that WAS production in a reactor treating synthetic domestic wastewater was reduced by 28% by treating part of the returned activated sludge with FNA at 2.0 mg N/L for 24 h. Recently, it has been demonstrated that methane production from a full-scale WAS, with FNA pre-treatment at 2.0 mg N/L for 24 h, was improved by approximately 30% at an anaerobic digestion time of 20 d in comparison with that from the WAS without

FNA pre-treatment.[11] The FNA-based sludge treatment technology was further proposed to be economically attractive[11-13], which poses a significant advantage over most of the current available cost-intensive technologies. However, a detailed understanding of the mechanisms involved in FNA treatment is still in need, which is essential for the optimization of the technology. It has been hypothesized that the improved sludge biodegradability was due to the enhanced cell lysis caused by FNA treatment.[9, 10, 12]

However, the sludge consists of not only cells, but also extracellular polymeric substances (EPS), which comprise different types of biopolymers (e.g. proteins and polysaccharides) to form the three-dimensional scaffold structure of the sludge aggregates and facilitate them function effectively. In fact, EPS can account for up to 33-42% of the volatile solids in sludge[12, 14] and their components and structure have significant impacts on sludge biodegradability.[15-18] Many studies have proven that both aerobic and anaerobic biodegradability of WAS was enhanced effectively by removing the EPS from the WAS flocs through lytic bacterial strains, bacteria secreted enzymes or surfactant.[19-22] Therefore, the enhanced cell lysis may not be the sole reason for the improved sludge biodegradability, and FNA may also break down EPS by reacting with the components and changing their chemical structures.

This study aims to verify the hypothesis that FNA breaks down EPS in activated sludge flocs, which accounts for the partial release of EPS into soluble phase and the

improvement of the sludge biodegradability in addition to the enhanced cell lysis. To verify this hypothesis, EPS extraction was used to investigate the working principles of the FNA-based sludge treatment technology. EPS extracted from WAS was treated with FNA at 2.0 mg HNO_2-N/L (260 mgNO_2^--N/L and pH 5.5), with untreated EPS and EPS treated at pH 5.5 alone as controls. The molecular weight (MW) distribution and the chemical structure of EPS were analyzed and compared by gel permeation chromatography (GPC) and Fourier transform infrared (FTIR), respectively. The same treatments were also directly applied to WAS and then the particle size distribution of sludge flocs was measured and compared.

2. Materials and methods

2.1. Sludge source

WAS used in this experimental study was collected from a local biological nutrient removal wastewater treatment plant treating domestic wastewater at three different time (27/08/2013, 04/05/2014 and 23/05/2014) with 2 L each time. Following collections, it was transported to the lab within 2 hours and stored at 4 ºC overnight prior to EPS extraction and sludge treatment as described in sections 2.2 and 2.3. The main characteristics of WAS were: total solids (TS) 9.6 ± 0.1 g/L, volatile solids (VS) 7.3 ± 0.1 g/L, total chemical oxygen demand (TCOD) 9.2 ± 0.0 g/L, soluble chemical oxygen demand (SCOD) 0.11 ± 0.01 g/L, and pH=7 ± 0.2 (with standard errors obtained from triplicate measurements of all samples).

96 **2.2. EPS extraction**

97 The sludge was firstly centrifuged at 2000 g, 4 ºC for 15 min and the supernatant was
98 decanted to remove the soluble microbial products in the bulk water. The pellet was
99 then dissolved to their original volume with extraction buffer consisting of 2 mM
100 Na_3PO_4, 4 mM NaH_2PO_4, 9 mM NaCl and 1 mM KCl at pH 7 with the similar ionic
101 strength to that in the original sludge according to [23] to extract both loosely and tightly
102 bound EPS. The modified cation exchange resin (CER) method (DOWEX 50x8,
103 20-50 mesh in Na^+, Sigma-Aldrich) was then used for EPS extraction, which is
104 capable of maintaining the biochemical properties of EPS components in comparison
105 with the chemical extraction methods[24]. The CER was added to the extraction beaker
106 with the ratio of 70 g/gVS with the redissolved sludge and the mixture was stirred at
107 600 rpm, 4 ºC for 6 hours. Afterwards, the mixture was settled for 5 mins for the
108 separation of the resin from the sludge. Then the mixture was centrifuged at 12000 g
109 at 4 ºC for 20 mins. The supernatant filtered through disposable Millipore filter units
110 (0.45 μm pore size) was the final EPS extract. EPS concentrations throughout the
111 study are expressed in bovine serum albumin (protein), D-glucose (carbohydrates) and
112 calf thymus (DNA) equivalents per gram VS. The yield of EPS extraction was 5%
113 (VS basis), which is comparable with the previous studies[23, 25]. The FTIR analyses on
114 both original sludge and EPS extract (data not shown) showed that their chemical
115 compositions are highly similar, suggesting that the EPS extract was sufficiently
116 representative for the chemical functional groups even though the extraction yield was
117 not very high. The main compositions were proteins (38.3 ± 1.8 mg/g VS) and

polysaccharides (19.1 ± 0.5 mg/g VS), accounting for up to 66% and 33% of the total EPS extract, respectively. The DNA concentration was 0.50 ± 0.02 mg/g VS, which is in consistency with previous studies[23, 25], indicating a minimal cell lysis during the extraction.

2.3. FNA treatment on EPS extract and WAS

The tests described below were carried out on all the three sludge samples collected at different time (see section 2.1).

In each set of tests, three batch reactors each with 100 mL EPS extract were used to investigate the effect of FNA on molecular weight distribution and chemical structures of the components in EPS extract. Two served as controls, with the other as the experimental reactor. The operating conditions are as summarised in Table 1. A nitrite stock solutions (40 g N/L) was added to the reactor to achieve the designed level of 260 mg N/L. Since the pH increased after FNA addition, 1 M HCl was used for pH control via a programmable logic controller. The FNA concentration achieved was estimated to be 2.0 mg HNO_2-N/L using the formula $S_{NO_2^- -N}/(K_a \times 10^{pH})$ with the K_a value determined as a function of temperature T (ºC) by $K_a = e^{-2,300/(273+T)}$ (22°C in this study)[23, 26]. The effectiveness of FNA on anaerobic methane production at different concentrations have been investigated. In combination with the economic analysis, FNA treatment at 2.0 mg HNO_2-N/L for 24 hours resulted in the highest methane production with significant economic advantages [11]. Therefore, these

conditions were selected in this study. After the 24-hour treatment, the EPS extract in each reactor was divided into two halves, with half for molecular weight distribution measurement and the other half freeze-dried for chemical structure analysis.

Table 1 Treatment conditions applied in this study

Treatment	FNA (mg N/L)	NO_2^--N (mg N/L)	pH	Treatment time (h)
Control I	0	0	6.8 - 7.0[a]	24
Control II	0	0	5.5±0.2[b]	24
FNA	2.0	260	5.5±0.2[b]	24

[a]pH was not controlled

[b]pH was controlled

In parallel to the above reactors, three other batch reactors each with 100 mL WAS, the same as that used for EPS extraction, were used to study the impacts of FNA treatment on particle size distribution of WAS. The WAS in the three reactors was subject to the same treatments conditions as applied to the EPS extract. The WAS collected after 24 hours treatment was analysed for its particle size distribution in less than two hours after the treatment completed.

2.4. Chemical analyses

Concentrations of proteins and DNA in the EPS extracts were analysed with Peirce™ Coomassie (Bradford) Protein Assay kit (Pierce Biotechnology, USA) and DNA

158 Quantification kit (Fluorescence assay using bisBenzimide H 33258, Sigma Aldrich,
159 USA), respectively. Carbohydrates were quantified with the Phenol-Sulfuric acid
160 method[27]. Bovine serum albumin (BSA), D-Glucose (Sigma Aldrich) and calf thymus
161 DNA were used to prepare standard curves in the same extraction buffer as EPS
162 extraction. To normalize the concentrations of the three major components in EPS, the
163 measured volume concentrations (mg BSA-, mg D-glucose- and mg calf-thymus-
164 equivalents/L) were then divided by the volatile solids concentration (g VS/L) in the
165 original sludge samples to obtain the mass-based concentrations (mg BSA-, mg
166 D-glucose- and mg calf-thymus- equivalents/g VS) All measurements were done in
167 triplicate. The concentrations of NO_2^-, NO_3^-, and NH_4^+ were determined using a
168 Lachat QuikChem8000 Flow Injection Analyzer (Lachat Instrument, Milwaukee,
169 Wisconsin). TS, VS, TCOD and SCOD was determined according to the standard
170 method[28].
171

172 **2.5. Analyses of molecular weight distribution and chemical structure of EPS**

173 The molecular weight (MW) distribution of EPS was analysed with Agilent Aqueous
174 Gel permeation chromatography 1260 Infinity System connected with refractive index
175 (RI) and ultraviolet (UV) detectors for data acquisition and equipped with Agilent
176 GPC/SEC software for operation and data analysis. Two Agilent Aqua gel PL
177 Aquagel-OH mixed-M, 8 µm columns were kept at 40°C with Milli-Q water as eluent
178 at a flow rate of 1 mL/min during the analysis. UV and RI detectors were used in
179 series to provide complementary information with regards to MW distribution of the

mixture consisting of UV non-absorbing (polysaccharides) and absorbing (protein, amino sugars and nucleic acids) constituents. The Pullulan Polysaccharide calibration kit (MW: 180, 667, 6,100, 9,600, 21,100, 47,100, 107,000, 194,000, 344,000, 708,000 g/mol) was used to establish the calibration curve.

The chemical structure of the components in EPS extract was analysed by Fourier transform infrared spectroscopy (FTIR) using Nicolet 5700 FTIR spectrometer with the resolution at 4 and Gain 8 with 64 scans in the spectral range of 400-4000cm^{-1}. The diamond smart accessory with DTGS Tec detector was used for analysing the sample at room temperature. The chamber was purged continuously with the nitrogen gas during the operation.

2.6. Analysis of particle size distribution of WAS flocs

The particle size distribution of sludge with and without FNA treatment was analysed by Mastersizer 2000 particle size analyzer (Malvern Co.) equipped with Hydro 2000MU sample dispersion unit with the measurement range from 0.02 to 2000 μm. Distilled water (2 L) was used as a liquid dispersant. Sludge samples were well mixed and then added to the dispersant until the laser obscuration reached the optimum range and then the particle size distribution was measured with the technique of laser diffraction, which is based on the principle that particles passing through a laser beam will scatter light at an angle that is directly related to their size. The measurement for each sample was repeated for 5 times to ensure that the results were reproducible.

3. Results

3.1. Effect of FNA on molecular weight distribution of EPS

The molecular weight distribution and chemical structure analyses were applied to the EPS extracted from the three batches of sludge. The results were reproducible and herewith only the results from 04/05/2014 were interpreted as a representative.

Fig. 1 shows the molecular weight distribution of EPS with and without FNA treatment. The corresponding molecular weights of the peaks at different retention times are listed in Table 2. Substantial peak shifts were observed from UV 254nm detector after FNA treatment. The peak at 15.36 min (in Control I) shifted to 15.67 min after FNA treatment, corresponding to the decrease of MW from 57,300 Da to 42,015 Da. Similarly, the peak at 17.12 min shifted to 17.60 min, representing the decrease of MW from 10,048 Da to 7,238 Da after FNA treatment. In the case of Control II, the decrease of the MWs in comparison with that in Control I was also observed but to a much less extent than that after FNA treatment, from 57,300 Da at 15.36 min to 52,149 Da at 15.46 min and from 10,048 Da at 17.12 min to 9,690 Da at 17.22 min, respectively. Besides peaks shifts, the intensity of the peak at around 17.60 min was significantly intensified after FNA treatment in comparison to that of Control I and Control II, indicating the breakdown of large molecules into smaller ones. In addition to the intensity changes, the shoulder peak at 16.25 min in Control I representing MW of 15,949 Da disappeared after FNA treatment, while no significant

224 change was observed in the case of Control II. Also, FNA treatment yielded more new
225 peaks in the range with MW lower than 180 Da. All the evidence collectively
226 suggested the breakdown of proteins, nucleic acids and/or amino sugars.
227
228 The RI detector showed a similar trend. Substantial peak shifts were observed from RI
229 detector after FNA treatment. The peak acquired from Control I at 15.05 min shifted
230 to 15.23 min, representing the decrease of MW from 32,732 Da to 29,945 Da after
231 FNA treatment. The more significant peak shift took place from 17.47 min to 17.80
232 min, implying the MW decreased from 2,971 Da to 2,525 Da after FNA treatment. In
233 the case of Control II, slight decrease of MWs in comparison with that in Control I
234 was observed but also to a much less extent compared to that after FNA treatment
235 from 32,732 Da at 15.05 min to 30,856 Da at 15.15 min and from 2,971 Da at 17.47
236 min to 2,889 Da at 17.52 min. The peak at around 17.80 min after FNA treatment was
237 substantially intensified compared to those of Control I and Control II. Besides the
238 peaks shifts, the new peak representing MW of 245 Da emerged only after FNA
239 treatment. These results collectively evidenced that FNA could also break down
240 polyssacharides.

241 **Table 2** Retention times and corresponding MWs of the peaks with and without FNA treatment

		Control I		Control II		FNA treatment	
	Peaks	Time (min)	Mw (Da)	Time (min)	Mw (Da)	Time (min)	Mw (Da)
UV 254nm	Peak 1	15.36	57300	15.46	52149	15.67	42015
detector	Peak 2	17.12	10048	17.22	9690	17.60	7238
	Peak 1	15.05	32732	15.15	30856	15.23	29945
RI detector	Peak 2	17.47	2971	17.52	2889	17.80	2525
	Peak 3	Not applicable		Not applicable		19.83	245

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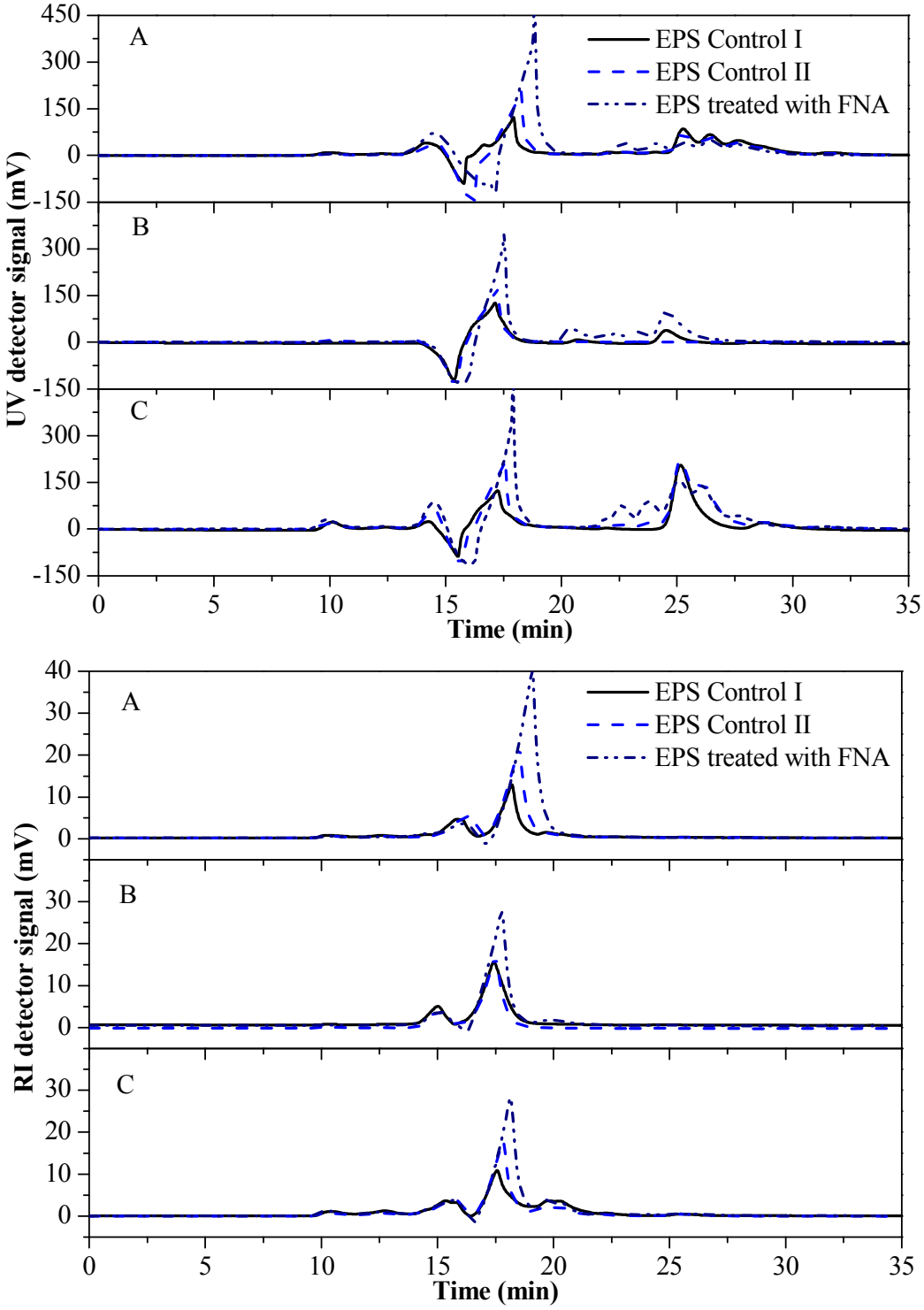


Fig. 1 GPC results of molecular weight distribution of EPS with and without FNA treatment. (UV detector: A 27/08/2013, B 04/05/2014, C 23/05/2014; RI detector: A 27/08/2013, B 04/05/2014, C 23/05/2014)

However, it is noticeable that the MW shifts from RI detector are not as distinct as those from UV detector, implying the main target of FNA in the EPS is UV absorbing substances, such as protein, amino sugars and nucleic acids.

3.2. Effect of FNA on chemical structures of EPS

The FTIR results revealed the chemical structure changes caused by FNA treatment as shown in Fig. 2. Four main characteristic functional groups were identified: 1700~1600 cm^{-1} for amide I region; 1500~1300 cm^{-1} for carboxylic group; 1200~900 cm^{-1} for carbohydrates and nucleic acid, and 900~600 cm^{-1} for fingerprint region. The broad band near 3300 cm^{-1} corresponds to the stretching of both υO-H and υN-H. The bands around 1640cm^{-1} and 1550 cm^{-1} represent υ_sC=O stretching and δN-H and υ_sC-N stretching, respectively, indicating the existence of amide I and amide II associated with proteins. The shoulder band around 1129 cm^{-1} represents δC-OH, δC-O, and υC-O associated with amino acids. The band around 1060 cm^{-1} corresponds to υC-OH of phosphorylated proteins and polysaccharides. The wide band around 950 cm^{-1} represents υ_{as} O-P-O stretching associated with nucleic acids. Bands at 600~900 cm^{-1} denote the existence of ring vibration associated with υC-C and υC-OH from aromatic amino acids (e.g. Phe, Trp and Tyr) and nucleotides[29]. The band assignment is summarised in Table 3.

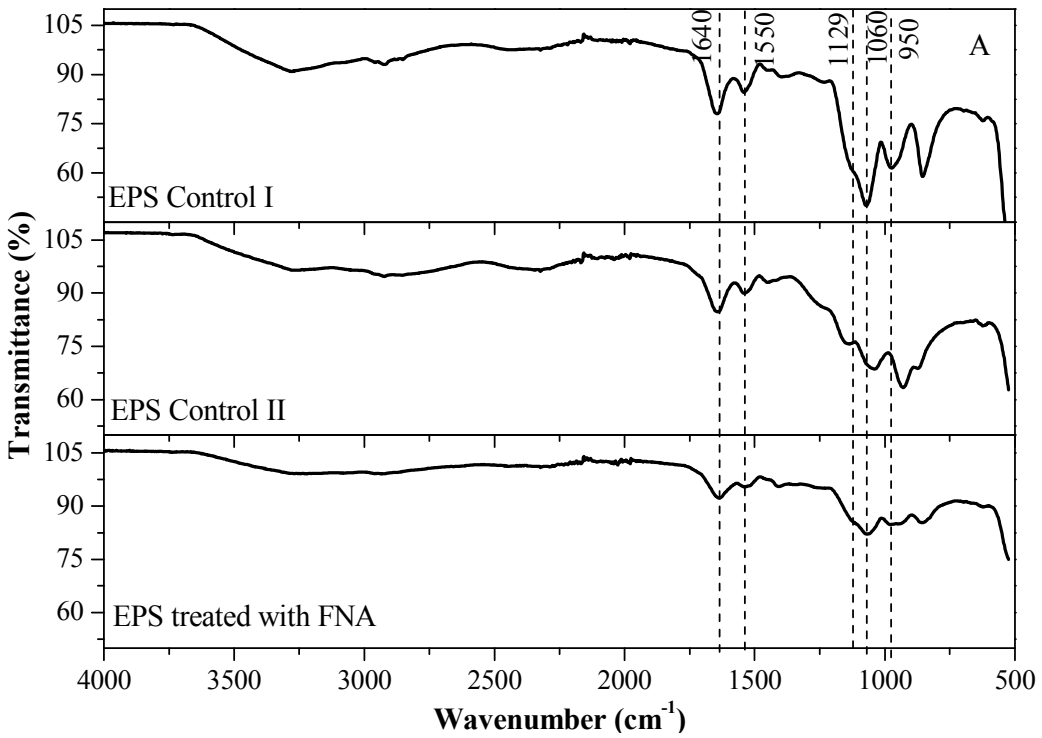
Table 3 FTIR Spectra Band Assignment [30-32]

Frequencies (cm^{-1})	Band assignment

3300	υO-H and υN-H associated with alcohols and amines
1640	υ_sC=O stretching (amide I) and υC=N stretching associated with proteins
1550	δN-H and υ_sC-N stretching in amide II associated with proteins
1129	δC-OH, δC-O, and υC-O associated with amino acids
1060	υO-H deformation, υC-O stretching, ring vibration υP=O, C-O-C and C-O-P in phosphodiesters and polysaccharides
950	υ_{as} O-P-O stretching associated with nucleic acids
900~600	Ring vibrations associated with υC-C and υC-OH from aromatic amino acids and nucleotides

The peak at 1550 cm^{-1} was weakened after FNA treatment, while in the case of Control II, no changes happened to this peak. For the shoulder peak at 1129 cm^{-1}, a slight peak shift was observed in the case of Control II while FNA treatment extended the shoulder band to an obvious peak in comparison to that in Control I. This is likely due to the deaminative depolymerisation of proteins and/or amino sugars by FNA and/or its derivatives[33, 34]. The band at 950 cm^{-1} split into two vibration bands near 950 cm^{-1} and 990 cm^{-1} only after FNA treatment, implying the presence of FNA-induced mutagenic changes of nucleic acids. It was reported that this mutagenesis was also accredited to the deamination of the amino bases in nucleic acids[32]. Slight impairment of the peaks between 600~900 cm^{-1} after FNA treatment

281 indicated that FNA is capable of destructing the ring structure, which likely improves
282 the biodegradability of the hardly biodegradable organic matters[35]. It should be noted
283 that the breakdown of the macromolecules is ascribed to the chemical reactions
284 between them and FNA and/or its derivatives instead of the microbial degradation.
285 This is because that all the EPS samples have been filtered through 0.45 μm pore size
286 disposable filters, which could remove the majority of the microorganisms. In
287 addition, previous studies have demonstrated that FNA at a concentration of parts per
288 million (ppm) level has a very strong biocidal effect on microorganisms.[9, 36] Therefore,
289 it is believed that, at the FNA concentration used in our study (2.0 mg HNO_2-N/L), a
290 residual amount of microorganisms that might have gone through the filters have been
291 inactivated and would not contribute to the breakdown of the macromolecules.
292



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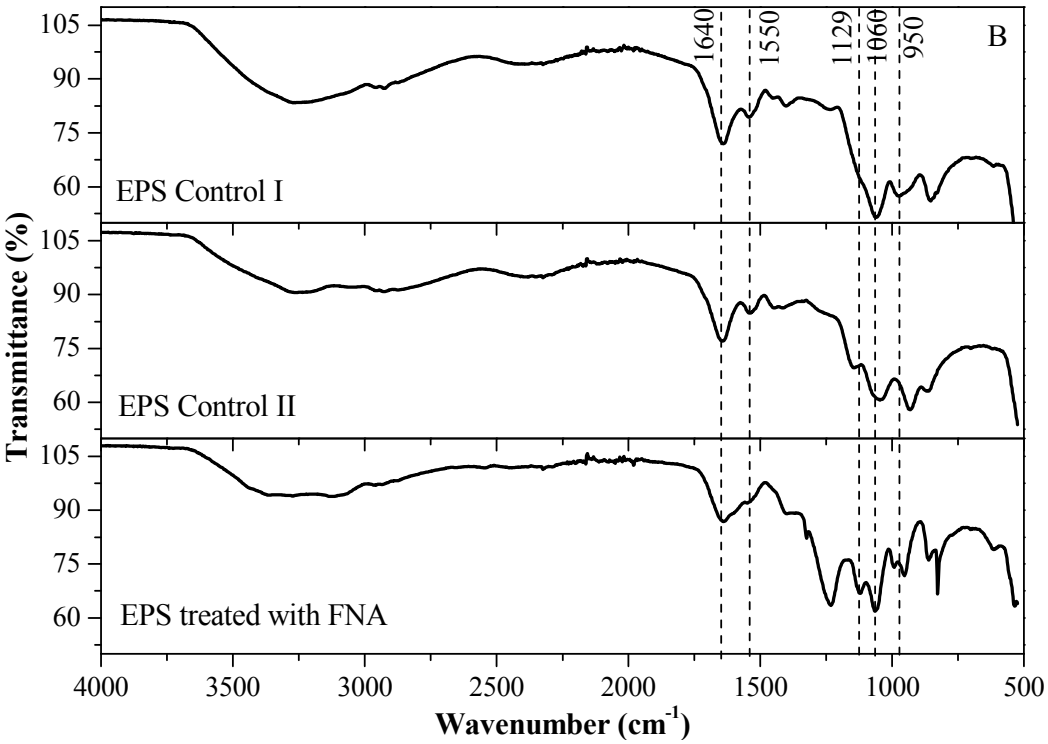

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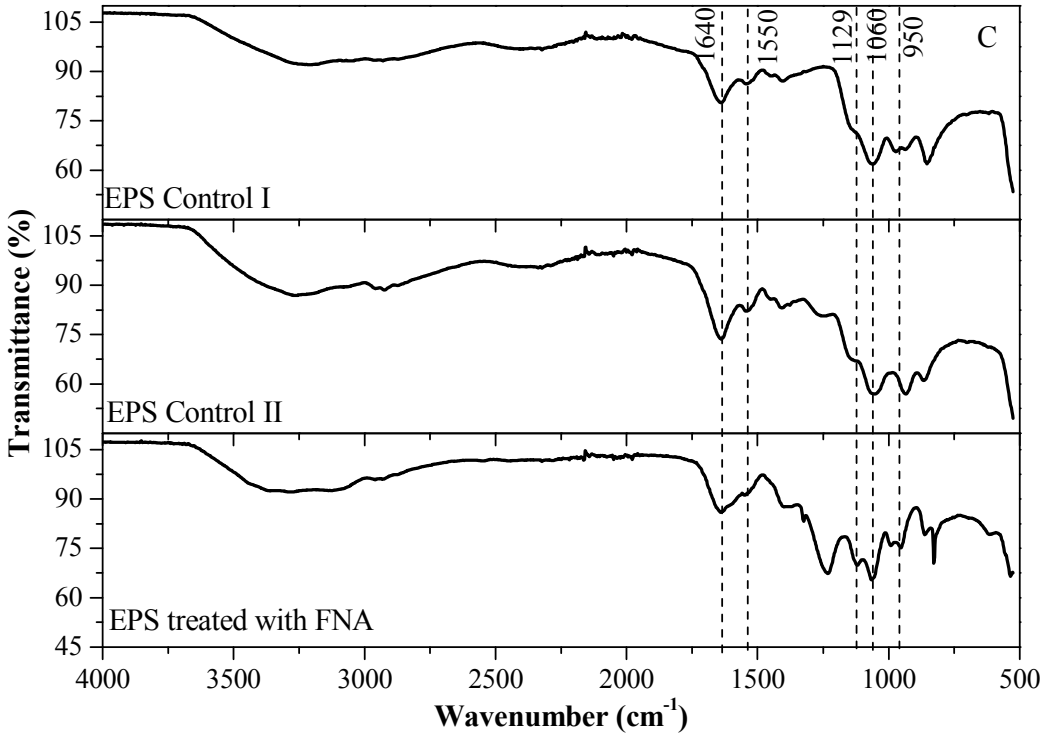

295

296 **Fig. 2** FTIR spectra of EPS with and without FNA treatment (A 27/08/2013, B

297 04/05/2014, C 23/05/2014)

3.3. Effects of FNA on particle size distribution of WAS flocs

Fig. 3 shows the sludge particle size distribution with and without FNA treatment. The d_{50} values are also shown in the figure. In comparison to the d_{50} in Control I (102 ± 2 μm), it remained without significant changes in the Control II (99 ± 1 μm) (p>0.05), whereas after FNA treatment, it was reduced significantly to 69 ± 1 μm (p<0.01), 32% and 30% lower than that in Control I and Control II. The shift of particle size distribution after FNA treatment indicated the drastic decrease in particle size. These imply that the sludge flocs were disintegrated, supporting that FNA led to the breakdown of EPS in flocs.

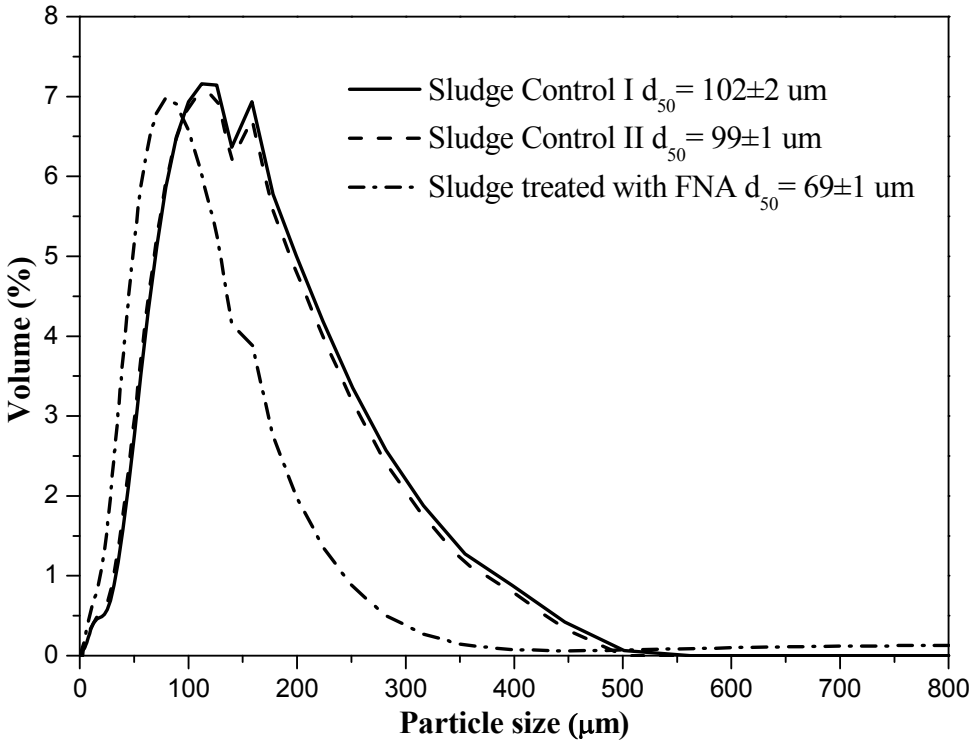

Fig. 3 Sludge particle size distribution with and without FNA treatment

4. Discussion

This study reveals for the first time that FNA breaks down EPS. This was evidenced by the changes in molecular weight distribution in the EPS extract and the chemical structures. The chemical structure analysis of EPS extract with and without FNA treatment revealed that the breakdown is due to the oxidative and deaminative depolymerisation of proteins, amino sugars and amino bases in nucleic acids by FNA or its derivatives. FNA has been reported to be able to cause oxidative deamination of NH_2 group in adenine or cytosine to ether group[36, 37] and chemically destroy DNA structure by converting adenine to hypoxanthine (which pairs with C), cytosine to uracil (which pairs with A) and guanine to xanthine (which still pairs with C)[32]. The deaminative cleavage of model amino sugar glycosides and glycosaminoglycuronans by FNA was also experimentally demonstrated[37]. Besides, it was also reported that the reactive nitrogen species (RNSs) derived from nitric oxide, which is one of the derivatives from FNA[38], could chemically depolymerize carbohydrates through deamination[39]. The results from our study are consistent with the previous studies. However, it should be noted that our study, for the first time, demonstrated the breakdown of the microbial produced molecules (i.e. EPS) by FNA while the previous studies focused on the chemistry between FNA and the synthetic pure macromolecules.

It has been demonstrated that extracellular proteins are strongly involved in the aggregation of bacteria into flocs[39, 40]. Therefore, the breakdown of proteins caused by

FNA treatment could result in the disintegration of EPS matrix. Furthermore, the breakdown of the macromolecules and ring structures due to FNA-induced deamination and oxidation potentially converted hardly biodegradable substances into more easily biodegradable ones[41], which likely contributed to the enhanced sludge biodegradability[11]. A recent study showed that the toxic metal removal from WAS with FNA treatment at 19.2 mg HNO_2-N/L (i.e. 20 mg NO_2^--N/L at pH 2) was significantly enhanced in comparison to that from WAS with acid treatment at pH 2 alone[42]. Further analysis verified that the improvement of toxic metal removal mainly resulted from the release of organically bound metals. This observation can be explained by the results reported in our study.

The FNA based sludge treatment technology has been experimentally demonstrated to be an effective and economically attractive method for improving sludge biodegradability[11]. This study significantly improved the understanding of the working principles of this technology, which is essential in the optimization of this technology. Additionally, it is also of importance in providing the fundamental knowledge for the development of new possible sludge treatment technologies using FNA in combination with other chemicals.

5. Conclusions

The effect of FNA on EPS from waste activated sludge was investigated through the molecular weight distribution and chemical structure analyses of the components in

355 EPS extract with and without FNA treatment. The main conclusions are:

356 (1) FNA could break down macromolecules in EPS, resulting in disintegration of
357 activated sludge flocs.

358 (2) The breakdown is mainly due to the oxidative and deaminative depolymerisation
359 of proteins, amino sugars and amino bases in nucleic acids by FNA and/or its
360 derivatives.

361

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