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1 **EFFECT OF OZONATION ON ANAEROBIC DIGESTION SLUDGE ACTIVITY**

2 **AND VIABILITY**

4 Jaime Chacana[1*], Sanaz Alizadeh[1], Marc-André Labelle[1,2], Antoine Laporte[3], Jalal

5 Hawari[1], Benoit Barbeau[1], Yves Comeau[1]

6 [1]Polytechnique Montreal, [2]WSP, [3]City of Repentigny

7 *jaime.chacana@polymtl.ca

8 **ABSTRACT**

9 The effect of ozonation of anaerobic sludge on methane production was studied as

10 a means to increase the capacity of municipal anaerobic digesters. Ozone doses

11 ranging between 0 to 192 mg O_3/g sludge COD were evaluated in batch tests with

12 a bench scale ozonation unit. Ozonation initially, and temporarily, reduced biomass

13 viability and acetoclastic methanogenic activity, resulting in an initial lag phase

14 ranging from 0.8 to 10 days. Following this lag phase, ozonation enhanced

15 methane production with an optimal methane yield attained at 86 mg O_3/g COD.

16 Under these conditions, the yield of methane and the rate of its formation were

17 52% and 95% higher, respectively, than those measured without ozonation. A

18 required optimal ozone dose could be feasible to improve the anaerobic digestion

19 performance by increasing the methane production potential with a minimum

20 impact on microbial activity; thus, it would enable an increase in the capacity of

21 anaerobic digesters.

22   *Keywords: A*naerobic digestion, sludge, ozone, extracellular polymeric substances,

23   mechanisms.

24   **1. Introduction**

25   Anaerobic digestion (AD) of primary and secondary sludge is commonly used for

26   sludge reduction, stabilization and energy recovery at municipal water resource

27   recovery facilities (WRRFs) (Appels et al., 2008). Sludge consists of a polymeric

28   network of organic and inorganic compounds, however, its actual composition

29   depends on the source of the sludge (Sheng et al., 2010). The presence of these

30   chemicals, including extracellular polymeric substances (EPS), e.g.

31   polysaccharides, proteins, lipids strongly influence the hydrolysis of sludge during

32   anaerobic digestion (Sheng et al., 2010). The hydrolysis of sludge requires long

33   hydraulic retention times (20 to 30 days), leading to moderate degradation

34   efficiencies (30 to 50%), translating into large volume digesters and high capital

35   expenditures (Foladori et al., 2010a).

36   Usually, the main factor limiting anaerobic digestion is the hydrolysis of particulate

37   matter. Improving anaerobic digestion through enhancing rate-limiting hydrolysis

38   can increase the degradability leading to improve anaerobic digestion performance

39   (Appels et al., 2008). A variety of treatment techniques have been studied to

40   enhance sludge hydrolysis by using thermal, chemical, mechanical and other

41   biological processes (Appels et al., 2008). Ozonation is one of the preferred

42   chemical treatments, which permits sludge reduction and it is effective in

43   enhancing methane production via the oxidation and solubilisation of sludge

44   (Weemaes et al., 2000). Ozonation of activated sludge prior to anaerobic digestion

45 (pre-ozonation) effectively enhances its anaerobic biodegradability, but it is not

46 effective with primary sludge (Carrère et al., 2010). Alternatively, the ozonation of

47 digested sludge in the recirculation loop of the anaerobic digester (post-ozonation)

48 has been shown to produce a significant increase in methane production (Battimelli

49 et al., 2003).

50 Previous studies demonstrated that ozonation has great potential to increase

51 biodegradation of activated sludge (Appels et al., 2008), but other studies showed

52 evidence of biomass destruction (Labelle et al., 2011; Chiellini et al., 2014). Further

53 investigation is required to establish the potential linkage between ozonation of

54 anaerobic digested sludge, methane production, and its biological response. A

55 better understanding of the mechanisms of sludge ozonation and its impact on

56 methane production and biological response may allow for better operational

57 control and design of an anaerobic digestion process integrated with post-

58 ozonation.

59 The objective of this study was to evaluate the effect of ozonation on the methane

60 production of anaerobic digested sludge, including the mechanisms involved in this

61 process. The specific objectives were to evaluate the impact of ozonation on the

62 methane yield and methane production rate in batch tests, and to evaluate the

63 microbial response of ozonated sludge by monitoring the microbial cell integrity,

64 the metabolism behaviour (key enzyme), the acetoclastic methane activity and the

65 intracellular reactive oxygen species (ROS) formed for various ozone dosages.

66

3

2. Material and method

2.1. Sludge ozonation

Anaerobic digested sludge was obtained from the Repentigny WRRF (Quebec) which treats 25 000 m^3/d using a chemically enhanced primary treatment (CEPT) process and stabilizes the sludge in a completely mixed mesophilic ($35°C$) anaerobic digester with a hydraulic retention time of 19 days. The collected sludge was passed through a 5 mm sieve to remove large debris, and then stored at 4°C until further use.

Ozone was generated by a pure oxygen ozone generator (Peak 2X, Pinnacle, USA). Ozonation of digested sludge was performed in a batch reactor. The gas flow rate was 6 L STP/min with an ozone mass concentration of about 12% by weight. The transferred ozone dose (mg/L) was calculated from the difference between the mass of ozone transferred (mass fed to the reactor minus the mass in the off gas) divided by the volume of sludge. Ozone dosages were normalized as mg O_3/mg COD by dividing the transferred ozone dosage by the initial total COD content of the sample.

Sludge ozonation was conducted on volumes of 2.2 L of digested sludge fed in a 3.8 L column and operated at room temperature. Using a peristaltic pump operating at a flowrate of 6 L/min, the sludge was recirculated through a venturi (484X, Mazzei, USA) where ozone was injected continuously. Higher ozone dosages required longer recirculation time. The contact time ranged from 0.0 to 6.1 minutes for ozone doses between 0 to 192 mg O_3/g COD. Sludge samples were

4

89 periodically collected during the operation of the ozonation system. Additionally, a

90 control was prepared to evaluate the effect of treatment without ozone injection.

91 2.2. Analytical methods

92 2.2.1. Ozone measurements

93 The inlet ozone concentration was measured using an ultraviolet ozone meter

94 (BMT 964, BMT Messtechnik GmbH, Germany) while ozone in the off gas was

95 measured using the standard KI method (Rakness, 2005). Dissolved ozone was

96 not measured; it was considered negligible as it was never detected during

97 preliminary tests.

98 2.2.2. EPS extraction and quantification

99 EPS were extracted from the control and ozonated samples based on the method

100 of EPS extraction of Liu and Fang. (2002) and Yu et al. (2008). First, 15 mL of the

101 sample was centrifuged at 2 000 g for 15 min at 4°C. The supernatant was

102 collected and filtered (S-Pak 0.45 µm filter, Millipore, USA) to measure soluble

103 EPS. The sludge pellet was re-suspended to its original volume using a phosphate

104 buffer saline (PBS) solution supplemented with 90 µL of formaldehyde (36.5% v/v)

105 and then incubated at 4°C for 1 hour under agitation. The suspension was

106 centrifuged at 5 000 g for 15 min at 4°C and the supernatant was collected and

107 filtered (0.45 µm) for measuring the loosely bound EPS (LB-EPS). The remaining

108 sludge pellet was re-suspended again with a PBS solution to its original volume

109 and incubated for 3 h at 4°C after the addition of 6 mL of a 1 M NaOH solution. The

110 suspension was then centrifuged at 12 000 g for 15 min at 4°C, the decanted

5

111   supernatant contained the tightly bound EPS fraction (TB-EPS). The residual

112   sludge pellet was re-suspended once again with a PBS solution to its original

113   volume (pellet fraction).

114   Proteins and polysaccharides were then measured in the samples before

115   extraction and in soluble EPS, LB-EPS, TB-EPS and pellet fraction. The protein

116   content in the samples was determined using the bicinchoninic acid (BAC) method

117   (Pierce© BCA Protein Assay Kit, Thermo Scientific, USA) with bovine serum

118   albumin (BSA) as the standard. The polysaccharide content of the extracts was

119   analyzed by the phenol-sulfuric acid method using glucose as a standard. Proteins

120   and polysaccharides were measured by a microplate reader (Synergy-HT, BioTek,

121   USA). Excitation–emission matrix (EEM) fluorescence spectra were obtained from

122   the extracts using a luminescence spectrometry (RF-5301pc, Shimadzu, Japan).

123   Samples for EEM analysis were diluted to a final COD of 30 mg COD/L with Milli-Q

124   water. The EEM spectra were collected with the scanning emission spectra (Em)

125   from 220 to 550 nm at 1 nm intervals by varying the excitation wavelengths (Ex)

126   from 220 to 400 nm at 10 nm sampling intervals. Excitation and emission slits were

127   set to 5 nm.

128   2.2.3. Biochemical methane potential

129   Methane yield and acetoclastic activity were evaluated by measuring the

130   biochemical methane potential (BMP) in 160 mL serological bottles incubated at

131   35°C based on Saha et al. (2011). A gas manometer (DG25, Ashcroft, USA) was

132   used to measure the biogas production and the methane gas content was

133   quantified with a gas chromatograph (GC-456, Bruker, USA) equipped with a

134 thermal conductivity detector (150°C). The modified Gompertz model was applied

135 to the cumulative methane production data to determine the maximum methane

136 production rate in the samples (Lay et al., 1996). Methane yield was evaluated

137 without substrate addition, and the acetoclastic activity test was fed with sodium

138 acetate solution. The methane production was evaluated at the standard

139 temperature and pressure (STP) of 0°C and 1 atm.

140 2.2.4. Characterization of biological response

141 Bacterial viability of anaerobic sludge was evaluated using the Live/Dead *Baclight*

142 bacterial viability kit (Molecular Probes, Invitrogen, Kit L13152) and the microplate

143 reader (Synergy-HT, BioTek, USA) using the modified protocol of Chen et al.

144 (2012). The fluorescence intensity of the stained bacterial suspensions (F_{cell}) was

145 determined at an excitation of 488 nm and detection at 635 nm (red) and 530 nm

146 (green), for red-fluorescent nucleic acid stain propidium iodide (PI) and green-

147 fluorescent nucleic acid stain SYTO 9, respectively. The green/red fluorescence

148 ratios ($R_{G/R}$) were used to compare the bacterial inactivation triggered by different

149 doses of ozone. Different proportions of fresh sludge (optimal viable cells) and

150 positive control, inactivated cells with alcohol treatment (2-propanol, 70%), were

151 used as standards. The viability calibration curve was obtained by linear regression

152 of the green/red fluorescence ratio ($R_{G/R}$) vs the percentage of viable cells.

153 The dehydrogenase activity was quantified by the protocol described by Von Mersi

154 and Schinner (1991). The technique uses soluble and colorless 2-(4-Iodophenyl)-3-

155 (4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) reduction to the red

156 insoluble iodonitrotetrazolium formazan (INF) as a tracer of active bacterial

7

157 electron transport systems (Caravelli et al., 2004). Briefly, triplicates of sludge

158 samples (0.5 g) were spiked with 0.75 mL of TRIS buffer (1M; pH 7.0) and 1 mL of

159 0.5% INT solution (9.88 mM), slightly mixed using a vortex for 30 seconds. After 2

160 hours incubation at 40 °C in the dark, the intracellular INF crystals were extracted

161 with 5 mL ethanol/N,N-dimethylformamide solution (1/1 v/v) and incubated for 1 h

162 at 40 °C in the dark. The concentration of developed formazan in the retained

163 supernatant of sludge was determined by a UV/vis spectrophotometer at 464 nm

164 using the extraction solution, ethanol/N,N-dimethylformamide solution (1/1 v/v) as

165 reference blank. INT-electron transport system activity was calculated using the

166 modified equation proposed by Yin et al. (2005) (equation 1)

$$INT–ETSA = D_{464} \cdot V/ k_i \cdot W \cdot t \tag{1}$$

167 Where INT-ETSA is the INT-electron transport system activity (mg INTF/g

168 biomass/h), D_{464} is the absorbance of the supernatant at 464 nm; V is volume of

169 solvent (mL), k_i is the slope of standard curve of absorbance at 485 nm vs INTF

170 concentration (O.D. mL/mg INTF), W is the weight of biomass (g) and t is the

171 incubation time (h).

172 ROS was determined using an established fluorescence assay (You et al., 2015).

173 The sludge samples were rinsed three times with 0.1 M phosphate buffer (pH 7.4)

174 and the pellets were re-suspended in 0.1 M phosphate buffer containing 50 μM

175 dichlorodihydrofluorescein diacetate (H2DCF-DA, Molecular Probes, Invitrogen).

176 The resulting mixture was incubated at 25 ± 1°C in the dark for 30 min. The

8

177 generated fluorescent fluorescein DCF was measured using a microplate reader

178 (Synergy-HT, BioTek, USA) at excitation of 488 nm and emission of 525 nm.

179 2.2.5. Other analytical methods

180 Chemical oxygen demand (COD) was measured using the HACH method (HACH

181 Reactor Digestion Method 8000). Soluble COD was determined on centrifuged

182 (10 000 g, 10 min) and filtered (S-Pak 0.45 µm filter, Millipore, USA) samples.

183 The morphology of blank and ozonated sludge were visualized using a scanning

184 electron microscope (SEM, JEOL JSM7600F). The sample preparation procedure

185 was adapted from Sheng et al. (2011). Sludge sample preparation included the

186 fixation with 2.5% glutaraldehyde in phosphate buffer for 30 min, followed by serial

187 ethanol dehydration. The gold-coated samples were observed with a high-

188 resolution SEM equipped with a field emission gun at a resolution of 1.4 nm at 1 kV

189 and an accelerating voltage of 0.1 to 30 kV.

190 2.6. Statistical analysis

191 Anaerobic biodegradability tests and EPS extraction were conducted in duplicate,

192 3D-EEM tests without replication and the other analyses in triplicate. The Student's

193 t-test was used to compare the quantitative variables considering a p value < 0.05

194 to be statistically significant. A nonlinear optimization by least squares procedure

195 was applied to calculate the maximum methane production by the Modified

196 Gompertz model (Lay et al., 1996).

197

9

3. Results and discussion

3.1. Effect of ozonation on COD solubilization and mineralization

The impact of ozonation on total COD was shown in Figure 1A. During ozonation, total COD was reduced from 15.0 to 12.3 g COD/L. This was a decrease of approximately 18% at 192 mg O_3/g COD. The decrease of COD by ozonation could be attributed mostly to the complete oxidation of a portion of the organic compounds to CO_2 and water (mineralization); this is based on previous studies for ozonation of activated sludge that reported a decrease of total organic carbon (TOC) similar to the reduction of COD, and also an increase of CO_2 in the residual gas of ozone reactor (Weemaes et al., 2000; Déléris, 2001).

Soluble COD increased significantly from 1.13 to 3.31 g COD/L (157 mg O_3/g COD) during ozonation, representing a solubilization of 15.7% (Figure 1A). Higher ozone doses resulted in an apparent decrease in the solubilized COD which may be due to increased mineralization. Solubilization effects observed in this study are consistent with the study of Weemaes et al. (2000) who reported 29% increase in COD solubilization of sludge exposed to 200 mg O_3/g COD. A comparison of the efficiency of sludge solubilization and mineralization in different studies is difficult since the performance depends on several factors including ozone injection conditions, ozone dosage and sludge characteristics (Foladori et al., 2010a). No significant solubilization and COD decrease were observed in the control.
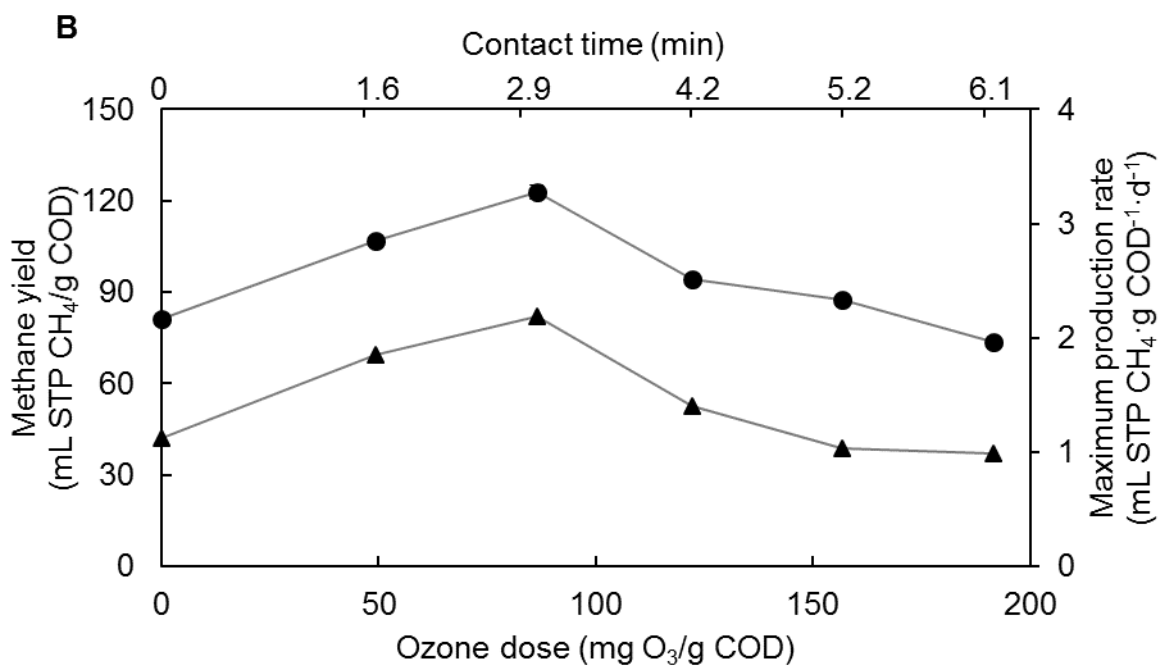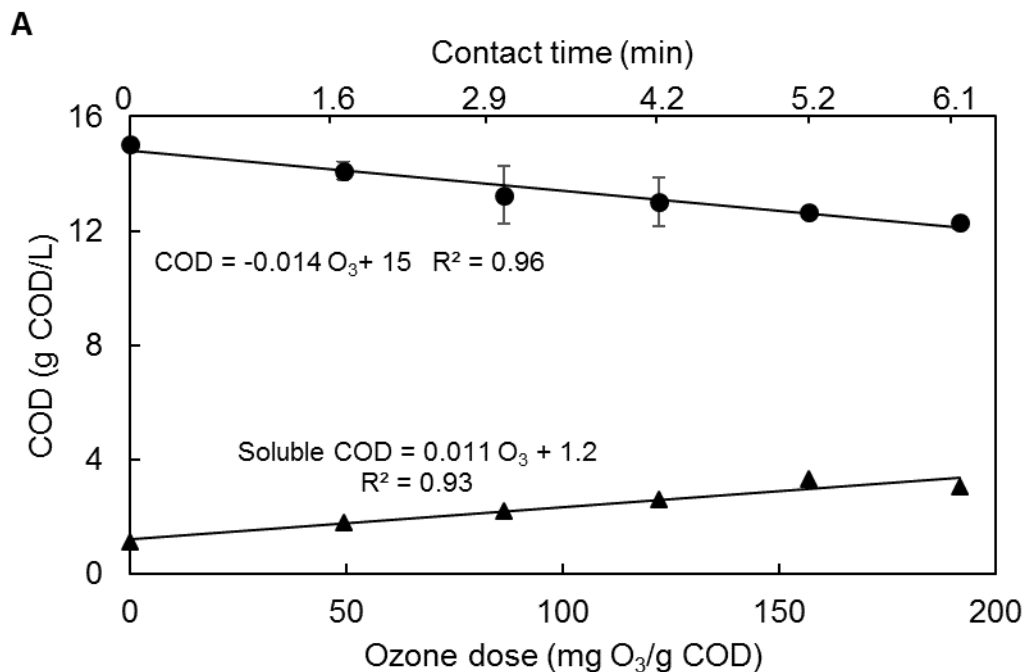
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A

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221   Figure 1: Effect of ozone dose and contact time on COD and methane production –
222   (A) total COD (●) and (▲) soluble COD; (B) Methane yield of ozonated sludge (●)
223   and Gompertz maximum production rate (▲).

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11

225   3.2. Effect of ozonation on methane production

226   The efficiency of ozonation on methane yield was evaluated in BMP assays using

227   ozonated sludges and controls (Figure 1B). Ozonation leads to a significant

228   increase in methane production and reaching a maximum yield of 123 mL STP

229   CH_4/g COD for an ozone dose of 86 mg O_3/g COD. In the absence of ozone,

230   methane production did not exceed 81 mL STP CH_4/g COD. The composition of

231   the biogas was not impacted significantly during ozonation. The average

232   composition of the biogas in both ozonated sludges and controls was 71.3%, 28.6,

233   and 0.05% for CH_4, CO_2 and H_2, respectively. These experimental findings

234   demonstrated that ozonation could increase methane production. Interestingly,

235   using doses of ozone higher than 86 mg O_3/g COD reduced the improvement in

236   methane production. Similar behavior was reported by Weemaes et al. (2000), who

237   found an optimal methane production for an ozone dose of 100 mg O_3/g COD

238   (80%), but also a higher ozone dose reduced the possitve effect on methane

239   production (30%) for activated sludge mixed with primary sludge.

240   The maximum methane production rate of samples was determined by fitting the

241   cumulative methane production data to the modified Gompertz model (Lay et al.,

242   1996). A good agreement between the experimental data and the modified

243   Gompertz model (R^2>0.95) was obtained. The maximum methane production rate

244   was 2.2 mL STP $CH_4 \cdot$g $COD^{-1} \cdot d^{-1}$ for an ozone dose of 86 mg O_3/g COD,

245   representing an increase of 94.5% relative to the untreated sludge (Figure 1B).

246   Ozone doses between 122 to 192 mg O_3/g COD did not change significantly the

247   maximum methane production rate compared to the untreated sample. The

12

248 maximum methane production rates of the current study are low compared to

249 Weemaes et al. (2000). These authors observed a methane production rate of 4.3

250 mL STP $CH_4 \cdot g$ $COD^{-1} \cdot d^{-1}$ for untreated sludge, while for the optimal ozone dose,

251 the production rate was 9.1 mL STP $CH_4 \cdot g$ $COD^{-1} \cdot d^{-1}$. This difference may be due

252 to the type of sludge used. Digested sludge has a low biodegradability since the

253 anaerobic digester has already removed readily biodegradable matter.

254 Ozonation can induce the release of soluble substances into the aqueous phase,

255 this phenomenon increases the accessibility of compounds to microorganisms, and

256 therefore, improves the anaerobic biodegradability of ozonated samples. The

257 maximum ozone dose tested (192 mg O_3/g COD) reduced methane yield and the

258 methane production rate, probably due to the complete oxidation of solubilized

259 matter caused by the mineralization. Therefore, mineralization should be

260 minimized, while organic matter solubilization should be maximised to enhance

261 methane production (Weemaes et al. 2000; Carballa et al., 2007).

262 3.3. Effect of ozonation on EPS

263 The effect of ozonation on the protein and polysaccharide content from different

264 extracted EPS fractions and pellets of anaerobic digested sludge is shown in

265 Figure 2A. For the un-ozonated sludge, the total content of proteins and

266 polysaccharides were 6.6 and 1.8 g/L, respectively, with almost 85% of both

267 polymer substances found in the pellet remaining after centrifugation, while the

268 bound EPS and soluble EPS accounted for only 8.6% and 6.2%, respectively. The

269 ratio of proteins and polysaccharides of extracted EPS (soluble EPS and bound

13

270　EPS) was 1.84, as compared with the reported ratios of 1.1 to 2.8 for digested

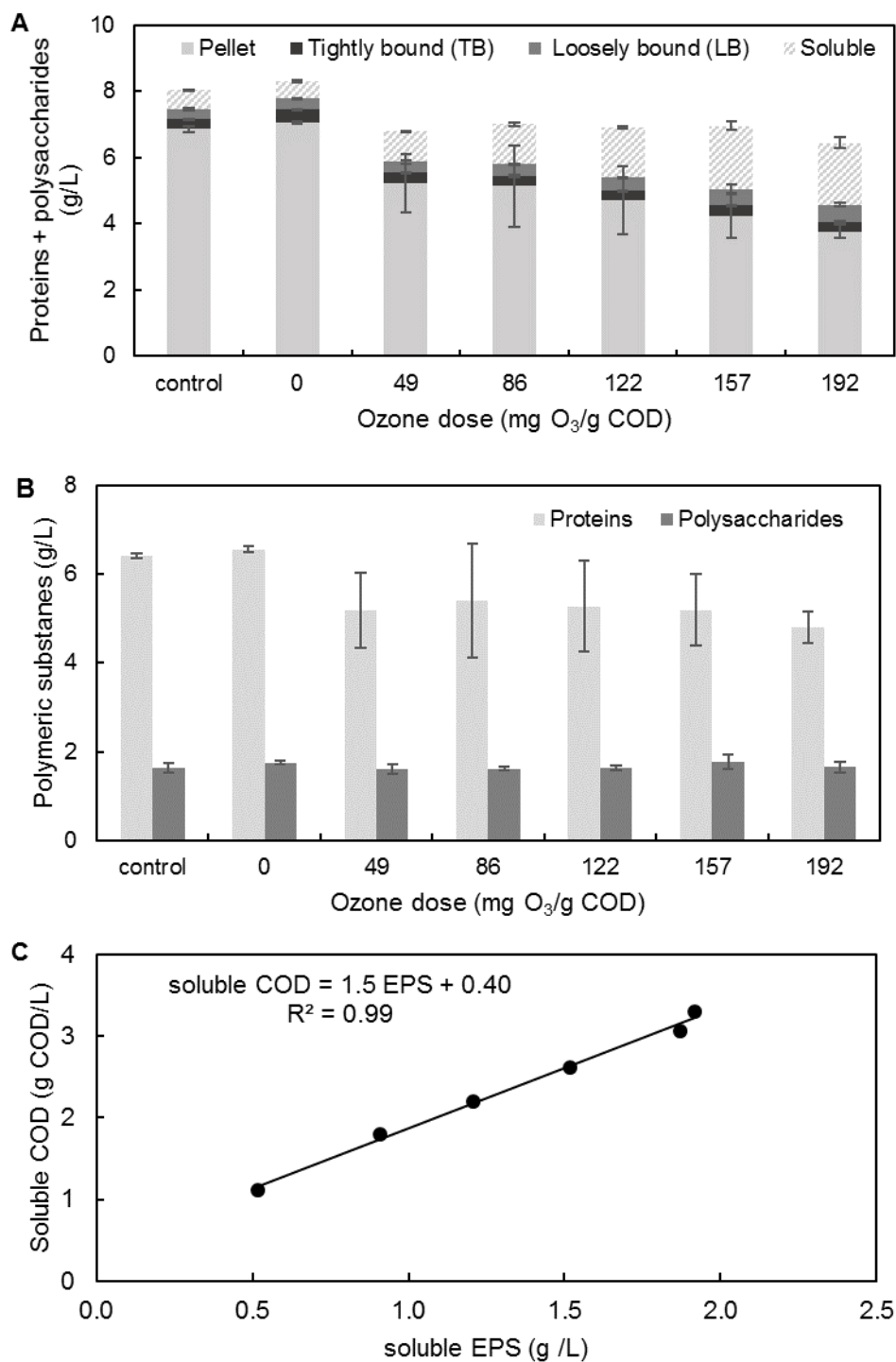271　sludge (Morgan et al., 1990).

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Figure 2: (A) Determination and distribution of EPS (proteins and polysaccharides) in extracted EPS fractions and pellet of digested sludge for an ozone dose between 0 to 192 mg O_3/g COD, (B) effect of ozonation on protein and polysaccharide content, (C) Correlation between soluble EPS and soluble COD.

15

280   A non-significant change in protein concentration was observed for ozone doses

281   between 0 to 157 mg O_3/g COD (Figure 2B). However, the protein content was

282   reduced by 27% for an ozone dose of 192 mg O_3/g COD. Oxidation can cause

283   structural modification of proteins ranging from fragmentation of the polypeptide

284   backbone to aggregation by cross-linking between amino acid residues (Davies,

285   2005). Furthermore, ozone can oxidize amino acid residues, such as cysteine,

286   tryptophan and tyrosine (Cataldo, 2003; Meng et al., 2016) which should usually be

287   quantified by the BCA method (Wiechelman et al., 1988). However, the by-

288   products of oxidation could not be quantified as proteins.

289   As for polysaccharides, no significant decrease in content was noted for doses up

290   to 192 mg O_3/g COD (Figure 2B). Polysaccharides were reported to react weakly

291   with ozone (Bablon et al., 1991). This is expected knowing that proteins have more

292   reactive functional groups (-NH_2, -SH, -COOH, amide linkages) than

293   polysaccharides (mostly –OH and ether linkages). Ozonation of β-D-glycosidic

294   linkages in polysaccharides leads to selective depolymerisation into short chain

295   polysaccharides and oligosaccharides (Wang et al., 1999). Using the phenol-

296   sulfuric acid method, these oligosaccharides will be detected as polysaccharides,

297   thereby, the total sugar content will remain constant.

298   For the pellet residues, measured amounts of proteins and polysaccharides were

299   significantly reduced during ozonation from 7.1 to 3.8 g/L at 192 mg O_3/g COD.

300   Total content of proteins and polysaccharides reduced from 8.3 to 6.5 g/L using an

301   ozone dose of 192 mg O_3/g COD. TB-EPS, LB-EPS and soluble-EPS content of

302   the sludge changed significantly upon exposure to ozone compared to the non

16

303   ozonated sample. TB-EPS decreased from 0.37 to 0.29 g/L for an ozone dose of

304   192 mg O_3/g COD whereas the amount of LB-EPS and soluble-EPS increased

305   linearly from 0.34 to 0.52 g/L (R^2 = 0.71) and 0.52 to 1.9 g/L (R^2 = 0.98),

306   respectively.

307   Ozonation was found to have a significant effect on the distribution of proteins and

308   polysaccharides in various fractions of the digested sludge. Initially, 85% of

309   proteins and polysaccharides were concentrated in the pellet fraction, but after

310   ozonation 59% remained in the pellet (192 mg O_3/g COD). On the other hand,

311   proteins and polysaccharides in the soluble fraction increased from 6.2 to 29%

312   after ozonation (192 mg O_3/g COD).

313   During ozonation, the concentration of EPS in the soluble layer increased while the

314   amount of proteins and polysaccharides from the pellet was reduced as the ozone

315   dose was increased suggesting that ozonation causes the release of EPS from the

316   inner layer to the outer layer. Protein release to the soluble phase was higher than

317   that of polysaccharides. The increase in EPS content in the soluble layer correlated

318   with the COD solubilization (Figure 2C). These results suggest that ozonation

319   disintegrates sludge flocs and releases COD, proteins and polysaccharides from

320   the pellet into the soluble phase. The control showed that mechanical friction of the

321   pump did not cause any significant effect on the protein and polysaccharide

322   content and its distribution in the different fractions.

323   Three-dimensional EEM spectroscopy was applied to characterize the EPS

324   extracted from untreated and treated sludge (192 mg O_3/g COD). Peaks at four

325   different locations were identified according to the literature (Chen et al., 2003).

17

326   The fluorescence peak positions and fluorescence intensity of the different EPS

327   fractions are detailed in Table 1 and Figure S1 (Supplementary Information). The

328   peaks were associated with the presence of aromatic amino acids, e.g.

329   tryptophane in proteins (peak A), fulvic acid-like (peak B), soluble microbial by-

330   products-like (peak C) and humic acid-like (peak D). EEM intensities of peaks

331   tended to decrease after ozonation. Intensity reduction of the fluorescence peaks

332   can be an indication of oxidation and removal of some of the molecular

333   functionalities responsible for fluorescence. Although protein content increased in

334   soluble EPS and LB-EPS, tryptophan and tyrosine are susceptible to oxidation by

335   ozone, thus, reducing the intensity of fluorescence peaks A and C (Figure S1).

336   Table 1: Impact of ozonation on peak intensities of the fluorescence spectra for
337   soluble EPS, LB-EPS, and TB-EPS fractions of anaerobic digested sludge (A =
338   tryptophan, B = fulvic acid-like, C = soluble microbial by-products-like, and
339   D=humic acid-like)

EPS fractions	Ozone dose mg O_3/g COD	Peak intensities			
		A	B	C	D
Soluble	0	340	1000	270	880
	192	200	540	140	720
LB-EPS	0	220	440	200	300
	192	67	180	140	180
TB-EPS	0	860	970	910	570
	192	490	590	650	430

340   3.4. Observations of samples by scanning electron microscopy

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341 SEM observations revealed a distinct difference in the morphology of the control

342 and the ozone treated sludge floc (Figure S2, Supplementary Information). The

343 untreated sludge samples consisted of smooth, dense and integrated structures,

344 with embedded cells in the sludge matrix. As the ozone dose increased, more

345 irregular porous and rough surface structures were observed in the treated

346 samples. Surface deformation and sludge floc disaggregation were observed in

347 sludge samples treated with a dose higher than 86 mg O_3/g COD. The morphology

348 modification of sludge agrees with the alteration of sludge properties, such as EPS,

349 which was confirmed by the release of soluble proteins.

350 3.5. Effect of ozonation on viability, enzymatic activity, ROS production and

351 acetoclastic activity of anaerobic sludge

352 3.5.1. Viability and dehydrogenase activity assay

353 Modified microbial activity of anaerobic sludge following ozonation was

354 characterized by the determination of the biomass viability and the dehydrogenase

355 activity (Figure 3A). The primary ozone dose of 49 mg O_3/g COD inhibited by 57%

356 the relative viability of cells. Ozone treatment between 49 and 122 mg O_3/g COD

357 significantly tailed off the viable biomass with intact membrane, coupled with a

358 higher ratio of inactivated cells. The ozone treatment at doses higher than 157 mg

359 O_3/g COD resulted in significant lysis of biomass with a relative viability of less

360 than 5%. Therefore, significant inactivation of active biomass was observed by

361 ozonation at all tested doses. Membrane integrity defines the potential metabolic

362 activity of the intact cells; therefore, cells with damaged membranes can be

363 classified as permeabilized/dead cells (Foladori et al., 2010b). The influence of

19

364   ozonation on bacterial viability consists of progressive degradation initiated with the

365   physical alteration of membrane permeability and cell integrity, followed by the lysis

366   reaction (Thanomsub et al., 2002). The bacterial cell membrane is comprised

367   dominantly of lipids with abundant C=C double bonds as well as proteins (Winter et

368   al., 2008; Arts et al., 2015). Ozone is a strong electrophile and thus, can easily

369   react with unsaturated lipids via their nucleophilic –C=C– functionality leading to

370   cellular membrane decomposition and the release of cellular components,

371   including EPS. It has been reported that oxidation of C=C double bonds in lipids

372   forms malondialdehyde (MDA) (Han et al., 2016) causing decomposition of the

373   cellular membranes resulting in cell disruption and subsequent leakage of cellular
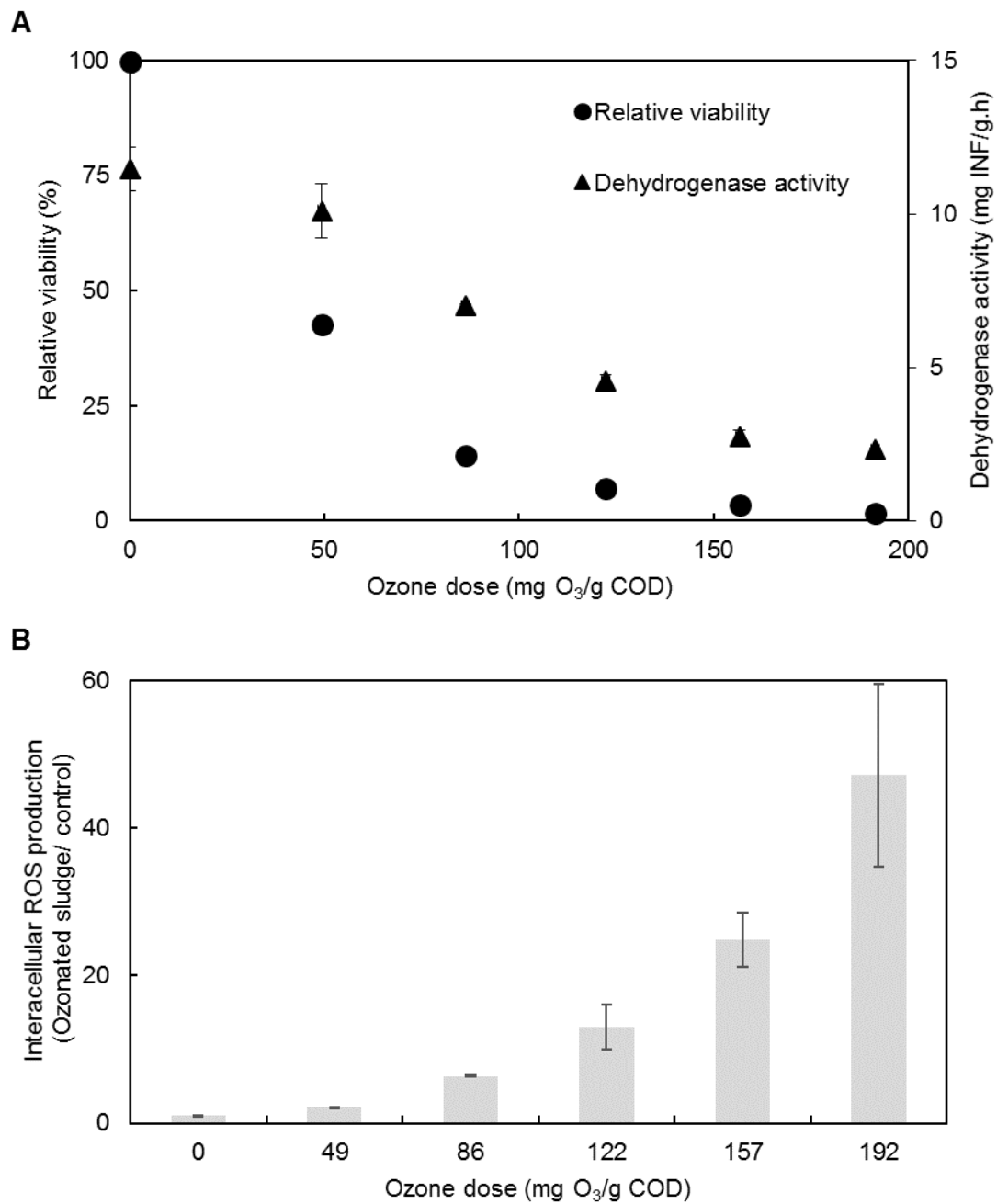
374   contents (Foladori et al., 2010b).

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377 Figure 3: Effect of ozone dose on (A) relative viability, dehydrogenase activity, and
378 (B) intracellular ROS production.
379

380   3.5.2. Intracellular ROS production

381   Ozonation induced ROS in treated sludge at each ozone dose (Figure 3B).

382   Intracellular ROS augmented with an increase in ozone dosage. The ROS

383   concentration was 46 times higher than the control at the highest ozone

384   concentration of 192 mg O_3/g COD. The phenolic and olefinic groups and proteins

385   in the lipid bilayers of the bacterial cell wall are the primary oxidative sites leading

386   to the formation of ROS, such as hydroxyl radicals ($OH^.$), peroxides ($RCOO^.$) and

387   superoxide radical anions ($O-O^{.-}$) (Pryor et al., 1991). Subsequent reactions of

388   ROS with cellular components, such as lipids, proteins and nucleic acid leads to

389   cell disruption and decomposition and causing the release of intracellular

390   components (Baier et al., 2005). Thus, the significantly higher intracellular ROS

391   above 86 mg O_3/g COD confirms the potential of oxidative stress to trigger cell

392   membrane damage and enzyme inhibition for ozonated sludge.

393   3.5.3. Acetoclastic methane activity

394   The acetoclastic methanogenic activity of sludge was used to determine the effect

395   of ozonation on the anaerobic biodegradability of sludge. The acetoclastic

396   methanogenic yield of control and ozonated sludge are illustrated in Figure 4A.

397   Acetoclastic activity after short-term exposure to ozone showed a lag phase, which

398   increased as ozone dose increased. The initial inhibition of acetoclastic activity was

399   consistent with the significant decrease of dehydrogenase enzymatic activity and

400   loss of intact viable cells measured at the beginning of experiment. Similarly, the

401   complete inhibition of respiratory activity of activated sludge has been reported at

402   100 mg O_3/g TSS (Chu et al., 2008). However, approximately 95% of the

22

403   theoretical methane production (350 mL STD CH_4/g COD) was achieved in the

404   samples over 14 days despite the presence of a lag phase of 0.8 to 10 days in the

405   initiation of activity for all ozonated sludge. Furthermore, the dehydrogenase

406   activity of sludge increased during the incubation (192 mg O_3/g COD) (Figure 4B).

407   The cell membrane disintegration, alteration of permeability and interaction of

408   membrane proteins and lipids with ozone can inhibit the acetoclastic activity of

409   sludge. The extension of the activity test, up to 80 days, demonstrated the recovery

410   of microbial activity of ozonated sludge due to the potential recovery of the

411   bacterial community.

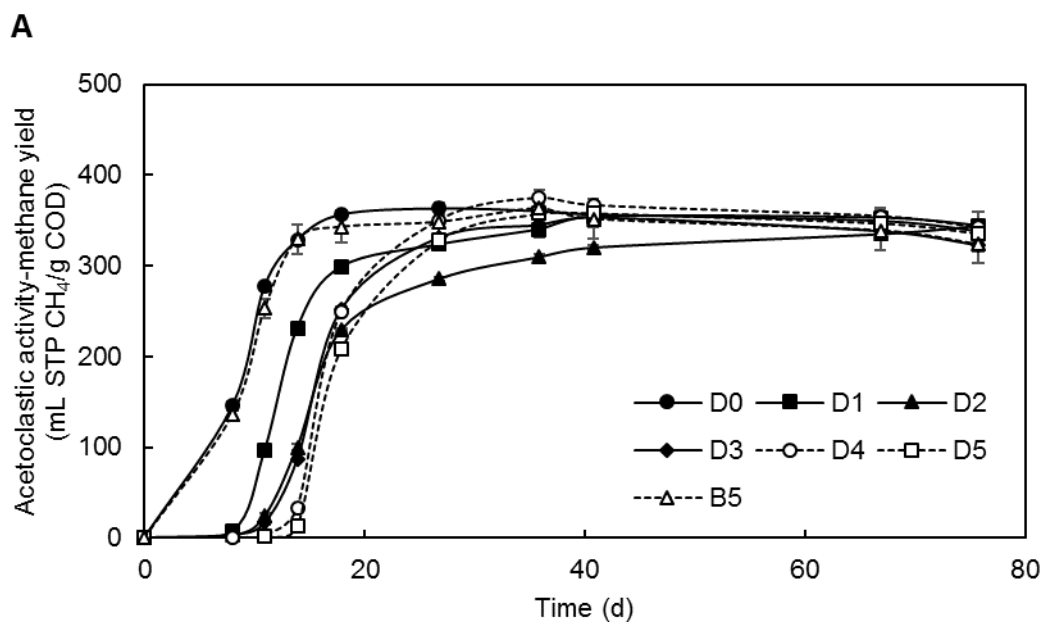23

A

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B

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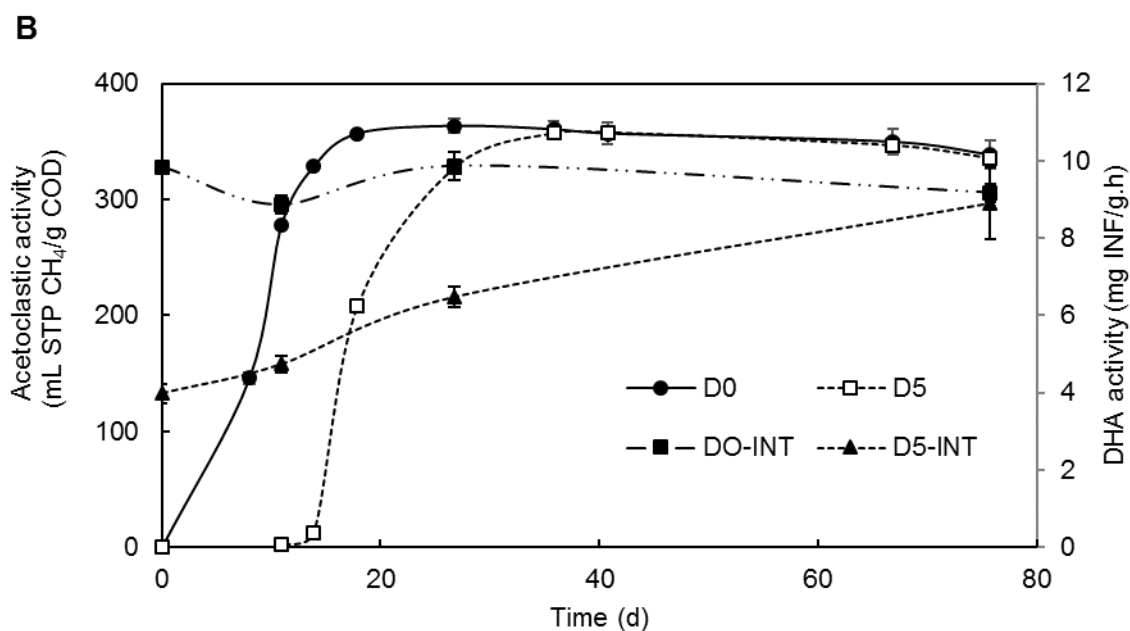
Figure 4: (A) Impact of exposure to ozone on acetoclastic methanogenic activity of anaerobic sludge, and (B) comparison of dehydrogenase activities and acetoclastic activity for D0 and D5 (D0=0 mg O_3/g COD, D1=49 mg O_3/g COD, D2=86 mg O_3/g COD, D3=122 mg O_3/g COD, D4=157 mg O_3/g COD, D5=192 mg O_3/g COD, B5= control).

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420   3.6. Potential mechanisms of improving of anaerobic biodegradability

421   Ozonation was shown to increase the solubilization of sludge mainly via partial

422   disintegration/solubilization of the sludge matrix and damage to the cell membrane

423   integrity. Ozonation can disintegrate the sludge matrix and release COD, proteins

424   and polysaccharides from the pellet into the soluble phase, thereby, promoting the

425   enhancement of methane production during anaerobic digestion. Furthermore, the

426   reduction in viability of the sample suggests that the broken cells can release

427   intracellular matter into the solution. The enhancement in methane production may

428   not only be ascribed to solubilization and it also can be influenced by the increase

429   of the biodegradability of organic products generated during ozonation, e.g. the

430   products of oxidation by ozone of olefins and aromatic compounds are more

431   biodegradable than their parent compounds (Hübner et al., 2015). As a result of

432   the increase in solubilization and biodegradability, anaerobic degradation can be

433   enhanced, improving methane yield and accelerating digestion time. An overdose

434   of ozone can reduce the methane production potential, probably due to the

435   potential mineralization of the solubilization matter. Additionally, an overdose of

436   ozone can minimize the viability of anaerobic biomass and enzymatic activity which

437   could have a negative impact on the stability of anaerobic digesters in a post-

438   treatment configuration.

439

4. Conclusions

440

441 The effect of ozonation on anaerobic digested sludge and its impact on microbial

442 response were evaluated by monitoring methane production, EPS, microbial

443 activity, viability and ROS. The EPS matrix was impacted by ozonation, resulting in

444 the release of COD, proteins and polysaccharides into the soluble phase.

445 Ozonation, initially and temporarily, reduced biomass viability and activity, but

446 following this lag phase, ozonation enhanced methane production. The optimized

447 ozone dose of 86 mg O_3/g COD increased the methane yield up to 52% and the

448 methane production rate up to 95%. Therefore, ozonation could be used to

449 increase the capacity of anaerobic digesters.

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581

Appendix. Supplementary information
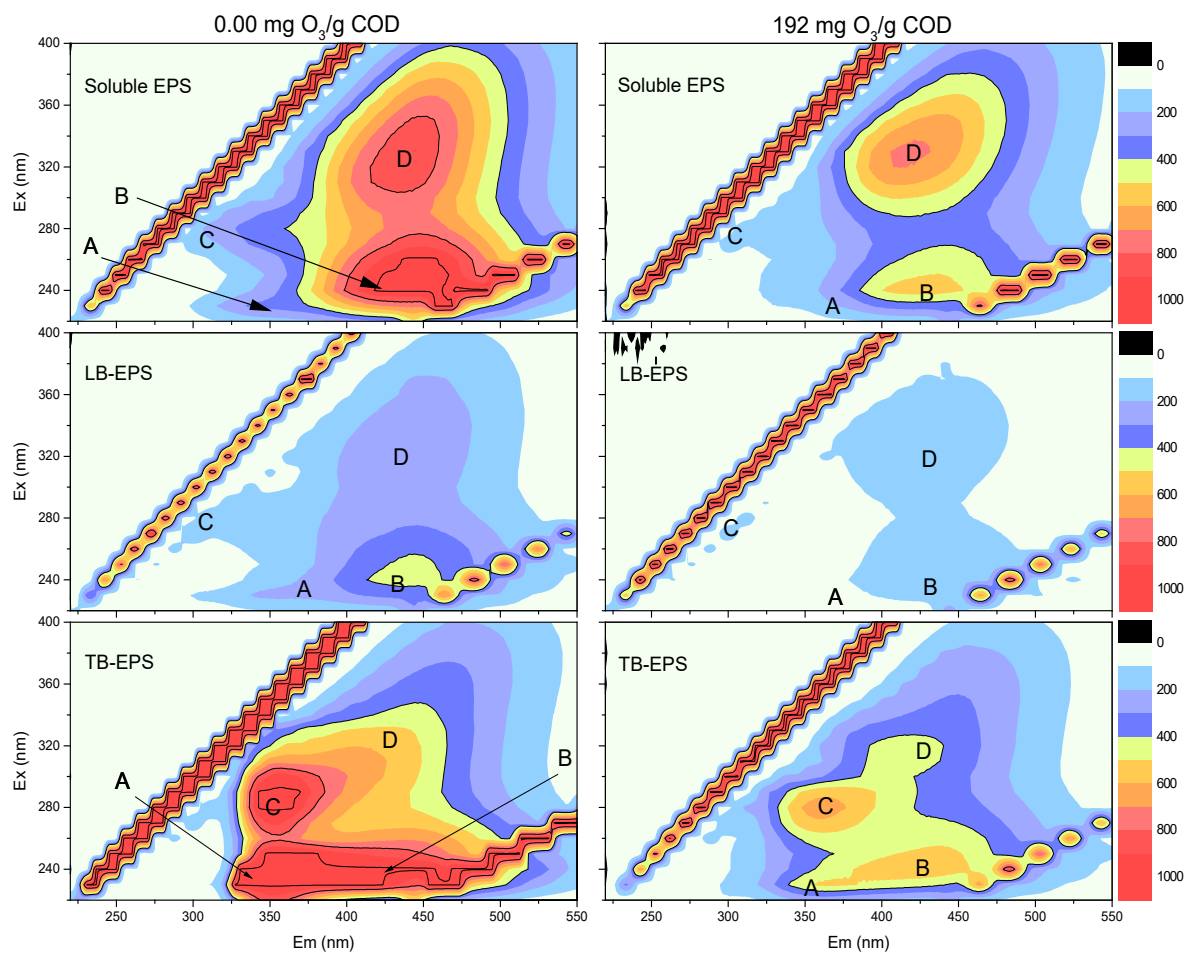
583
584 Figure S1: EEM spectra of the extracted EPS fractions for untreated and treated
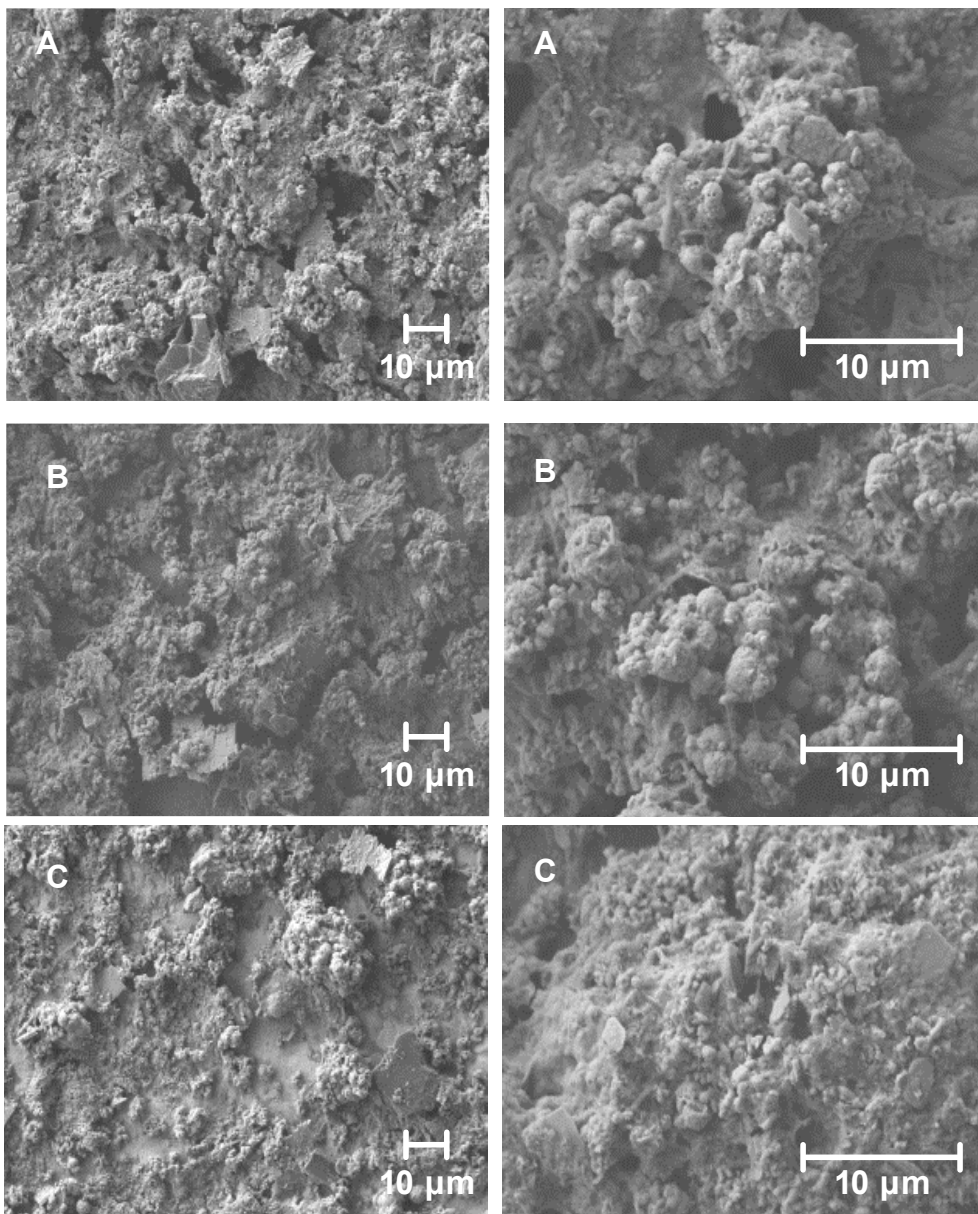
585 sludge (192 mg O_3/g COD).

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588   Figure S2: Scanning electron micrographs imaging of anaerobic sludge exposed to 0 mg O_3/g COD (A), 86 mg O_3/g COD (B), and 192 mg O_3/g COD (C).