1 **Probing the intracellular organic matters released from the photocatalytic**
2 **inactivation of bacteria using fractionation procedure and**
3 **excitation-emission-matrix fluorescence**

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14

15 **Abstract**

16 Photocatalysis provides a “green” and effective strategy for water disinfection. During the
17 photocatalytic disinfection process, intracellular organic matter (IOM) from bacterial cells may be
18 released into the bulk solution. In this study, the role of released IOM in the photocatalytic bacterial
19 inactivation was investigated by fractionation procedure and fluorescence excitation-emission-matrix
20 (EEM) combined with paralleled factor analysis (PARAFAC) approaches. The normal bacterial cells
21 treated by TiO₂-UVA in the presence and absence with fractionated IOM results implied that the
22 released IOM would be either absorbed on the surface of the photocatalysts or reacted by the
23 photo-generated reactive species, and thereby affecting the kinetics of photocatalytic
24 bacterial inactivation. Fluorescence EEM-PARAFAC results showed that two components (C1 and
25 C3) associated with tryptophan- and tyrosine-like proteins were released. While another two
26 components (C2 and C4) were the oxidation products, and their intensities were found to negatively
27 correlate with those of C1 and C3, respectively. Microtox[®] test results indicated that toxicity
28 occurred during the photocatalytic bacterial inactivation process. The toxicity was found to decrease
29 after the bacteria were completely inactivated, and completely removed if provided a sufficient
30 reaction time. Of particular interest is that a significant high linear correlation was observed
31 between the toxicity and the maximum fluorescence intensity of C4. The results and information
32 obtained in this study will be important for further developing photocatalysis in water/wastewater
33 disinfection.

34 **Keywords**

35 Photocatalysis; *Escherichia coli*; Intracellular organic matters; Fractionation procedure; 3-D

36 fluorescence

37

38 1. Introduction

39 Photocatalysis has emerged as a promising disinfection alternative for chlorination in
40 water/wastewater treatment (Shannon *et al.*, 2008). Unfortunately, the mechanisms and kinetics of
41 photocatalytic bacterial inactivation have not been fully understood. Well accepted mechanism is
42 that the photo-generated reactive oxygen species (ROSs) attack the cell membrane, followed by the
43 release of intracellular organic matters (IOM) and their oxidation, and eventually leading to
44 cell inactivation (Maness *et al.*, 1999; Carre *et al.*, 2014). In addition to the mechanism by which
45 bacterial cells are inactivated, the released IOM and their transformation products need careful
46 assessment and consideration, due to the fact that the quality of organic matter could be an important
47 parameter of water.

48 The release of organic matters into the bulk solution from the bacterial cells during the
49 photocatalytic inactivation was well-evidenced by many studies by showing an increase in bulk total
50 organic carbon (TOC) content (Leung *et al.*, 2008). However, only TOC content is not adequate
51 enough to fully understand the characteristics of the released organic matters. Given that the
52 microbial inactivation is typically the last process of a water/wastewater treatment plant, the
53 characteristics of the organic matters in the resultant water after photocatalytic treatment might be of
54 particular importance to the consumers of drinking water or the quality of receiving water
55 systems (Dietrich and Burlingame, 2015). A comprehensive study of the released organic matters from
56 bacterial cells during the photocatalytic inactivation process is needed.

57 The release profile of organic matters from live bacterial cells during the photocatalytic
58 inactivation of bacteria is difficult to study because it is likely a complex process. For instance, the

59 organic matters in the bulk could be either originated from the intracellular regions, namely
60 intracellular organic matters (IOM), or the cell membrane. The released organic matters from inside
61 the bacterial cells would likely influence the inactivation of the remaining cells differently
62 compared with the cell membrane debris due to their different chemical properties. Moreover, the
63 behavior of the released organic matters are rather dynamic; they could be either further degraded by
64 the ROSsor adsorbed onto the surface of photocatalysts and thus making their characterization and
65 quantification biased. Recently, Li *et al.* (2014) and Korak *et al.* (2015) developed a fractionation
66 procedure to get in-depth knowledge of algae-derived IOM in the water treatment process such as
67 filtration and disinfection. They fractionated the living cells into non-living IOM using physical
68 method (e.g. freeze-thaw cycles or sonication) and subsequently subjected to *in vitro* treatment. This
69 fractionation procedure could aid in the investigation on how IOM plays a role in the corresponding
70 treatment and draw insight into the evolution profile of the IOM. To the best of our knowledge, no
71 previous study has applied fractionation procedure to study the released organic matters in the
72 photocatalytic bacterial inactivation.

73 The structural and compositional information on the organic matters in water have typically
74 been obtained with bulk parameters including TOC, UV absorbance and SUVA values (Hu *et al.*,
75 2003). More specific parameters such as malondialdehyde (MDA) (Maness *et al.*, 1999), carboxylic
76 acids (Pigeot-Remy *et al.*, 2011; Pigeot-Remy *et al.*, 2012), carboxylated proteins (Thabet *et al.*, 2014)
77 and N-nitrosodimethylamine (Wang *et al.*, 2014) have been successfully characterized the organic
78 matters in disinfection process, with an attempt to probe their possible relationship with disinfection
79 kinetic and toxicity of the treated water. Nevertheless, these methods are usually suffered from

80 relatively low detection limit, or require expensive equipment with chemical reagents and complex
81 preparation procedures. As such, the lack of robust real-time approach to evaluate the composition
82 and properties of the released organic matters from the bacterial cells during the photocatalytic
83 inactivation process remains a major challenge for a precise and comprehensive understanding of this
84 issue.

85 Recently, fluorescence excitation-emission-matrix (EEM) spectroscopy has received much
86 attention on studying of environmental samples due to its effective and sensitive nature in analysis of
87 the fluorescent organic matter fractions (fluorophores), based on their characteristic fluorescence
88 intensities and differences in the wavelength maxima (Hudson *et al.*, 2007; Ishii and Boyer, 2012).
89 However, the overlap of the peaks was usually observed in EEM contour, thus making the
90 interpretation of EEM contour invalid solely based on visual inspection approach. The parallel factor
91 analysis (PARAFAC) is a widely used method to mathematically separate organic matters into
92 chemically independent fluorescence components. Fluorescence EEM combined with PARAFAC has
93 been successfully applied to probe the transformation of dissolved organic matters in environmental
94 water samples (Baghoth *et al.*, 2011; Meng *et al.*, 2013; Valencia *et al.*, 2014; Yang *et al.*, 2014).
95 Despite its wide usage and increasing applications, so far, EEM-PARAFAC has not been used to
96 characterize the organic matters released from the cells during photocatalytic bacterial inactivation.

97 The primary objectives of this study are to evaluate the released organic matter during the
98 photocatalytic bacterial inactivation process in order to (i) better understand how the released organic
99 matters have an impact on the photocatalytic bacterial inactivation mechanisms; (ii) provide in-depth
100 information on the quality of resultant water using fractionation procedure and fluorescence EEM

101 combined with PARAFAC factor analysis; and more importantly, and (iii) assess the toxicity of water
102 resulted from photocatalytic bacterial inactivation.

103

104 **2. Methods**

105 **2.1 Cell cultures and fractionation procedure**

106 *Escherichia coli* (*E. coli*) K-12 was used as model bacteria and cultured according to our
107 previous studies (Gao *et al.*, 2012; Wang *et al.*, 2012). In brief, fifty mL cells were cultured in 50
108 mL Nutrient Broth (Lab M, Lancashire, UK) solution at 37 °C for 18 h to reach stationary phase with
109 a cell density of $\sim 2 \times 10^9$ colony forming unit per milliliter (cfu/mL). The culture was then and
110 washed with sterilized saline (0.9% NaCl) solution twice by centrifugation at 13000 rpm for 10 min
111 to remove metabolites prior to use.

112 In the fractionation procedure, the pellet was subsequently resuspended in 50 mL ultrapure
113 water, and lysed through 30 cycles of short burst of 30 seconds sonication via a sonicator probe
114 (Vibra Cell, Sonics & Materials, USA) at 50% intensity (600 W maximum), followed by intervals of
115 30 seconds for cooling. During the whole lysis process, the suspension was immersed in ice-water
116 bath for cooling. The suspension containing the lysates was then centrifuged at 13,000 rpm for 10
117 min to separate different fractions. The supernatant fraction was gently filtered by a sterile 0.2 μ m
118 syringe filter (Acrodisc 4612) and the organic matters in the filtrate were designated as IOM
119 standard. The pellet was washed twice with ultrapure water and designated as cell debris.

120

121 **2.2 Photocatalytic experiments**

122 The photocatalytic inactivation experiments were conducted in a photocatalytic reactor by
123 using a UVA lamp (emission peak at 365 nm) as light source and 100 mg/L TiO₂(P25, Degussa,
124 German) as the photocatalyst with the volume of reaction mixture of 50 mL. To investigate how the
125 different cellular fractions affect the photocatalytic inactivation of bacteria, the as-prepared fractions
126 of IOM or/ and cell debris were externally spiked into a $\sim 2 \times 10^7$ cfu/mL (7 log) live *E. coli*
127 suspensions to adjust to a final equivalent cell density of $\sim 2 \times 10^8$ cfu/mL (8 log) for photocatalytic
128 inactivation experiments. The photocatalytic inactivation of *E. coli* cells alone in concentrations of
129 $\sim 2 \times 10^7$ cfu/mL and $\sim 2 \times 10^8$ cfu/mL were also conducted as control experiments. At different time
130 intervals, one mL aliquot was collected from each experiment and serially diluted with sterilized
131 saline solution; 0.1 mL of the diluted sample was then immediately spread onto nutrient agar (Lab
132 M, Lancashire, United Kingdom) plates. Subsequently, the agar plates were incubated at 37°C for 24
133 h to determine the number of surviving cells (in cfu). All the above experiments were carried out in
134 triplicates.

135 Besides, the absorbance and fluorescence EEM spectra evolution of IOM and cell debris
136 treated by photocatalysis were monitored as compared to the released organic matters from
137 photocatalytic inactivation of live *E. coli* cells. At different time intervals, three mL aliquots of the
138 suspensions were taken from the each reaction mixture and filtered through 0.2 µm syringe filter for
139 analyses.

140

141 2.3 Chemical measurements and spectroscopic analyses

142 The total organic carbon (TOC) and total nitrogen (TN) of the bulk samples obtained from the

143 photocatalytic inactivation of 8 log *E. coli* cells, as well as the filtrate in the fractionation procedure
144 were analyzed using a TOC-TN analyzer (TOC-V, Shimadzu, Japan). Cell debris derived from the
145 fractionation procedure of 50 mL cells were freeze-dried and then the TOC concentration in the cell
146 debris was measured by a TOC Analyzer (Shimadzu, Japan) combined with a solid sample
147 combustion unit (Shimadzu, Japan). The protein concentrations were also measured according to
148 Bradford assay (Zor and Selinger, 1996) using bovine serum albumin (BSA) as a standard.

149 The absorption spectra of the bulk samples were obtained by scanning the absorbance at the
150 wavelengths from 200 to 600 nm via a UV-visible spectrophotometer (LabTech, USA) at a
151 resolution of 1 nm.

152 The three-dimensional EEM spectra of the bulk samples were obtained by a fluorescence
153 spectrometer (F-7000, Hitachi, Japan) with excitation wavelengths in the range of 200-500 nm in 5
154 nm intervals and with emission wavelengths in the range of 290-550 nm in 5 nm intervals. The
155 emission and excitation slits were fixed at 10 nm, and the scanning speed was 12000 nm/min.
156 Second-order Rayleigh scattering in the EEM measurements was eliminated using a 290 nm
157 emission cutoff filter. According to suggested protocols of the manufacturer, the instrument was
158 corrected for excitation and emission prior to use. The fluorescence intensities of each sample were
159 calibrated to Raman unit (R.U.) through dividing each intensity by the integrated intensities of
160 Raman peak (371-428 nm) of ultrapure water at excitation of 350 nm (Lawaetz and Stedmon, 2009).
161 The inner filter effect was minimized by diluting the samples to their UV absorbance at 254 nm less
162 than 0.2 cm⁻¹. The contour plots of the EEM dataset were visualized using Matlab 7.0.

163

164 2.5 Statistic analysis

165 *PARAFAC analysis.* The PARAFAC analysis of the EEM datasets is based on well-established
166 principles (Baghoth *et al.*, 2011). In brief, an alternating least squares algorithm is applied to
167 minimize the sum of squared residuals in a trilinear model, thus allowing the deconvolution of the
168 overlapped EEM spectra into independent components as depicted in the following equation:

169

$$170 \quad x_{ijk} = \sum_{f=1}^F a_{if} b_{jf} c_{kf} + \varepsilon_{ijk} \quad i=1, \dots, I; j=1, \dots, J; k=1, \dots, K; \quad (1)$$

171

172 where x_{ijk} is the fluorescence intensity of the i th sample at the k th excitation and j th emission
173 wavelength; a_{if} is directly proportional to the concentration of the f th fluorophore in the i th sample
174 (defined as scores), b_{jf} and c_{kf} are estimates of the emission and excitation spectra respectively for
175 the f th fluorophore (defined as loadings), F is the number of fluorophores (components) and ε_{ijk}
176 is the residual element, representing the unexplained variation in the model. PARAFAC analysis
177 was performed using the DOMFlour toolbox of the Matlab software package, which can decompose
178 the EEM spectrum into relatively independent fluorescent components and can perform residual
179 errors and split-half diagnostics. The DOMFlour tool box and tutorial can be downloaded freely via
180 http://www.models.life.ku.dk/al_domfluor (Stedmon and Bro, 2008). In this study, PARAFAC
181 modeling between two and nine components were generated. Finally, according to the residuals and
182 split-half analysis of the PARAFAC modeling, the number of components could be determined.

183 *Cluster analysis.* In this study, the cluster analysis was applied to the EEM datasets using the
184 minimum-distance method, as employed in the Matlab 7.0. A shorter distance between clusters

185 indicates a greater similarity. The data point of each EEM dataset was normalized by the sum of its
186 dataset to qualitatively reveal the similarities of the shape of EEM regardless of the concentrations
187 of the fluorophores in the samples. Details of the procedures can be found in the Fig. S1 in
188 Supporting Information.

189 Pearson's correlation analysis was carried out using SPSS 18 software (SPSS, Chicago) and
190 calculated correlation coefficient R was used to evaluate the correlation between two parameters.
191 Linear regression was performed by Origin 8.5.

192

193 **2.6 Toxicity test.**

194 The Microtox® test was used to assess the total toxicity of the treated water from photocatalytic
195 inactivation of normal *E. coli* cells at different reaction time. This test takes the reduction in
196 bioluminescence of the marine bacterium *Vibrio fischeri* after exposure to toxic compounds as
197 endpoints and was carried out according to instructions provided by the manufacturer using a
198 Microtox 500 analyzer (Azur Environmental, USA) coupled with a Microtox-Omni™ software.
199 This test was chosen because it is a simple, rapid (15 min), and reproducible acute toxicity test. For
200 each test, the bioluminescence of *Vibrio fischeri* exposed to duplicates of 4 concentrations (29.3%,
201 44%, 66.0%, and 99.0% of sample) plus a blank (0% of sample) was measured. The obtained data
202 was used to calculate the EC50, which is the median sample concentration that causes a 50%
203 reduction in bacterial bioluminescence. Toxicity units (TU) were used to standardize results from
204 the following equation:

205

$$TU = \frac{100}{EC_{50}(15 \text{ min})} \quad (2)$$

207

208 3. Results and discussion

209 3.1 Impact of IOM and cell debris on photocatalytic inactivation kinetics

210 Fig. 1a compares the photocatalytic inactivation kinetics of bacteria cells in the presence and
 211 absence of external bacterial fractions. It was observed that in the absence of external bacterial
 212 fractions, both the inactivation curves of the 7 and 8 log initial cell density exhibit similar shapes,
 213 which can be roughly divided into three stages. In the first 20 min for the 7 log and 30 min for 8 log
 214 initial cell density (Stage I), both inactivation kinetics were subjected to a log-linear decay in cell
 215 density, suggesting first order exponential decreases in cell populations. Thereafter, the reaction
 216 processes decelerated for a certain period (from 30 min to 2 h for 8 log cell density, from 20 min to
 217 60 min for 7 log cell density) (Stage II), followed by another log-linear decay in cell density until
 218 complete inactivation (Stage III). Previously, many studies observed that a deceleration at the end of
 219 the reaction, which is known as the “tailing” (Marugan *et al.*, 2008; Chong *et al.*, 2010). The reason
 220 for the tailing has not yet well understood. One possibility was proposed as that variation in
 221 bacterial population that became resistant to the photocatalysis (Lambert and Johnston, 2001). In
 222 principle, the major ROS in the TiO₂-UVA system is the highly active hydroxyl radical (•OH) with a
 223 relatively high redox potential of +2.8 eV (Dalrymple *et al.*, 2010), that can virtually oxidize all
 224 forms biomolecules. Besides, we also observed completely inactivation with prolonged reaction
 225 time (Stage III). As such, the generation of ROS-resistant bacteria is not likely to occur. A different
 226 possibility was that the competition for the ROS between the remaining viable cells and the organic

227 matters released from the lysed cell (Benabbou *et al.*, 2007). In addition, there is also possibility
228 that there is a competition for the photon between the photocatalysts and released organic
229 matters (Marugan *et al.*, 2010). Collectively, the deceleration of photocatalytic inactivation efficiency
230 highlighted the significance of released organic matters in affecting photocatalytic bacterial
231 inactivation process.

232 To investigate how different fractions of bacterial cells affect the photocatalytic inactivation,
233 the IOM and cell debris were initially spiked into the reaction mixtures with an equivalent cell
234 density of 2×10^8 cfu/mL (8 log). For instance, cells of 2×10^7 cfu/mL initial density (7 log) were
235 added with IOM or/and cell debris of equivalent cell density of 1.8×10^8 cfu/mL, corresponding to
236 a total equivalent cell density of 2×10^8 cfu/mL. Under all conditions, the bacterial cells could be
237 completely inactivated within 4 h, but different fractions had different effect on the photocatalytic
238 inactivation kinetics (Fig. 1a). It can be seen that a lag or plateau in the first 30 min of the reaction,
239 known as “shoulder”, appeared in the inactivation curves with initial addition of IOM and the
240 inactivation efficiency was greatly reduced during all the reaction time, indicating that the presence
241 IOM greatly inhibited the photocatalytic inactivation efficiency. In contrast, the addition of cell
242 debris did not confer a “shoulder” in the inactivation curves, but the inactivation followed a first
243 order decay and achieved complete inactivation within 2 h. This result suggested that the cell debris,
244 which is mainly from the cell membrane and cell wall, play a moderate role in inhibiting the
245 photocatalytic inactivation. This could be reasoned by the fact the cell debris are mainly composed
246 of fatty acids, which were widely regarded to be susceptible to peroxidation (Dalrymple *et al.*, 2010).
247 A radical chain reaction could be initiated by a radical due to its reaction with an unsaturated fatty

acid in the presence of oxygen, leading to the formation of a peroxy radical, which in turn can react with other nearby lipid molecules to generate additional lipid radicals. Besides, the cell debris was less dispersed in water than the soluble IOM fraction. Therefore, it is reasonable to infer that the cell debris would not compete as much ROS as IOM in the photocatalytic inactivation experiments and hence played a moderate role in decelerating the photocatalytic inactivation efficiency. With the addition of both IOM and cell debris, the “shoulder” extended to 2 h. In general, the extent of inhibition for photocatalytic inactivation followed: IOM + cell debris > IOM > cell debris. This trend highlighted that the IOM play a major role in the deceleration of photocatalytic bacterial inactivation process.

257

3.2 Evolution of the bulk parameters

To monitor the evolution of the released organic matters during the photocatalytic bacterial inactivation, the bulk parameters including protein, TOC and TN concentrations were measured along with time (Fig. 1b and c). As shown in Fig. 1b, trace amount of protein (~0.7 mg/L) were detected in the bulk solution at $t = 0$ min, which was probably due to the release of soluble microbial product (SMP) from metabolic activity (Ni *et al.*, 2011). The bulk protein concentration first dropped to 0.3 mg/L at $t = 20$ min, and then soared up at $t = 30$ min. It was worth noted that, from $t = 0$ min to $t = 30$ min (Stage I), approximately 3 log (99.9%) cells were inactivated (Fig. 1a). Considering the mechanism that the cell membrane is the primary target for the attack by ROS, the increased amount of protein in bulk was likely attributed to the leakage of intracellular protein through the damaged cell membrane, and the rate of leakage was faster than that of damage. From t

269 = 30 min to $t = 2$ h (Stage II), the bulk protein concentration slightly increased to a maximum of 3.7
270 mg/L at $t = 1$ h and then underwent a drastic drop. Taken the deterioration of bulk protein and
271 deceleration of bacterial inactivation rate together at Stage II, we can conclude that the released
272 protein could compete for the photo-generated ROS and subsequently lead to a deceleration of
273 inactivation efficiency. After the cells were completely inactivated ($t = 4$ h), we observed a
274 fluctuated increasing trend of the bulk protein concentration. This increase can be ascribed to the
275 ROSs continuously attack the inactivated cell skeleton thus rendered more protein being released
276 into the bulk. A drop in protein concentration at $t = 8$ h was possibly due to the released protein
277 subsequently being adsorbed by the photocatalysts or photocatalytically degraded. Typically, the
278 complete disruption of cell skeleton by photocatalysis required much longer time than completely
279 inactivation as demonstrated by the TEM and SEM images in our previous studies (Gao *et al.*, 2012;
280 Wang *et al.*, 2012; Shi *et al.*, 2014).

281 TOC and TN are important indexes of water quality. As seen in Fig. 1c, the bulk TOC and TN
282 both increased and reached a maximum at $t = 6$ h (16.8 mg C/L for TOC and 9.3 mg N/L for TN). It
283 is worth noting that the maximum bulk TOC did not exceed either the TOC of IOM or that of cell
284 debris (21.0 and 17.4 mg/L for IOM and cell debris, respectively, with an equivalent cell density of 2
285 $\times 10^8$ cfu/mL) as shown in Table 1, which indicated neither IOM nor cell debris were completely
286 released into the bulk at $t = 6$ h. Afterward, the TOC started to decrease gradually, whereas TN
287 remained leveled off, suggesting that the bulk TOC could be further mineralized while the TN could
288 not be removed by photocatalysis in the presence of dissolved oxygen. In principle, the
289 photocatalytic oxidation end-products of carbon and nitrogen will be carbon dioxide (CO₂) and

290 nitrate (NO_3^-), respectively. Since CO_2 can volatilize into air whereas the NO_3^- will be retained in
291 solution, the photocatalytic bacterial inactivation process will finally end up in nitrogen-rich
292 solution as indicated by a consistent increasing TN/TOC ratio with increasing reaction time (Fig.
293 1c). It is of significance to note that nitrogen is an essential element for the growth of
294 microorganisms, and is one of the major reasons to cause eutrophication (Conley *et al.*, 2009).
295 Therefore, post-treatment might be required to tackle the dissolved nitrogen to avoid potential risks
296 such as growth of microorganism in pipeline or eutrophication in natural environment.

297

298 **3.3 Changes in bulk solution absorption spectra of *E. coli* cells, IOM and cell debris by** 299 **photocatalysis**

300 The absorption spectra allow one to readily estimate the water quality such as aromaticity or
301 the presence of nitrate/nitrite (Rieger *et al.*, 2004; Matilainen *et al.*, 2011). Fig. 2 shows the changes
302 in bulk absorption spectra of *E. coli* cells, IOM and cell debris treated by photocatalysis along with
303 irradiation time. As for the *E. coli* cells (Fig. 2a), generally, the intensities in the region of 200 – 250
304 nm, which was mainly responsible for the nitrate/ nitrite (peak at 220 nm) (Rieger *et al.*, 2004),
305 increased along with increasing time, probably suggesting that the oxidation of organic nitrogen
306 into nitrate/nitrite, which was then released into the bulk. For the untreated IOM (Fig. 2b), there was
307 a peak at ~ 254 nm, which was mainly due to $\pi - \pi^*$ transitions of aromatic ring (Matilainen *et al.*,
308 2011). This peak decayed substantially at the very beginning of the photocatalytic reaction,
309 suggesting that the aromatic chromophores were preferentially oxidized by ROSs. This is in
310 agreement with a previous report on the ability of photocatalysis to attack the aromatic ring of

tryptophan by a mechanism of hydroxylation initiated by the photo-generated $\bullet\text{OH}$ (Elsellami *et al.*, 2010). In bacterial cells, the aromaticity mainly results from proteins that contain aromatic amino acids such as tryptophan, tyrosine and phenylalanine (Nelson and Cox, 2004). These amino acids are essential for lives and their high susceptibility may possibly play a role in the mechanism photocatalytic bacterial inactivation, besides lipid peroxidation (Carre *et al.*, 2014). The bulk absorbance of cell debris treated by TiO_2 -UVA exhibited the same trend as the live *E. coli* cells but with a relatively low intensity, suggesting the bulk organic compounds might mainly originate from the IOM other than the cell debris. A shoulder peak at 225-250 nm appeared and increased in the bulk absorbance of cell debris along with reaction time, indicating the formation of peroxidized phospholipid (Kim *et al.*, 1987; Souza *et al.*, 2011) as a result of lipid peroxidation. This phenomenon was not observed in the *E. coli* cells samples which could be attributable to the high intensities of the broad major peak at 200-250 nm in the bulk of the *E. coli* samples. Additionally, we observed no absorbance for all the samples in the region of 300-400 nm, which covers the peak of UVA light source in this study (Fig. S2). Therefore, the possibility that the released organic matters would compete for photons with the photocatalysts and lead to deceleration of the inactivation rate is negligible.

327

3.4 Fluorescence EEM spectra

In this study, the fluorescence EEM spectra of the bulk samples of *E. coli* cells, IOM and cell debris treated by TiO_2 -UVA were obtained to get in-depth insight into the evolution of the released organic matters, and the EEM contours of the each treatment along with time are illustrated in Fig.

332 3. Before irradiation ($t = 0$ h), two distinct peaks presented at Ex/Em of 280/330 and 220/330 nm
333 were observed for all the three samples. According to the previous studies, these two peaks were
334 associated with the protein-like substances (Hudson *et al.*, 2007). Generally, changes in the
335 fluorescence intensities and shape in the EEM contour plots were observed along with time for each
336 photocatalytic experiments. Specifically, after 1 h of irradiation, new peaks located at Ex/Em of
337 280/430, 220/415 nm appeared, accompanied by the decay of the original peaks in the bulk *E. coli*
338 cells. Similar trend was observed for the IOM, whereas there were three distinct news peaks located
339 at Ex/Em of 285/405, 250/415 and 220/405 nm. As for the cell debris, three new peaks located at
340 Ex/Em of 210/415, 240/415 and 280/415 nm appeared at the early stage of irradiation ($t = 10$ min)
341 and remained steady until the end of the experiment. These results suggested that the degradation of
342 proteins and formation of fluorescence EEM byproducts occurred during the photocatalytic
343 treatments, and different fractions had different potential in the formation of byproducts.
344 Interestingly, intensities of new peaks in the *E. coli* cells and IOM were both observed to decrease
345 after 6 h, suggesting that the byproducts could be further degraded upon photocatalysis.

346

347 **3.5 PARAFAC analysis**

348 To get more detailed information of the fluorophores changes of the photocatalytic
349 experiments, PARAFAC analysis was applied to decompose the fluorescence EEM data. Four
350 components were identified and validated by residual analysis (Fig. S3) and half-split analysis (Fig.
351 S4). As shown in Fig. 4, components 1 (C1) exhibited a two peaks Ex/Em of 225/335 and 280/335
352 nm, which was associated with tryptophan-like proteins (Hudson *et al.*, 2007). Component 2 (C2)

353 had three peaks located at Ex/Em of 210/415, 240/415 and 280/415 nm. Components 3 (C3) had
354 characteristic peaks at Ex/Em of 270/320 and 220/320, which was attributed to tyrosine-like
355 proteins (Hudson *et al.*, 2007). Components 4 (C4) had a prominent peak Ex/Em at 280/405 nm.
356 Tryptophan and tyrosine-like proteins (C1 and C3) are important constituents of microorganisms
357 and they were commonly found in microbial systems using EEM-PARAFAC analysis (Ni *et al.*,
358 2009; Xu *et al.*, 2013). The detailed classification of C2 and C4 remained unknown and will be
359 discussed in the following sections.

360 First of all, we noted that the $F_{\max}$ of C1 and C3 in the IOM decreased remarkably (from 59.1
361 to 32.7 and from 37.8 to 2.9 R.U., respectively) after mixing with TiO₂ nanoparticles (Table 1 and
362 Fig. 5), demonstrating that considerable amount of IOM protein had relatively high absorption
363 ability on the TiO₂ nanoparticles, which is in agreement with our previous findings that protein are
364 preferentially absorbed on the TiO₂ nanoparticles surfaces to form nanoparticle-protein ‘corona’.
365 Considering this phenomenon, we could postulate that the deceleration of the inactivation of
366 bacteria inactivation rate by released and spiked IOM (“tailing” and “shoulder”) in Fig. 1a could be
367 attributed to two possibilities: (i) inhibit direct contact between nanoparticles and bacteria by
368 absorbing on the TiO₂ nanoparticles surface; (ii) compete for ROS with residual bacteria cell
369 membrane.

370 The maximum fluorescence intensities ($F_{\max}$) of the four components of each photocatalytic
371 experiments as a function of time are shown in Fig. 5. It was found that the $F_{\max}$ of C1 for *E. coli*
372 cells and cell debris fluctuated and generally exhibited an increasing trend similar to that of protein
373 evolution (Fig. 1b). This result implied that bulk C1 could be a rough indicator of protein that are

374 being released into bulk, considering the fact that tryptophan is an important building block of
375 protein. In contrast, C3 was found to decrease along with irradiation time for *E. coli* cells and cell
376 debris, indicating a faster rate of degradation or adsorption than that of release for tyrosine-like
377 protein. It was also found that $F_{\max}$ of C2 and C4 in the fractions of IOM and cell debris were close
378 to 0 R.U., indicating they were not original components of IOM or cell debris. Interestingly, we
379 observed a significant increase in $F_{\max}$ for C2 and C4 upon irradiation, followed by peak off with
380 prolonged irradiation time. These results indicated that C2 and C4 mainly originated from the
381 photocatalytic oxidation of the cellular components and they could be further degraded by
382 photocatalysis. We also observed that C2 and C4 existed in the control experiments of *E. coli* cells
383 (with TiO_2 under dark, without TiO_2 under light and without TiO_2 under dark) initially (Fig. S5),
384 and their intensities exhibited a slightly increasing trend but significantly lower than those of the
385 photocatalytic treated IOM. The occurrence of C2 and C4 in the non-photocatalytic treated samples
386 could be explained by the oxidative stress induced by environmental stress (i.e. the presence of
387 nanoparticles, UVA irradiation or starvation) to the bacteria (Mandel and Silhavy, 2005;
388 Pigeot-Remy *et al.*, 2012; Manke *et al.*, 2013). In addition, C2 was found to initially present in the
389 bulk of *E. coli* cells with a $F_{\max}$ of ~ 0.4 R.U. and this background could be due to the oxidative stress
390 induced by oxygen-respiring activity of the live bacteria (Imlay, 2013). However, the $F_{\max}$ of C2 in
391 the *E. coli* cells fluctuated along with photocatalytic reaction time and remained below the
392 background value, suggesting that C2 was not a suitable parameter to indicate photocatalytic
393 oxidation.

394 Indeed, for the photocatalytic treatment of *E. coli* cells and cell debris, the concentrations of

each organic compound existed in the bulk solution is rather dynamic and could be determined by a combination effect of release, adsorption and degradation, and thus complicating the interpretation of the evolution of the PARAFAC EEM components along with irradiation time. Therefore, it is of particular interest to analyze the data obtained from IOM fractions because the releases of organic substances from insoluble fractions of cells were eliminated. The Pearson correlation analysis of the PARAFAC EEM components of IOM showed that C1 was negatively correlated with C2 ($R = -0.557$, $p < 0.05$) whereas C3 negatively correlated with C4 ($R = -0.638$, $p < 0.05$). Considering the fact that fluorophores typically contain aromatic groups (Lakowicz, 2013) and that the negative correlation of C1 and C2, and that of C3 and C4, C2 and C4 were possibly originated from the photocatalytic modification byproducts of C1 and C3, respectively, that still maintained aromatic groups (fluorophores). A previous study on the degradation of pure tryptophan by TiO_2 -UVA reported that there were formation of aromatic intermediates through (i) hydroxylation on the aromatic ring by $\bullet\text{OH}$ considering an electrophilic attack; (ii) elimination of amino groups by e^- ; and (iii) removal of the carbon that bearing carboxylic acid and amino group by $\bullet\text{OH}$ attack (Elsellami *et al.*, 2010). Considering that change of substituents on the aromatic groups would lead to the change of the fluorescence profile, therefore, the C2 and C4 were very likely the aromatic intermediates as a result of the photo-generated ROS attack on C1 and C3 in a mechanism. So far, the detailed structures of C2 and C4 remained unknown and merited further study in the future.

Cluster analysis of the dataset obtained from photocatalytic experiments of each fraction and bacteria (Fig. S6) indicates that the released organic substances during the photocatalytic inactivation of *E. coli* cells mainly derived from the photocatalytic degradation products of IOM

other than the cell membranes. This is in good agreement with the PARAFAC analysis results, in which the C4 exhibits similar evolution profile and relatively high intensities in the bulk *E. coli* cells and IOM, while C4 in the bulk cell debris showed slightly increase along with irradiation time. Therefore, the domination of C4 might be used as an indicator of photocatalytic damage to cell membrane as well as the degradation of the released IOM.

421

3.6 Toxicity test

The toxicity of the treated water from photocatalytic inactivation of normal *E. coli* cells at different reaction time were assayed. Table 3 shows the EC50 results together with their respective toxicity units. No toxicity (EC50 > 99%) were detected at the beginning of the photocatalytic treatment (0-30 min). It is noteworthy that toxicity appeared after 1 h (EC50 = 91.3%) and increased until a maximum at t = 4 h (EC50 = 55.74%), which corresponded to the time necessary to the complete inactivation of bacteria (Fig. 1a). Afterward, a decrease in toxicity was observed and finally complete detoxification was achieved. These results suggest that some toxic by-products were formed during the photocatalytic bacterial inactivation process, and these generated by-products could be further degraded/detoxified by this treatment.

To quantitatively investigate whether there is any relationship between the toxicity and four fluorescent components, we performed linear regression for the toxicity unit and $F_{\max}$ using the linear fit equation:

435

$$Y = A \times X + B \quad (3)$$

437

438 Where Y and X are the $F_{\max}$ of the four fluorescent components and toxicity unit, respectively.

439 Results indicate that only C4 exhibit a significant ($R^2 = 0.8253$, $p < 0.05$) linear correlation with the

440 toxicity unit and is shown in Fig. 6. The fit parameters A and B were determined as 2.49904 and

441 -2.04789 with the intervals of [1.63216, 3.36592] and [-3.12142, -0.97437], respectively, at a

442 confidence level of 95%. Thus, it can be deduced that the maximum fluorescence intensity of C4

443 can be used as a quantitative indicator of toxicity.

444 During the past decades, the photocatalytic bacterial inactivation mechanism has attracted

445 considerable research interests and different depths of knowledge have been obtained, however,

446 little attention has been pay to the final product of photocatalytic disinfection, which purpose is to

447 get clean and safe water. One major concern regarding the potential risks of photocatalytic

448 disinfection technologies is to release toxic by-products. Results of this study demonstrated that

449 toxicity occurred during the photocatalytic disinfection process, even at the time when the bacteria

450 were completely inactivated. More crucially, the toxicity can be migrated if provided a longer

451 reaction time. Of particular interest is the link between the toxicity and fluorescence component C4.

452 As a result, monitoring the fluorescent components of water could be a valuable surrogate for

453 photocatalytic bacterial inactivation or other hydroxyl radical ($\bullet\text{OH}$)-based disinfection

454 technologies.

455

456 **4. Conclusions**

457 This study for the first time systematically investigates the organic matters released from the

458 photocatalytic bacterial inactivation through fractionation procedure and spectroscopic analysis.

459 The major conclusions can be drawn as follows:

460 ● The released organic matters would be either absorbed on the surface of the photocatalysts
461 or consume the photo-generated ROSs to decelerate the inactivation of the remaining cells
462 as reflected by a plateau in the inactivation curve (“tailing”).

463 ● EEM-PARAFAC analysis identified four fluorescence components during the
464 photocatalytic inactivation of bacteria. Two components (C1 and C3) were attributed to
465 tryptophan and tyrosine-like proteins from the original cells, while the other two (C2 and
466 C4) were postulated to be their oxidation by-products, respectively. More importantly,
467 these by-products were subjected to photocatalytic degradation with prolonged reaction
468 time.

469 ● Toxicity occurred during the photocatalytic bacterial inactivation. Interestingly, we found
470 that the toxicity shows a high linear relationship with the C4.

471 ● The 3-D fluorescent fingerprint could be used as a surrogate to rapidly detect IOM release
472 and their oxidation as well as toxicity in the photocatalytic bacterial inactivation.

473

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605

606 **Figure Captions**

607 **Fig. 1** (a) Photocatalytic inactivation kinetics of bacterial cells in the presence and absence of
608 external spiking bacterial fractions of IOM or cell debris under TiO₂-UVA; (b) Evolution of
609 the bulk protein concentrations and (c) bulk TOC, TN concentrations, TN/TOC ratio during the
610 photocatalytic inactivation of 8 log *E. coli* cells under TiO₂-UVA. Bars represent the standard
611 deviation of three replicates.

612 **Fig. 2** Changes in the bulk absorbance spectra of (a) *E. coli* cells, (b) IOM and (c) cell debris treated
613 by TiO₂-UVA.

614 **Fig. 3** Changes in the EEM spectra of (a) *E. coli* cells, (b) IOM and (c) cell debris treated by
615 TiO₂-UVA.

616 **Fig. 4** EEM contours of the four components obtained by the PARAFAC analysis.

617 **Fig. 5** Changes of maximum fluorescence intensity ($F_{\max}$) for the four components of (a) *E. coli* cells,
618 (b) IOM, and (c) cell debris treated by TiO₂-UVA.

619 **Fig. 6** Linear regression between the toxicity unit and maximum fluorescence intensity ($F_{\max}$) of C4.

620

621

622 **Fig. 1**

623
624
625

(a) *E. coli* cells

(b) IOM

(c) Cell debris

Raman Unit

632 **Fig. 4**

633

634

637 **Fig. 6**

638

639

640 **Table 1. The TOC concentration of the cell debris and bulk parameters and $F_{\max}$ of the four**
641 **PARAFAC components of the IOM obtained from the fractionation procedure. The cell debris**
642 **and IOM were in anequeivalent cell density of 2×10^8 cfu/mL.**

Cell debris	IOM						
mg/L	Bulk parameter (mg/L)			PARAFAC components (Raman Unit)			
TOC	TOC	TN	Protein	C1	C2	C3	C4
17.4	21.0	8.5	15.9	59.1	0	37.8	0.5

643

644 **Table 2. Pearson correlation between the $F_{\max}$ of the four PARAFAC components for IOM**
645 **treated by TiO_2 -UVA.**

	C1	C2	C3	C4
C1	1	-0.557*	0.641*	-0.460
C2		1	0.252	-0.130
C3			1	-0.638*
C4				1

646 * Correlation is significant at the 0.05 level.

647

648 **Table 3. Toxicity of various samples according to Microtox[®] test.**

Time (h)	EC50 (%)	Toxicity unit	95% Confidence interval	R ²
0	> 99	1.00	NA	NA
0.16	> 99	1.00	NA	NA
0.33	> 99	1.00	NA	NA
0.5	> 99	1.00	NA	NA
1	91.3	1.10	84.59-98.53	0.988
2	70.75	1.41	67.46-74.2	0.995
3	71.88	1.39	67.46-76.58	0.987
4	55.74	1.79	51.32-60.55	0.982
6	63.02	1.59	54.93-72.3	0.918
8	> 99	1.00	NA	NA
10	> 99	1.00	NA	NA

649